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Localization of Tyrosine-Phosphorylated Proteins in the Normal Rat Kidney

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Summary. Protein tyrosine phosphorylation is responsible for activating or repressing the activity of proteins involved in numerous signal transduction pathways in a living organism. Using immunochemical methods with two well characterized monoclonal anti-phosphotyrosine antibodies, we examined the distribution and localization of tyrosine-phosphorylated proteins expressed in the three major compartments of the normal rat kidney: the glomerulus, cortex, and medulla. Western blotting analysis revealed that tyrosine-phosphorylated proteins were predominantly expressed in the glomerulus, compared with the cortex and medulla. The intensity of tyrosine-phosphorylated protein bands was augmented by using orthovanadate — an inhibitor of protein tyrosine phosphatase — during sample preparation, and the bands were undetectable when the antibodies were absorbed with phosphotyrosine but not with phosphoserine or phosphothreonine. Immunofluorescence microscopy indicated that the phosphotyrosine staining was intense along glomerular capillary walls and sparse along parts of the tubuli. Immunoelectron microscopy further showed that phosphotyrosine immunoreactivity was predominantly localized at the basal membranes of podocyte foot processes. In addition, some immunogold labeling was also observed at cell-matrix attachment sites of glomerular endothelial cells and mesangial cells as well as at basal membranes of epithelial cells of proximal tubules, distal tubules, and collecting ducts. In conclusion, tyrosine-phosphorylated proteins are predominantly and constitutively expressed in the normal rat glomerulus, especially in the basal membranes of podocyte foot processes, suggesting tyrosine-phosphorylated proteins could play an important role in maintaining the unique organization of foot processes and in the glomerular ultrafiltration there.

Key words— kidney; glomerulus; tyrosine phosphorylation; podocyte; foot process.

INTRODUCTION

Tyrosine phosphorylation is one of the most important protein post-translational modifications which is responsible for activating or repressing the activity of proteins involved in numerous signal transduction pathways in a living organism. In normal cells, the tyrosine phosphorylation of proteins by receptor and non-receptor protein tyrosine kinases (PTKs) is critical for the signal transmission from the plasma membrane to the plasma, or even to the nucleus, where the transcription of certain target genes can be regulated.[1,2] The importance of PTKs in cellular signaling has led to extensive research on the roles of tyrosine-phosphorylated proteins in cellular physiological or pathological processes.

The kidney consists of three major compartments: the glomerulus, cortex, and medulla. The glomerulus is a highly specialized structure that functions as the basic filtering section in the kidney. The filtration barrier between the blood and urinary space is composed of fenestrated endothelial cells, glomerular basement membrane (GBM), and slit diaphragms between the foot processes of podocytes. The podocyte is a

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Abbreviations– ECM, extracellular matrix; GBM, glomerular basement membrane; PTKs, protein tyrosine kinases; PVDF, polyvinylidene difluoride.

terminally differentiated cell that covers the outer aspect of GBM, forming the final and the most important barrier for the plasma ultrafiltration. Previous reports have suggested that signal transduction plays a crucial role in physiological and pathological processes of the kidney,[3,4,5,6] but the details in the signal transduction pathways occurring in the kidney have not been clearly studied.

The present study examined the distribution and localization of tyrosine-phosphorylated proteins in the normal rat kidney through immunochemical methods using two commonly used, authentic monoclonal anti-phosphotyrosine antibodies. Our results demonstrated that tyrosine-phosphorylated proteins are predominantly expressed in the glomerulus as compared with the cortex and medulla, especially in the basal membranes of the podocyte foot processes.

MATERIALS AND METHODS

Animals

Male Wistar rats were purchased from Charles River Japan (Atsugi, Kanagawa) and used in all experiments at the age of eight weeks. The present study was approved by the Animal Committee at Niigata University School of Medicine (approved number, 161), and all animals were treated according to the Guidelines for Animal Experimentation of Niigata University.

Antibodies

Two mouse monoclonal anti-phosphotyrosine antibodies were used in this experiment: P-tyr-100 was obtained from Cell Signaling Technology (Danvers, MA, USA), and 4G10 was from Upstate Cell Signaling Solutions (Charlottesville, VA, USA).

Sample preparation

Rats were anesthetized with diethyl ether, and kidneys were perfused through the abdominal aorta with phosphate-buffered saline (PBS) before removal. Sodium orthovanadate, an inhibitor of protein tyrosine phosphatase, was added to PBS at 1 mM when indicated. Glomeruli were isolated from kidney cortices by the standard sieving method. The isolated glomeruli, cortex, and medulla were homogenized with a Polytron homogenizer in a lysis buffer (1 % Nonidet P-40, 0.1 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA-2Na, 1 mM Na_3VO_4, 25 mM NaF, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin A), followed by centrifugation at 14,000 g for 20 min. at 4 °C to remove the insoluble materials. The supernatants were used as samples for Western blotting. Preliminary experiments indicated that almost all the proteins immunoreactive with anti-phosphotyrosine antibodies were recovered in the supernatants as examined by immunoblotting. Protein was quantified by Lowry's method as modified by Peterson.[7] The whole experiment was repeated at least three times.

Western blotting

Samples of the glomerulus, cortex, and medulla were separated on 10 % sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels with a protein load of 10 μg, and immediately transferred to a polyvinylidene difluoride (PVDF) membrane (Immobiline-P transfer membrane, Millipore, Bedford, MA, USA) in a semi-dry apparatus. The transferred PVDF membrane was incubated with the primary antibody, P-tyr-100 or 4G10, at 4 °C overnight, followed by the secondary antibody, Mouse EnVision (Dako, Hamburg, Germany) at room temperature for two h. When the effects of preabsorption of the anti-phosphotyrosine antibody with phosphorylated amino acids were examined, phosphotryosine, phosphoserine, or phosphothreonine were included at 1 mM in the incubation medium for the primary antibody with the transferred PVDF membrane. The detection of immunoreactivity was performed with an enhanced chemiluminescence detection system, ECL plus (GE Healthcare, Chalfont, St, Giles, UK).

Immunofluorescence microscopy

The indirect immunofluorescence technique was applied to frozen kidney sections. Rat kidneys without prior perfusion were immediately snap-frozen at –80 °C and sectioned at a thickness of 3 μm in a cryostat. Frozen sections were fixed in 2% paraformaldehyde in PBS for five min., blocked with 10% goat normal serum for 30 min., then incubated with the primary antibody P-tyr-100 or 4G10 at 4 °C overnight, and then with the second antibody, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, for one h at room temperature. As negative controls, the frozen sections were first incubated with PBS in the absence of the primary antibody. Immunofluorescence of the sections was observed with an Olympus microscope (BX50) equipped with epi-illumination optics and appropriate filters (Olympus Corp.,Tokyo).

Immunoelectron microscopy

Immunoelectron microscopy analysis for normal rat kidneys was performed according to the procedure previously described.[8,9] In brief, 1-mm^3 tissue blocks from paraformaldehyde-lysine-periodate (PLP) perfused

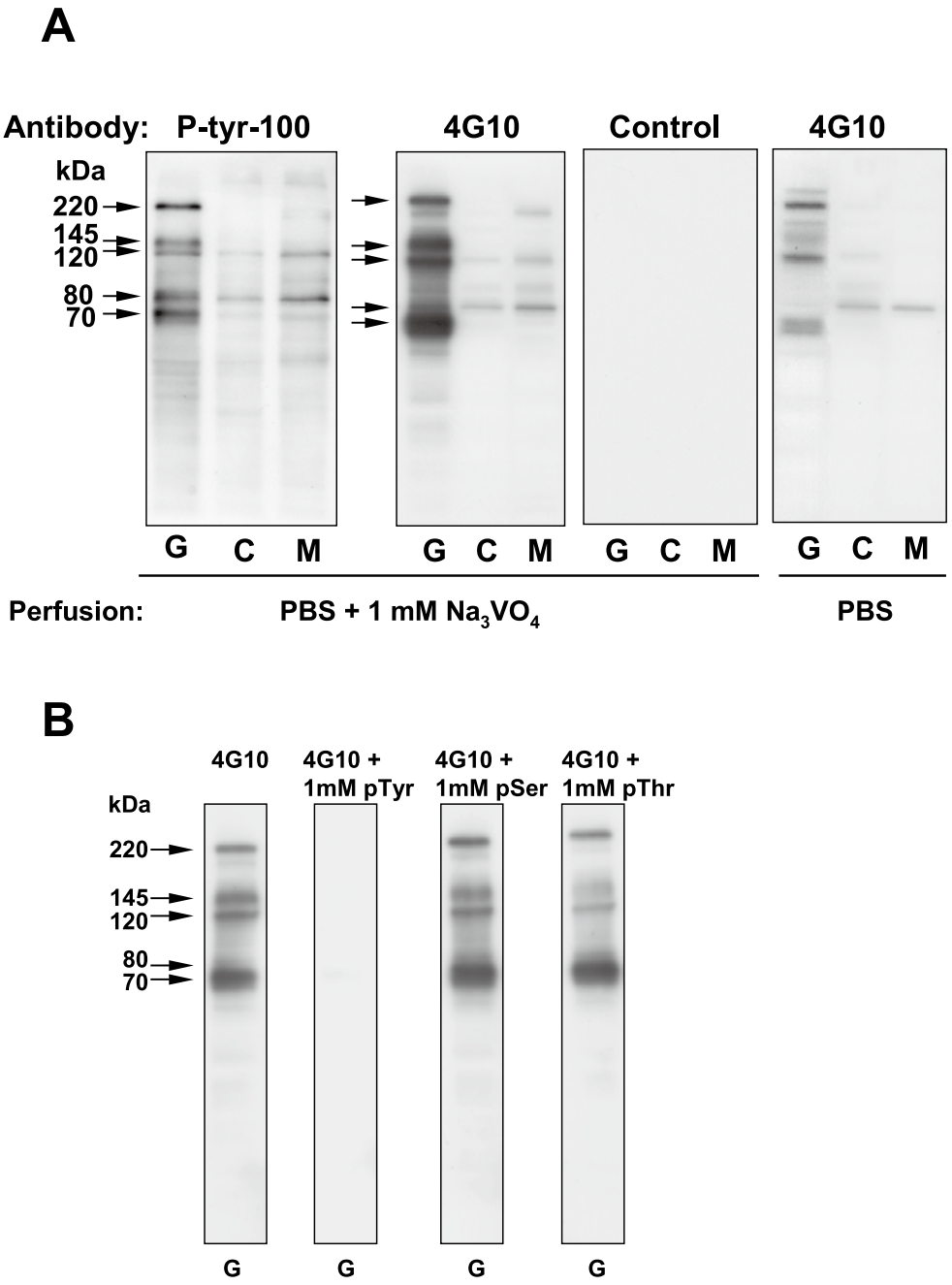

Fig. 1. Immunoblotting analysis of tyrosine-phosphorylated proteins in the glomerulus (*G*), cortex (*C*) and medulla (*M*) of the normal rat kidney. A mouse monoclonal antibody for phosphotyrosine, P-tyr-100 or 4G10, was used as the primary antibody. In panel **A**, immunoreactive proteins in the protein extracts from kidneys perfused with PBS in the presence or absence of sodium orthovanadate (Na_3VO_4) are shown. Major immunoreactive bands with molecular masses of 220 145, 120, 80, and 70 kDa are indicated by *arrows*. Immunoblotting without the primary antibody did not bring about any immunoreactive bands (Control). In panel **B**, immunoblotting analysis of tyrosine-phosphorylated proteins in the glomerular extract in the presence of 1 mM each of three phosphorylated amino acids — phosphotyrosine (pTyr), phosphoserine (pSer), or phosphothreonine (pThr) — is shown. The phosphorylated amino acids were added to the incubation medium for the primary antibody with transblotted PVDF membranes. The anti-phosphotyrosine antibody 4G10 was used in this experiment. A similar result was obtained with P-Tyr-100. (data not shown)

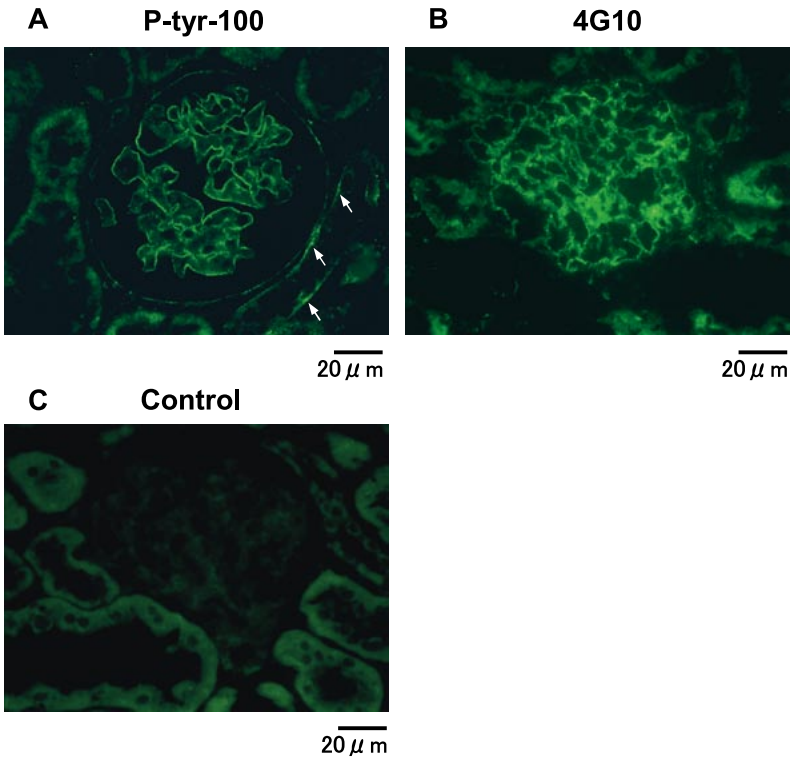

Fig. 2. Immunofluorescence micrographs of frozen sections of the normal rat kidney cortex with anti-phosphotyrosine antibodies; P-tyr-100 **A** and 4G10 **B**. The frozen sections were prepared from snap-frozen kidney tissues without prior perfusion. **C.** A micrograph without using the primary antibody is also shown as a negative control. Staining with P-tyr-100 and 4G10 is prominent along the glomerular capillary walls. Sparse staining can be observed along the basolateral membranes of some tubular epithelial cells ***(arrows)***.

kidneys were placed in the PLP fixative at 4 °C overnight, dehydrated, and then embedded in hydrophilic methacrylate resin. The prior perfusion of kidneys with PBS was omitted in this experiment. Ultrathin sections collected on nickel grids were stained with the primary antibody P-tyr-100, followed by 15 nm gold-labeled goat anti-mouse IgG (Fc) (AutoProve EM GAM IgG G15, GE healthcare).

RESULTS

Tyrosine-phosphorylated proteins in normal rat kidneys

The tyrosine-phosphorylated proteins in the normal rat glomerulus, cortex, and medulla were analyzed by Western blotting using mouse monoclonal antibody P-tyr-100 or 4G10 (Fig. 1A). Tyrosine-phosphorylated proteins were detected mainly in the glomerulus. Three intense bands with molecular masses of 220, 145, and 70 kDa were almost exclusively found in the glomerulus. Besides these intense bands, two other bands with molecular masses of 120 and 80 kDa were also detected, which were additionally found in the cortex and medulla. The intensity of the immunoreactive bands was augmented by the perfusion of PBS with orthovanadate, suggesting the involvement of tyrosine kinase activity in the phosphorylation of these proteins. The results of Western blotting analysis were very similar with both anti-phosphotyrosine antibodies. Specific detection of tyrosine-phosphorylated proteins was further confirmed by abolishment of the immunoreactive bands in the glomerulus after preabsorption of the 4G10 antibody with phosphotyrosine but not with either phosphoserine or phosphothreonine (Fig. 1B). Similar results were obtained with the P-tyr-100 antibody (data not shown).

Immunolocalization of tyrosine-phosphorylated proteins in normal rat kidneys

The localization of tyrosine-phosphorylated proteins was examined by immunofluorescence microscopy by using the anti-phosphotyrosine antibody P-tyr-100

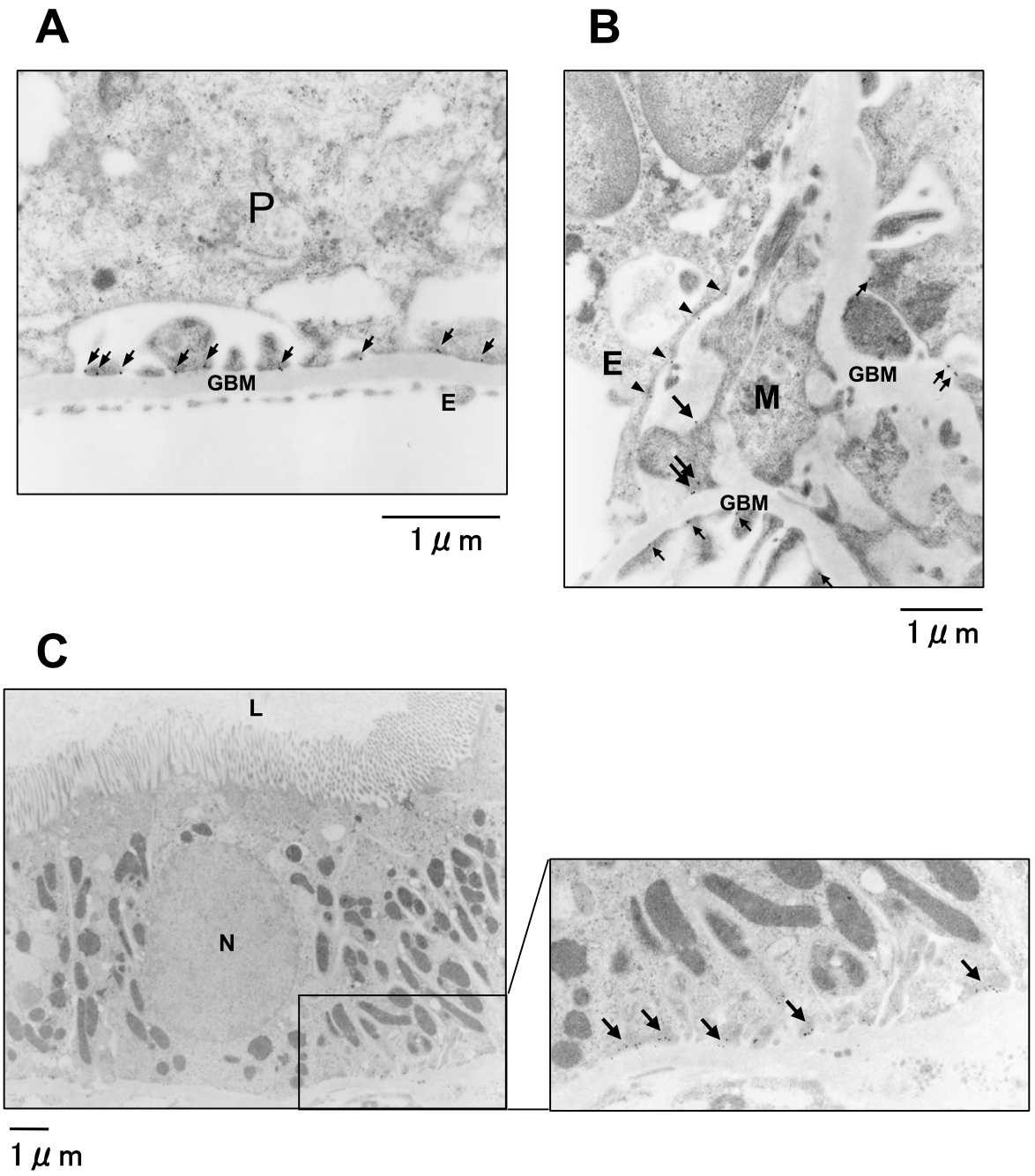

Fig. 3. Immunogold localization of phosphotyrosine with anti-phosphotyrosine antibody P-tyr-100. The immunogold particles were predominantly confined to the basal membranes of podocyte foot processes (**A** and **B**, *small arrows*). The kidneys perfused with PLP fixative were used in this experiment as shown in the "Materials and methods". No prior perfusion with PBS was carried out in this experiment. Some of the immunogold particles can be observed in the cell-matrix contact sites of endothelial cells (**B**, *arrowheads*) and mesangial cells (**B**, *big arrows*). Additionally, immunogold particles appear sparsely on the basolateral membranes of some proximal tubular epithelial cells (**C,** *arrows*). Symbols: *P,* podocyte; *GBM,* glomerular basement membrane; *E,* endothelial cell; *M*, mesangial cell; *N,* nucleus; *L,* lumen.

(Fig. 2A) or 4G10 (Fig. 2B). Immunofluorescence staining with antibody P-tyr-100 and 4G10 indicated that immunoreactivity was intense along the glomerular capillary walls and less intense in the mesangium. It should be noted that the staining was also detected along the basolateral membranes of some tubular epithelial cells (Fig. 2A, *arrows*).

The immunolocalization of tyrosine-phosphorylated proteins was further examined by immunoelectron microscopy (Fig. 3). The glomerular capillary wall is comprised of three layers: fenestrated endothelium, GBM, and podocyte foot processes. The immunogold particles were largely confined to the basal membranes of the podocyte foot processes (Fig. 3A and 3B, *small arrows)*. In addition, some of the immunogold particles were observed at the cell-matrix attachment sites of the fenestrated endothelial cells (Fig. 3B, *arrowheads*) and the mesangial cells (Fig. 3B, *big arrows*). Immunogold particles were also detected at the basolateral membranes of proximal tubular epithelial cells (Fig. 3C, *arrows*), epithelial cells of distal tubules, and collecting ducts (data not shown); these were much less prominent in comparison with those observed in the podocyte foot processes.

DISCUSSION

This study examined the expression and immunolocalization of tyrosine-phosphorylated proteins in the normal rat kidney using those specific anti-phosphotyrosine antibodies which have been generally employed to detect tyrosine-phosphorylated proteins. Western blotting analysis indicated that tyrosine-phosphorylated proteins were predominantly found in the glomerulus when compared with the cortex and medulla. Five intense bands with molecular masses of ~ 220, 145, 120, 80, and 70 kDa were observed as major tyrosine-phosphorylated proteins in the normal rat glomerulus by the Western blotting analyses using the two authentic anti-phosphotyrosine antibodies. The specific detection of tyrosine-phosphorylated proteins was confirmed by the increased tyrosine phosphorylation of these proteins in the glomerulus isolated from kidneys perfused with orthovanadate, a protein tyrosine phosphatase inhibitor, and by the complete abolishment of the Werstern blotting signals in the presence of phosphotyrosine but not of phosphoserine or phosphothreonine in the antibody. In addition, immunoblotting without the primary antibody did not occur any immunoreactive bands.

Among the five major immunoreactive bands, the 220, 145, and 70 kDa bands were almost exclusively detected in the glomerulus, while the two bands with respective molecular masses of 120 and 80 kDa were detected in all three compartments with the less intense immunoreactivity in the cortex. Although a more conclusive explanation should await the identification and localization of proteins consisting of these bands, these immunoreactive proteins may represent tyrosine-phosphorylated proteins commonly expressed in the glomerulus and the medulla. The less intense immunoreactivity in the cortex may be attributable to their predominant localization to the glomerulus but lack of or very low expression in the proximal and distal tubules as well as collecting ducts in the cortex as suggested by immunoelectron microscopy (Fig. 3).

The immunofluorescence staining for phosphotyrosine indicated a predominant immunolocalization along the glomerular capillary walls. It was noted that the staining patterns of P-tyr-100 and 4G10 were different: the staining along capillary walls with 4G10 was more discontinuous than that with P-tyr-100, and the staining at the mesangial area was apparently more intense with 4G10 while there was no reactions or very weak staining with P-tyr-100. As shown in the results of Western blotting, the two anti-phosphotyrosine antibodies, P-tyr-100 and 4G10, detected almost the same sets of tyrosine-phosphorylated proteins, but exhibited different preferences towards some of these proteins. Thus, the different staining pattern between the two antibodies could be explained by their different preference for tyrosine-phosphorylated proteins.

Consistent with the observations by immunofluorescence staining, immunoelectron microscopy for phosphotyrosine revealed that immunogold particles were found mainly in the basal membranes of podocyte foot processes with sparse distribution at the cell-matrix attachment sites of the glomerular endothelial cells and the mesangial cells (Fig. 3).

The prominent distribution of tyrosine-phosphorylated proteins in the glomerulus and their predominant localization to the basal membranes of foot processes of podocytes, in conjunction with their localization to the cell-matrix contact sites of glomerular endothelial cells and mesangial cells, all strongly suggest that some components of the focal adhesion complex are tyrosine-phosphorylated in the normal rat kidney. Focal adhesion is the specialized site of cell attachment to the extracellular matrix (ECM), where integrins — integral membrane adhesion molecules — link the actin cytoskeleton to the ECM, and has been long investigated as a model system for cell-matrix interactions.[10,11,12] Focal adhesion serves at least two fundamental cellular functions: 1) the transmission of force or tension at adhesion sites to maintain strong attachments to the underlying ECM; and 2) functioning as signal centers from which numerous intracellular pathways emanate to regulate cell growth, survival, and gene expression.[13,14] Although this structure is prominent in many adherent cell types grown in culture but rarely observed *in vivo*,

the focal adhesion complex exists in the podocyte foot process of the glomerulus and anchors the basal domain of the foot process to the GBM. The focal adhesion complex in the podocyte consists of the integral membrane protein integrin $\alpha_3\beta_1$ heterodimer, talin, vinculin, and paxillin, as well as a number of signaling molecules.[15,16] Many focal adhesion components are tyrosine kinase substrates, with several being tyrosine kinases themselves, and it has been postulated that tyrosine phosphorylation participates in regulation of the assembly and disassembly of the focal adhesion complex.[17]

Although the tyrosine-phosphorylated proteins detected in the present study are yet to be identified, it is not difficult to speculate that at least some of them are tyrosine-pshophorylated forms of components comprising the focal adhesion complex, especially in the podocyte foot process. The tyrosine-phosphorylated proteins may play crucial roles in maintaining the unique organization of the foot process and in glomerular ultrafiltration under physiological conditions. The identification of the tyrosine-phosphorylated proteins in this study is now in progress by mass spectrometric analysis.

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REFERENCES

1) Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**: 203-212, 1990.
2) Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S: Oncogenes and signal transduction. *Cell* **64:** 281-302, 1991.
3) Yaoita E, Yao J, Yoshida Y, Morioka T, Nameta M, Takata T, Kamiie J, Fujinaka H, Oite T, Yamamoto T: Up-regulation of connexin43 in glomerular podocytes in response to injury. *Am J Pathol* **161**: 1597-1606, 2002.
4) Benzing T: Signaling at the slit diaphragm. *J Am Soc Nephrol* **15**: 1382-1391, 2004.
5) Huber TB, Benzing T: The slit diaphragm: a signaling platform to regulate podocyte function. *Curr Opin Nephrol Hypertens* **14**: 211-216, 2005.
6) Mundel P, Shankland SJ: Podocyte biology and response to injury. *J Am Soc Nephrol* **13**: 3005-3015, 2002.
7) Peterson GL: A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* **83**: 346-356, 1977.
8) Kihara I, Yaoita E, Kawasaki K, Yamamoto T: Limitation of podocyte adaptation for glomerular injury in puromycin aminonucleoside nephrosis. *Pathol Int* **45**: 625-634, 1995.
9) Kamiie J, Nameta M, Ma M, Takata T, Fujinaka H, Yoshida Y, Yaoita E, Yamamoto T: Localization and expression of the aquaporin-1 water channel in mesangial cells in the human glomerulus. *Arch Histol Cytol* **65**: 83- 90, 2002.
10) Burridge K, Chrzanowska-Wodnicka M: Focal adhesions, contractility, and signaling. *Annu Rev Cell Dev Biol* **12**: 463-519, 1996.
11) Jockusch B, Bubeck P, Giehl K, Koremker M, Moschner J, Rothkegel M, Rudiger M, Schluter K, Stanke G, Winkler J: The molecular architecture of focal adhesions. *Annu Rev Cell Dev Biol* **11**: 379-416, 1995.
12) Critchley DR: Focal adhesions -the cytoskeletal connection. *Cur Opin Cell Biol* **12**: 133-139, 2000.
13) Schwartz M, Schaller M, Ginsberg M: Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Biol* **11**: 549-599, 1995.
14) Howe A, Aplin A, Alahari S, Juliano RL: Integrin signaling and cell growth control. *Curr Opin Cell Biol* **10**: 220-231, 1998.
15) Adler S: Characterization of glomerular epithelial cell matrix receptors. *Am J Pathol* **141**: 571-578, 1992.
16) Kriedberg JA, Donovan MJ, Goldstein SL, Rennke H, Shepherd K, Jones RC, Jaenisch R: Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development* **122**: 3537-3547, 1996.
17) Carragher NO, Frame MC: Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. *Trends Cell Biol* **14:** 241-249, 2004.