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Tyrosine protein kinase activity in renal brush-border membranes

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Tyrosine protein kinase (TPK) activity was detected in rat renal brush-border membranes (BBM) using poly(Glu^{80}Na,Tyr^{20}) as a substrate. Maximal TPK activity required prior detergent dispersion of the membranes with 0.05% Triton X-100 and the presence of vanadate, a potent inhibitor of phosphotyrosine protein phosphatases, in the phosphorylation medium. Optimal conditions for measurement of TPK activity were 10 mM of $MgCl_2$ and $MnCl_2$, at 30°C and pH 7.0. TPK activity was inhibited by genistein, with a IC_{50} value of 15 μM, while no inhibition was observed in the presence of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), an inhibitor of serine-threonine kinases. TPK activity was enriched 4-fold in the BBM fraction relative to cortex homogenate. It was co-enriched with BBM enzyme markers, but not with those of the basolateral membrane (BLM). The endogenous substrates of TPK in brush-border and basolateral membranes were determined by Western blot analysis using an antiphosphotyrosine monoclonal antibody (PY20). Various phosphotyrosine-containing proteins were found in the BBM (31, 34, 46, 50, 53, 72, 90, 118 and 170 kDa) and in the BLM (37,48, 50, 53, 72, 90, 130 and 170 kDa). Addition of exogenous insulin receptor to BBM and BLM increased the phosphorylation of most of the substrates. Solubilization of the TPK activity from BBM with 0.5% CHAPS and subsequent gel filtration on Superdex 75 yielded two peaks of tyrosine protein kinase activity with apparent molecular masses of 49 and 66 kDa. These results provide evidence for a non-receptor TPK activity associated with the renal tubular luminal membrane.

Introduction

Tyrosine protein kinase (TPK) activity was first discovered in association with transforming gene products of a number of sarcoma-inducing retroviruses and has been postulated to be responsible for the transforming ability of these agents [1]. The supposition that this activity was confined to viral products was dispelled by two findings: (1) the capacity of the cellular receptors for growth factors, insulin and somatomedin C, to catalyze phosphorylation of tyrosine residues; and (2) the existence of a group of proto-oncogenes encoding membrane-associated non-receptor tyrosine kinases in normal tissues [2–4]. The relationship between oncogenic transformation, growth factor receptors, and increased activity early in embryogenesis has linked the TPK activity to the regulation of cell growth, differentiation and development. In normal cells, protein tyrosine phosphorylation is a rare event since less than 0.1% of all phosphoproteins are phosphorylated on tyrosine residues [5]. For this reason, TPK activity was studied mostly in carcinomas and transformed cell lines, in which it is overexpressed [2]. However, TPK activity has been identified in various normal mammalian tissues by using different synthetic substrates containing tyrosine residues [6–8].

The renal proximal tubule represents an interesting model for examining tyrosine phosphorylation enzymatic systems in a normal tissue. Proximal tubular cells are characterized by the differentiation of the plasma membrane into two distinct components, the luminal brush-border membranes (BBM) and the antiluminal basolateral membranes (BLM) which differ by their protein composition and the mechanisms by which metabolites enter and exit the cell [9–10]. Protein phosphorylation has been studied extensively in plasma membranes of the renal cortex. It has been demonstrated that the cyclic AMP-dependent protein kinase was predominantly localized in BBM [11] while a cal-

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Abbreviations: BBM, brush-border membrane; BLM, basolateral membrane; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; poly(GT), poly(Glu^{80}Na,Tyr^{20}); PY20, anti-phosphotyrosine monoclonal antibody; PTPase, phosphotyrosine-specific protein phosphatase; TPK, tyrosine protein kinase; IR, insulin receptor.

cium-activated, phospholipid-dependent protein kinase was present in both BLM and BBM [12,13]. Studies on specific binding of ^{125}I-insulin on the cortex plasma membranes have established that the insulin receptors were located predominantly in the BLM [14]. A low-affinity, high capacity binding of insulin has been demonstrated in BBM, distinct from the high-affinity insulin receptors associated with a TPK activity in BLM [15]. Nevertheless, no investigation on the TPK activity and the phosphotyrosine-containing proteins in the BBM have been reported and we must consider the possible existence of TPK activity in the renal BBM.

Synthetic polymer substrates are useful to evaluate and to partially characterize the total TPK(s) found in a subcellular fraction and they are currently being used as substrates during the purification of different TPKs from various tissues [16–19]. In this report, we demonstrate the presence of a TPK activity localized in the brush-border membrane of normal proximal tubule cells of the kidney by using a synthetic copolymer of glutamate and tyrosine, poly(Glu^{80}Na,Tyr^{20}), and identify the phosphotyrosine-containing proteins of BBM.

Experimental procedures

Chemicals

[γ-^{32}P]ATP and an anti-phosphotyrosine monoclonal antibody (PY20) were purchased from ICN Biomedicals (Irvine, CA). Poly(Glu^{80}Na,Tyr^{20}) (poly(GT)) was from Sigma (St. Louis, MO). Other chemicals were of the highest purity available commercially. Purified insulin receptor was generously provided by Dr. B. Posner and Ms. Céline Brulé [20].

Preparation of brush-border and basolateral membranes

The experiments were performed on Sprague-Dawley male rats weighting 200–300 g. BBM were prepared according to the method of Booth and Kenny [21]. BLM were isolated with a self-orienting Percoll gradient method [22]. Purified membranes were resuspended in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5), and stored in liquid nitrogen. Protein concentration was determined with the method of Bradford [23].

Poly(Glu^{80}Na,Tyr^{20}) phosphorylation assays

BBM vesicles were diluted (to 2 mg protein/ml) to obtain a solution containing 60 mM mannitol, 25 mM Hepes-Tris (pH 7.5) and preincubated for 1 h at 4°C with 0.05% (v/v) Triton X-100 and proteinase inhibitors (bacitracin (10 μg/ml), aprotinin (2 μg/ml), pepstatin A (10 μg/ml), chymostatin (10 μg/ml)). TPK activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into poly(GT), as described by Bourassa et al. [16]. Assays in which poly(GT) was deleted were performed and used as blanks. The assay was linear with respect to the amount of membrane protein and time, up to 15 μg and 15 min, respectively (data not shown). Results were expressed as the mean ± S.D. of three distinct experiments done in triplicate.

Phosphorylation of endogenous membrane proteins

Phosphorylation of endogenous proteins was carried out as described for the exogenous phosphorylation assays except that poly(GT) was omitted and unlabelled ATP was used instead of [γ-^{32}P]ATP. The reactions were stopped by the addition of SDS sample buffer [24]. The samples were then heated for 3 min at 100°C and proteins were separated by SDS-PAGE with 9% polyacrylamide gels and a Mini Protean slab system (Bio-Rad). Phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) were used as molecular weight standards. The gels were electrophoretically transferred to Immobilon-P (Millipore, MA) with a Mini Protean slab system (Bio-Rad). Blots were blocked with 3% BSA and 1% ovalbumin in saline buffer overnight at 4°C, washed three times with 0.05% Tween-20 in the same buffer, and incubated overnight at 4°C with ^{125}I-labelled anti-phosphotyrosine (PY20) at a concentration of 10^6 cpm/ml [25]. Duplicate blots were incubated with PY20 in the presence of 5 mM phosphotyrosine. The blots were then washed in saline buffer three times and exposed to Fuji film at −80°C for 1–3 days.

FPLC gel filtration of tyrosine protein kinase activity from brush-border membranes

The TPK activity associated with BBM was solubilized by extracting the BBM proteins (10 mg/ml) with 0.5% CHAPS, 1 mM EDTA, and 20 mM Tris-HCl (pH 8.0). After incubation of the membranes for 1 h at 4°C, the suspension was centrifuged at 100000 × *g* for 30 min. Over 90% of the TPK activity, measured by the poly(GT) phosphorylation assay, was solubilized. The supernatant was applied to a Superdex 75 column eluted with 20 mM Tris-HCl (pH 8.0) and 0.05% CHAPS at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected. Bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) were used as molecular weight standards.

Results

Tyrosine-specific protein kinase activity

The TPK activity associated with BBM was measured by following the phosphorylation of an exogenous substrate, poly(GT). Enzymatic activity was assayed after 1 h of preincubation at 4°C in the presence of Triton X-100. Pretreatment of BBM with 0.05 and 0.5% (v/v) Triton X-100 increased the TPK activity

TABLE I

Effect of pretreatment of brush-border membranes with Triton X-100 on the TPK activity

Brush-border membranes were preincubated for 1 h with or without Triton X-100 in 25 mM Hepes-Tris (pH 7.5), 60 mM mannitol and proteinase inhibitors. The reaction mixture was incubated for 10 min at 30°C and contained 10 μg of membrane protein, 50 μg of poly(GT), 10 μM [γ-^{32}P]ATP (3 μCi), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 200 μM Na_3VO_4, 0.02% Triton X-100 and 25 mM Hepes-Tris (pH 7.0).

Triton X-100 (% v/v)	Tyrosine kinase activity (pmol/min per mg protein)
0	44 ± 6
0.05	83 ± 11
0.50	56 ± 7

1.9- and 1.2-fold, respectively (Table I). Pre-treatment of the membranes with 0.05% Triton X-100 was thus used in all following assays.

The effects of $ZnCl_2$, EDTA, vanadate and molybdate, which are inhibitors of various PTPases, were then studied. Phosphorylation of exogenous substrate was allowed to proceed for 5 min and the incubation medium was then diluted in the presence or absence of PTPase inhibitors. In the absence of PTPase inhibitors, phosphorylation of the poly(GT) was decreased by approx. 50% after 30 min of incubation (Fig. 1). Vanadate or molybdate, by inhibiting PTPases, stimulated the phosphorylation 2.25- and 1.25-fold, respectively (Fig. 1). The PTPase system associated with BBM was not affected by $ZnCl_2$ or EDTA. To prevent the dephosphorylation of the exogenous substrate, the assays were thus performed in the presence of vanadate. As shown in Fig. 2, a 2-fold increase in basal TPK activity was obtained in the presence of vanadate, while okadaic acid, a specific inhibitor of phosphoserine/threonine phosphatases [26], was without effect.

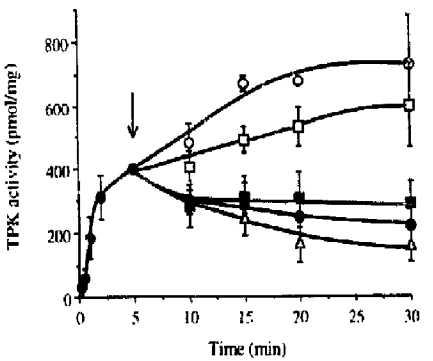

Fig. 1. Effects of PTPase inhibitors on the rate of incorporation of ^{32}P in poly(GT). The reaction mixture contained 10 μg of membrane protein, 50 μg of poly(GT), 10 μM [γ-^{32}P]ATP (3 μCi), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.02% Triton X-100 and 25 mM Hepes-Tris (pH 7.0). After 5 min of incubation at 30°C, inhibitors were added (↓) and the incubation was allowed to proceed for the indicated times. The final concentrations were 200 μM for vanadate (○), 5 μM for molybdate (□), 100 μM for $ZnCl_2$ (■) and 1 mM for EDTA (△). Control contained no inhibitor (●).

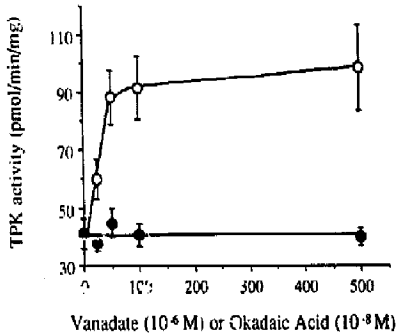

Fig. 2. Effects of vanadate and okadaic acid on the phosphorylation of poly(GT) catalyzed by BBM. TPK activity was assayed in the presence of vanadate (○) or okadaic acid (●) under the conditions described in Table I.

Since kinase activity is strongly dependent upon Mg^{2+} and/or Mn^{2+} as cofactors [2,3], TPK activity was also assayed in the presence of these two cations. Both cations caused the stimulation of TPK activity (Fig. 3A). $MnCl_2$ was much more effective than $MgCl_2$ as a cofactor. TPK activity was optimal when both $MgCl_2$ and $MnCl_2$ were present at a concentration of 10 mM. The temperature dependence of phosphorylation showed a maximum at 30°C (Fig. 3B). TPK activity showed a maximal activity at pH 7.0 whereas it was much less active at acidic and basic pH values (Fig. 3C). Phosphorylation of poly(GT) by the TPK associated with BBM was not stimulated by cyclic AMP (100 μM), cyclic GMP (100 μM) or Ca^{2+} (100 μM) (data not shown).

Recently, genistein, an isoflavone compound isolated from the fermentation broth of *Pseudomonas sp.*, was identified as a highly specific inhibitor of tyrosine kinases [27]. As shown in Fig. 3, addition of genistein to the phosphorylation medium caused a dose-dependent inhibition of the TPK activity with a half-maximal effect at about 15 μM. This concentration of genistein is similar to that required to inhibit the EGF receptor, $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$ [27]. In contrast, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), an inhibitor of serine-threonine kinase [28], had no effect on TPK activity (Fig. 4).

Since receptors with TPK activity are present in the BLM [14], we have determined whether the measured TPK activity was truly a component of the BBM by comparing the enrichment factors of BBM and BLM

for enzymatic markers and TPK. γ-Glutamyl transpeptidase was highly enriched in the BBM used in this study while Na^+/K^+-ATPase (BLM marker) was not enriched (Table II). TPK activity associated with BBM

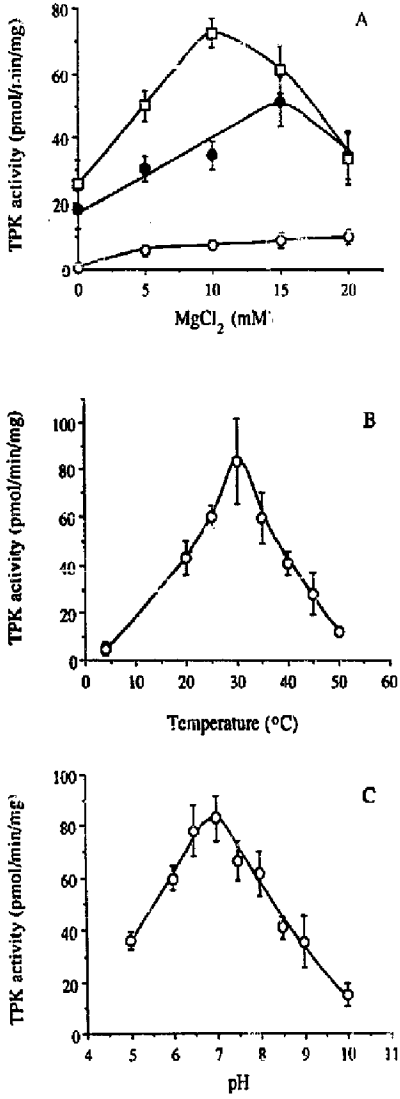

Fig. 3. Biochemical properties of the TPK activity of the brush-border membrane. (A) The effect of Mn^{2+} and Mg^{2+} on TPK activity was assayed at different concentrations of $MgCl_2$ with 0 (○), 5 (●) and 10 mM (□) of $MnCl_2$. (B) TPK activity was assayed at 4 to 50°C. (C) The effect of pH was studied with 25 mM of the following buffers: Mes-Tris (pH 5.0–6.5), Hepes-Tris (pH 7.0–8.5), CHES-Tris (pH 9.0–10.0). Other conditions were as described in Table I.

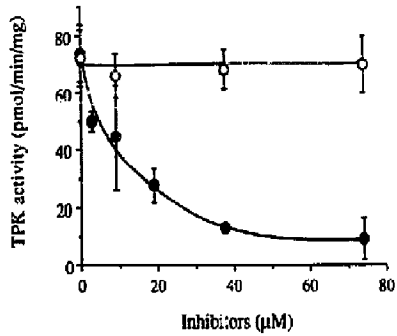

Fig. 4. Effects of genistein and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) on the phosphotyrosylation of poly(GT) catalyzed by BBM. TPK activity was assayed in the presence of genistein (●) or H7 (○) under conditions described in Table I.

was enriched 4-fold over the activity found in the cortex homogenate whereas that found in purified BLM was only enriched 2-fold.

Detection of the endogenous substrates of the tyrosine protein kinase

Phosphotyrosine-containing proteins of BBM and BLM were detected by Western blot analysis using an iodinated anti-phosphotyrosine monoclonal antibody, PY20. Fig. 5 shows representative profiles of phosphorylation obtained for the BBM and the BLM. A variety of phosphotyrosine-containing proteins was observed in both plasma membranes (Table III). Duplicate blots incubated with phosphotyrosine showed a complete abolition of the labelling (not shown), in agreement with the reported specificity of PY20 for phosphotyrosine [25].

To determine whether the endogenous substrates of these plasma membranes could be phosphorylated by receptor-TPKs, we added a purified insulin receptor (IR) preparation to the phosphorylation mixture containing BBM or BLM (Fig. 5). Phosphorylation per-

TABLE II

Comparative enrichment of TPK activity and plasma membrane marker enzyme activities for the BBM and BLM

Membranes were preincubated for 1 h with 0.05% Triton X-100 in 25 mM Hepes-Tris (pH 7.5), 60 mM mannitol and proteinase inhibitors. TPK activity was assayed as described in Table I.

Fractions	Enrichment		
	γ-glutamyl transpeptidase	Na^+/K^+-ATPase	TPK
BBM	14 ±2.5	1.5±0.3	4.0±1.0
BLM	0.9±0.2	16.0±2.4	2.0±0.5

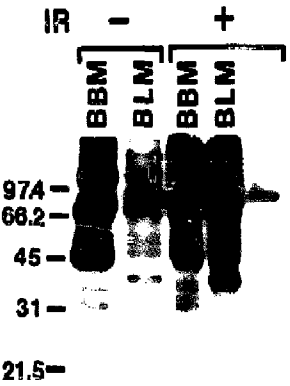

Fig. 5. Tyrosine-phosphorylated proteins from BBM and BLM. Autoradiogram of the immunoblot performed with the phosphotyrosine-specific antibody PY20. TPK activity was assayed in the presence (+) or in the absence (−) of purified insulin receptor (IR). Numbers refer to the molecular weights of the standard proteins. TPK activity and Western blot were assayed as described in Experimental Procedures.

TABLE III

Phosphorylation of the proteins of BBM and BLM by endogenous TPKs and by exogenous insulin receptor

Phosphorylation of BBM and BLM substrates was evaluated by densitometric scanning of the phosphotyrosine-containing proteins detected by immunoblot. Purified insulin receptor was added to phosphorylation mixture containing BBM or BLM. The increase in phosphorylation was calculated from the area under each peak of the densitometric scanning of the immunoblot. The results are given as means ± S.D. of five experiments. n.d., not detectable.

Substrates (kDa)	% of total phosphorylation		x-fold stimulation by IR	
	BBM	BLM	BBM	BLM
170 ± 5.0	7.3 ± 2.4	15.0 ± 5.0	3.0 ± 0.5	3.2 ± 0.6
135 ± 3.5	n.d.	16.6 ± 4.0	n.d.	3.0 ± 0.9
118 ± 2.2	19.5 ± 4.8	n.d.	1.6 ± 0.5	n.d.
90 ± 1.5	7.5 ± 1.5	21.6 ± 3.0	2.8 ± 1.2	2.7 ± 1.4
72 ± 3.0	23.2 ± 6.0	20.6 ± 6.0	1.5 ± 0.3	2.0 ± 0.5
53 ± 1.5	5.0 ± 1.2	4.2 ± 1.0	2.0 ± 0.5	2.0 ± 0.6
50 ± 1.2	6.1 ± 1.5	4.0 ± 1.0	2.2 ± 0.5	3.0 ± 1.5
48 ± 1.0	n.d.	6.2 ± 2.3	n.d.	2.7 ± 1.3
46 ± 2.0	19.2 ± 3.6	n.d.	1.5 ± 0.2	n.d.
37 ± 1.5	n.d.	8.0 ± 3.5	n.d.	3.6 ± 2.3
34 ± 0.5	3.2 ± 1.0	n.d.	2.0 ± 0.5	n.d.
31 ± 1.0	4.3 ± 1.8	n.d.	1.8 ± 0.4	n.d.

formed with the preparation of IR alone revealed a phosphotyrosine-containing protein of 90 kDa which corresponds to the autophosphorylated IR β-subunit. Addition of IR increases the phosphorylation of most of the substrates (Table III). The range of stimulation of the phosphorylation by IR varies from 1.5-fold for the 46- and 72-kDa substrates of the BBM to 3.6-fold for the 37-kDa substrate of the BLM.

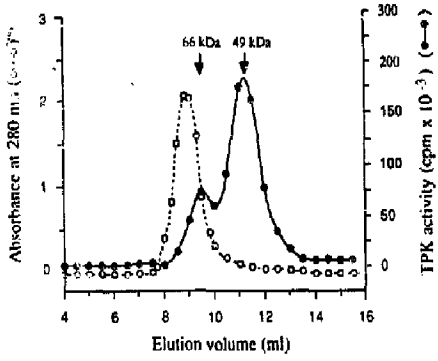

Fig. 6. Gel filtration of solubilized BBM on Superdex 75. The extract of BBM (1 ml, 9 mg of protein) was chromatographed on a Superdex 75 column and eluted as described in Experimental Procedures. The TPK activity was assayed by the phosphorylation of poly(GT), as described in Table I. The position of the peaks of TPK activity was evaluated from the elution profile of standard proteins.

Determination of the apparent molecular masses of tyrosine protein kinase(s) of brush-border membrane

Following solubilization of BBM with 0.5% CHAPS, a profile of TPK activity was obtained by FPLC on Superdex 75 (Fig. 5). Two distinct peaks of activity with molecular masses of approximately 49 and 66 kDa were detected. Most of the TPK activity (80%) eluted as proteins of approx. 49 kDa and was enriched at this stage about 20-fold compared to the activity found in the membranes.

Discussion

TPK activity associated with the brush-border membrane of rat proximal tubules was characterized by measuring the phosphorylation of an exogenous substrate, poly(GT), previously shown to be an excellent substrate for this class of kinases [16–19]. Intact BBM did not phosphorylate poly(GT) as efficiently as membranes solubilized with 0.05% Triton X-100; this may suggest that the catalytic site of the TPK is present on the inside of the BBM vesicles, since intact BBM are oriented predominantly right-side-out [29]. This is in agreement with the localization of the catalytic domain of most TPKs on the cytoplasmic side of plasma membranes [1]. The TPKs detected in BBM appear to be very similar in some of its biochemical characteristics to several other TPKs. It is most active near physiological pH, requires both Mg^{2+} and Mn^{2+} for maximal activity and is sensitive to genistein, a specific inhibitor

of the TPKs. We found that addition of vanadate, an inhibitor of PTPase [30], was necessary to measure an optimal membrane-dependent phosphorylation of poly(GT). The coexistence of TPK and PTPase in BBM is consistent with the model of protein phosphorylation-dephosphorylation which seems one of the essential regulatory systems for maintaining the intracellular phosphotyrosine content at low levels [5]. Inhibition by vanadate and insensitivity to EDTA were also reported for the PTPases found in crude preparations of rabbit kidney plasma membranes [31]. However, in this latter case PTPase was found to be sensitive to $ZnCl_2$, in contrast with the PTPase activity reported in this paper.

More than 99% of the amino acids phosphorylated in a normal cell are serine and threonine residues [5], and it is well established that the renal BBM has serine/threonine phosphorylation-dephosphorylation systems [32]. In order to study a possible contribution of these non-TPK activities to the phosphorylation of poly(GT), membranes were incubated in the presence of okadaic acid, a specific inhibitor of serine-threonine phosphatases, or in the presence of H-7, an inhibitor of serine-threonine kinases. These inhibitors had no effect on the incorporation of ^{32}P into poly(GT) (Figs. 2 and 4). Consequently, these results exclude the possible participation of the endogenous phosphorylation of serine/threonine substrates in the evaluation or in the regulation of TPK activity.

The BBM preparations were almost free of BLM, suggesting that the BBM-associated TPK activity is not due to a contaminating BLM enzyme. This is also supported by the strong phosphorylation of poly(GT) obtained with BBM (82 ± 10 pmol/min per mg of BBM proteins) compared with that obtained with BLM (35 ± 7 pmol/min per mg of BLM proteins). These values correspond to enrichment factors of 4 and 2 over the homogenate, respectively. On the basis of these results, TPK activity appears to be an intrinsic component of the BBM of the rat proximal tubule.

Although some substrates with similar molecular weights were found in the BBM and BLM, the major substrates recognized by the BBM-associated TPK (46 and 118 kDa) were distinct from those recognized by the BLM-associated TPK (90, 135 and 170 kDa). This suggests that the TPKs associated with these plasma membranes could work through distinct cellular pathways.

Addition of an exogenous insulin receptor (IR) to the BBM and BLM increases the phosphorylation of most substrates. Although proteins of both the BBM and the BLM could be phosphorylated by this receptor tyrosine kinase, the substrates of the BBM had a lower reactivity. Moreover, the stimulation by IR obtained for the major substrates in the BBM (46, 72 and 118 kDa) was only 1.5-fold, while it was much stronger for the major BBM-associated substrates: 2.7-, 3.0- and 3.2-fold for the 90, 135 and 170 kDa proteins. These differences suggest that the tyrosine-containing proteins associated with the BBM could be substrates of another class of TPKs.

Receptors with tyrosine kinase activity are known to be high-molecular weight proteins; molecular masses of 170, 185, > 300 and > 350 kDa were estimated for EGF, PDGF, IGF-I and insulin receptors, respectively [4]. In the present study, gel filtration of solubilized BBM revealed two peaks of TPK activity with apparent molecular masses of 49 and 66 kDa. The smaller sizes of the molecular masses reported here indicate that these kinase activities are distinct from the receptor tyrosine kinases. This is in agreement with the known segregation of receptors to the basolateral side of these cells [14]. The similarity of the apparent molecular masses (49 and 66 kDa), evaluated for the TPK by gel chromatography and two major phosphotyrosine-containing proteins of the BBM (46 and 72 kDa) may represent an autophosphorylation of the TPKs. However, further investigations will be necessary to confirm this interpretation.

In spite of several reports on the TPK of normal and some transformed cells, their role is still unclear. Involvement in cell transformation and in regulation of cell growth has been suggested but the physiological function of the non-receptor TPK activity in normal cells remains largely unknown [1]. Because the generation and maintenance of the polarized cell phenotype is a multistage process [33], alterations by genetic mutations or environmental factors at any stage in development could have direct consequences on tissue function. Interestingly, approx. 85% of human tumors are carcinomas that are derived from epithelia, and many carcinomas are characterized by morphological changes in cell polarity [33].

The mechanisms implicated in the regulation of the transmembrane transport processes involved in the reabsorption of important solutes from the glomerular ultrafiltrate remain largely unknown, but protein phosphorylation has been suggested in the regulation of the activity of the Na^+/phosphate co-transporter [34–36]. Further characterization of the BBM proteins, which undergo tyrosine phosphorylation, should provide information regarding the physiological functions of the TPK activity associated with BBM.

Acknowledgments

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