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Highly polarized expression of carbohydrate-binding protein p33/41 (annexin IV) on the apical plasma membrane of epithelial cells in renal proximal tubules

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Abstract

p33/41 is a Ca^{2+}-dependent carbohydrate-binding protein and is identical to annexin IV, a member of the annexin protein family. The localization of p33/41 in bovine kidney specimens was investigated immunohistochemically by use of specific polyclonal antibodies. The most interesting finding on immunostaining was that p33/41 was highly concentrated in the apical plasma membrane of the epithelial cells in the proximal tubules contrary to the distribution throughout the cytoplasm in the papillary ducts and papilla epithelium. The enrichment of p33/41 in the apical membrane was confirmed by immunoblotting of the brush border membrane fraction prepared from a kidney homogenate. Sequential extraction with EDTA and Triton X-100, and a partition experiment with Triton X-114 revealed that most p33/41 associates with the renal brush border membrane in a Ca^{2+}-independent manner and is integrated into the membrane like intrinsic membrane proteins.

Key words: Lectin; Annexin; Brush border membrane; Kidney; Immunohistochemistry

1. Introduction

A number of carbohydrate-binding proteins, lectins, have been identified in various animal tissues and body fluids. Lectins are counterpart receptors of carbohydrate chains on glycoconjugates, and the interaction between a lectin and a specified carbohydrate chain is one of the critical forms of recognition concerning various biological events, such as cell adhesion [1], complement activation [2], molecular clearance [3], and cell growth regulation [4]. In recent studies, most lectins isolated from animal sources were classified into two groups, C-type and S-type lectins, based on the amino acid sequence similarity in each carbohydrate recognition domain (CRD) and the divalent cation requirement for expression of lectin activity. We have purified a Ca^{2+}-dependent carbohydrate-binding protein, p33/41, from bovine kidney extracts by means of two-step affinity chromatography on fetuin and heparin columns. Although p33/41 binds to sialylated oligosaccharides and sulfated polysaccharides, its carbohydrate specificity and/or the carbohydrate structures of endogenous ligands remain to be elucidated in detail [5]. Partial amino acid sequences of p33/41 highly corresponded to those of annexin IV, but not to those of C-type or S-type CRDs [6]. Therefore, we cloned a cDNA for p33/41 and expressed the cDNA in *E. coli*. Sequencing of the cDNA, and analysis of the immunoreactivity and carbohydrate-binding activity of the recombinant protein showed that p33/41 is identical with annexin IV (in preparation for publication). These findings revealed that p33/41 is a new type of carbohydrate-binding protein belonging to the annexin protein family.

The annexins comprise a growing family of Ca^{2+}-dependent phospholipid- and/or membrane-binding proteins which are distributed in a wide range of tissues in animals and plants. These proteins consist of four or eight conserved repeating units of 70–80 amino acids in length, each of which contains a 17-amino acid consensus sequence, and an N-terminal segment, which exhibits the greatest variation in sequence and length between annexin family proteins. Annexins are claimed to have particular functions in cell regulation. In vitro they are anti-inflammation proteins that inhibit phospholipase A_2 activity, because they sequester the substrate phospholipids from phospholipase A_2 [7]. Furthermore, the annexins exhibit anti-coagulant activity [8], calcium channel activity [9], and cyclic phosphate phosphohydrolase activity [10], and function in the membrane fusion process, exocytosis [11], and membrane-cytoskeleton interaction [12]. However, the precise functional mechanisms underlying their individual biological roles remain to be elucidated.

Now, the main issue is whether the lectin activity of p33/41 (annexin IV) is biologically meaningful or just a

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Abbreviations: CRD, carbohydrate-recognition domain; PVDF, polyvinylidene difluoride.

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non-specific cross-reaction property. If p33/41 plays a role as a lectin in the kidneys, it must be localized where it will encounter glycoconjugates. Regarding this point, information about localization of p33/41 in the kidneys is extremely important for biological evaluation of p33/41 as a lectin. Therefore, in the present study, we investigated the distribution of p33/41 by means of immunohistochemistry using specific polyclonal antibodies.

2. Materials and methods

2.1. Materials

The reagents for SDS-PAGE and 4-chloro-1-naphthol were purchased from Wako Pure Chemicals (Osaka, Japan). Triton X-100 and Triton X-114 were purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and Nacalai Tesque, Inc. (Kyoto, Japan), respectively. Horseradish peroxidase-conjugated anti-rabbit IgG antibodies (goat) were obtained from Kirkegaad and Perry Laboratories, Inc. (Gaithersburg, MD, USA). *N*-Octyl β-D-glucoside was purchased from Dojindo Lab. (Tokyo, Japan). Polyvinylidene difluoride (PVDF) membranes and Mayer's Hematoxylin solution were purchased from Toyo Roshi Kaisya, Ltd. (Tokyo, Japan), and Sigma Diagnostics (St. Louis, MO, USA), respectively.

2.2. Electrophoresis and immunoblotting

SDS-PAGE was performed by the method of Laemmli [13]. Immunoblotting was carried out by use of rabbit polyclonal anti-p33/41 antibodies and secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG antibodies). The reactive band was visualized with 4-chloro-1-naphthol and H_2O_2, as previously described [6].

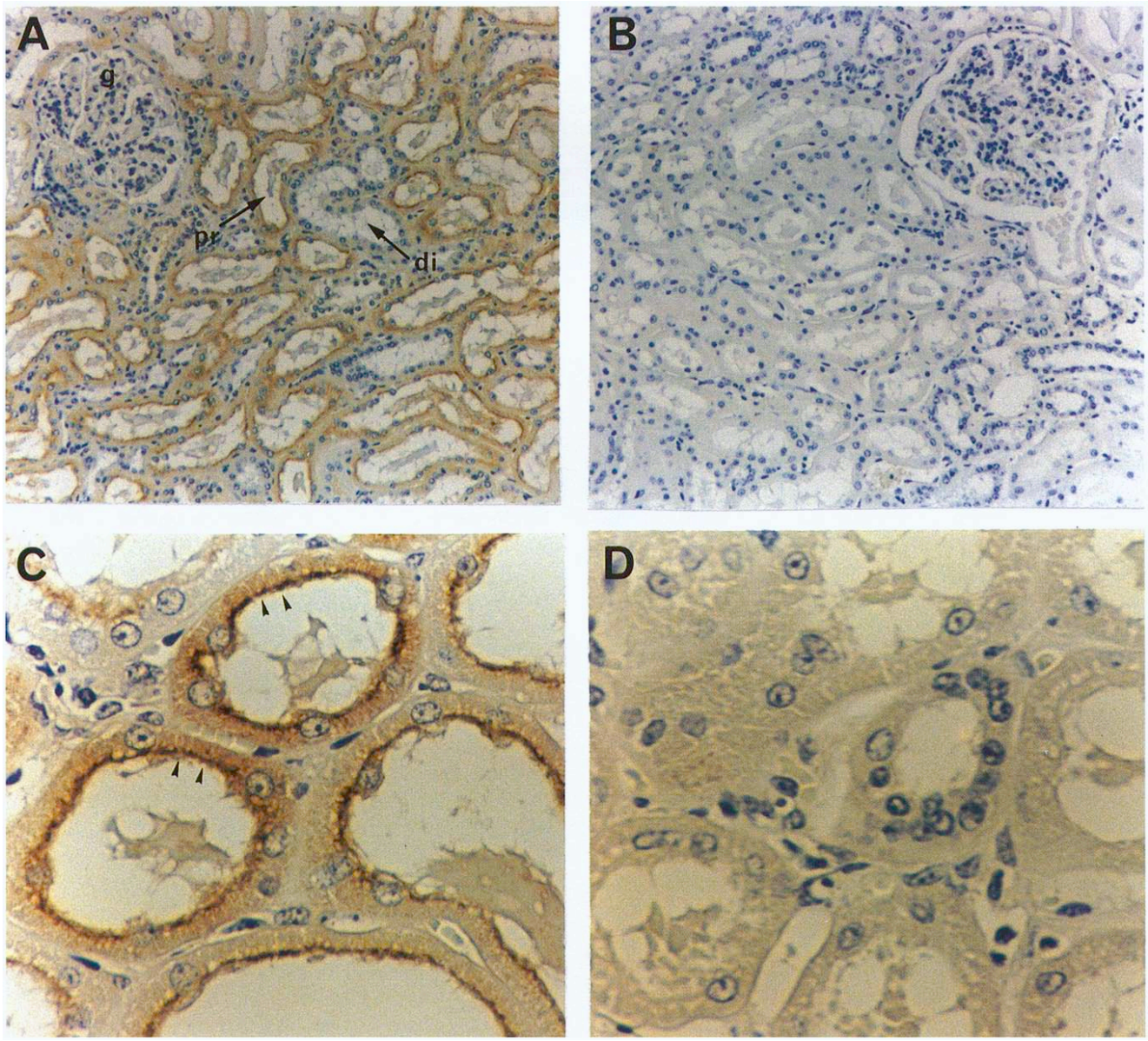

Fig. 1. Immunohistochemical staining of p33/41 in the renal cortex. A,B (×40), C,D (×160). Formalin-fixed paraffin-embedded sections were immunostained with specific polyclonal antibodies to p33/41 (A,C) or preimmune normal rabbit IgG (B,D). Immunoreactions were detected with horseradish peroxidase-conjugated second antibodies and the substrate, 3,3′-diaminobenzidine tetrahydrochloride. Strong staining was observed in the proximal tubules (pr), but not in the distal tubules (di) or glomeruli (g) in A. The positive reaction on the apical surfaces of the proximal tubules is indicated by arrowheads in C.

2.3. Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded bovine kidney were deparaffinized with xylene and then rehydrated with a graded series of ethanol (100–70%). The sections were rinsed with distilled water and washed with PBS, and then treated with 3% H_2O_2 for 7 min to block endogeneous peroxidase. Non-specific binding to sections was blocked by incubation with 0.5% defatted milk and 1% normal goat serum. The sections were incubated with rabbit polyclonal anti-p33/41 antibodies or preimmune normal rabbit IgG for 1h and then washed in PBS. After incubation with the secondary antibodies and washing in PBS, the sections were developed with 3,3′-diaminobenzidine tetrahydrochloride and H_2O_2, and then stained with Hematoxylin.

2.4. Preparation of membrane fractions

The bovine renal brush-border membrane fraction was prepared by the following procedures, based on the method of Turner and Silverman [14]. All steps were carried out at 4°C. Renal cortex was removed by scraping with a razor blade, suspended in extraction buffer (comprising 10 mM HEPES buffered with Tris to pH 7.4, 30 mM mannitol, and 10 mM $CaCl_2$) to a final dilution of 1 g cortex/30 ml, and then homogenized. After incubation for 15 min, the resulting initial homogenate was centrifuged at 3,000 × *g* for 15 min and the pellet was discarded. The supernatant was centrifuged at 43,000 × *g* for 20 min, and the resulting pellet was resuspended in suspension medium (comprising 10 mM HEPES buffered with Tris to pH 7.4, and 100 mM mannitol) and passed through a 22-gauge needle. After recentrifugation at 43,000 × *g* for 20 min, the pellet was suspended in suspension medium (1.3 ml/g original cortex), and then successively passed through a 22-gauge needle and a 26-gauge needle. This final fraction was snap frozen in ethanol and dry ice, and then stored at −80°C until use. The purity of the final fraction was monitored by assaying the activities of marker enzymes known to be characteristic of brush-border microvilli (alkaline phosphatase), baso-lateral membranes (Na^+,K^+-ATPase), and endoplasmic reticulum (glucose-6-phosphatase). The specific activity of only alkaline phosphatase was enriched, about 10-fold, relative to in the initial homogenate. Protein concentrations were measured using of the DC Protein Assay (Bio-Rad, Melville, NY, USA), with bovine serum albumin as a standard.

2.5. Extraction of the brush border membrane fraction and subfractionation with Triton X-114

The brush border membrane fraction was vortexed in TN buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, and 100 kallikrein U/ml aprotinin) containing 2 mM EDTA, 1% Triton X-100, 1% Triton X-114 or 45 mM *n*-octyl β-D-glucoside. After centrifugation at 43,000 × *g* for 20 min, the remaining proteins in

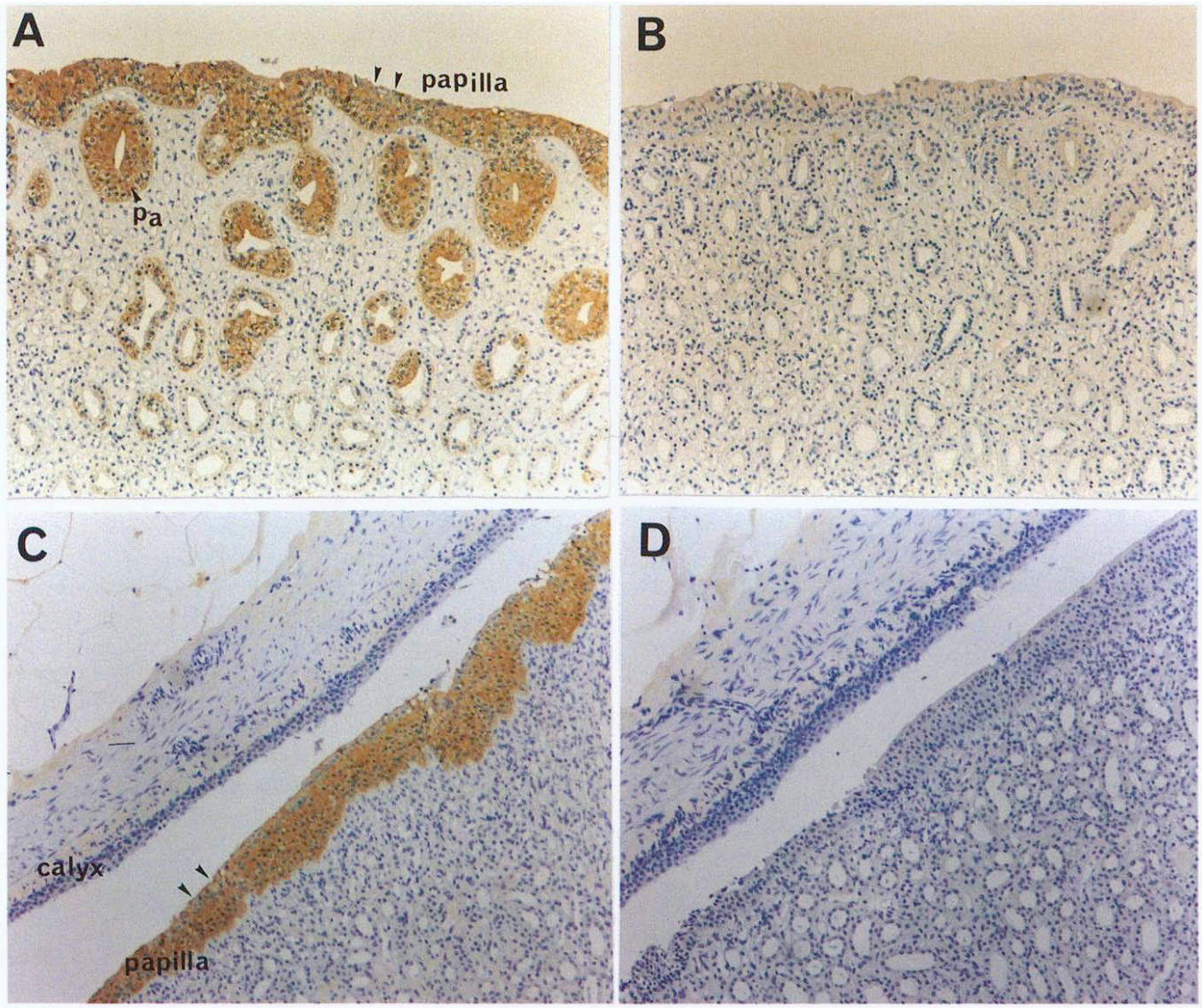

Fig. 2. Immunohistochemical staining in the renal medulla (× 25). A and C were stained with specific polyclonal antibodies to p33/41, and B and D were stained with preimmune normal rabbit IgG. In the renal papilla and calyx, the positive reaction in the cytoplasm of papillary tubules (pa) and the epithelium of renal papilla (papilla) is indicated by arrowheads. The transitional epithelium of renal calyx (calyx) was not stained.

the pellets were solubilized with TN buffer containing 2% (w/v) SDS. Subfractionation of the extracts containing 1% (v/v) Triton X-114 was performed by the following procedure. The extracts were incubated at 30°C for 5 min until the solutions became cloudy, and then were centrifuged at 600 × *g* for 5 min at room temperature to separate the upper (aqueous) and lower phases. The individual upper phases were saved, and equal volumes of buffers containing 1% (v/v) Triton X-114 were added to the lower phases, followed by mixing, incubation at 30°C for 5 min, and then centrifugation as above. The upper phases were discarded, and the lower phases were used as the detergent-rich phases.

3. Results and discussion

3.1. Localization of p33/41 in kidneys

Formalin-fixed and paraffin-embedded sections of three different portions of each of five normal bovine kidneys were stained immunohistochemically using specific polyclonal antibodies to p33/41. Figs. 1 and 2 show the results of immunohistochemistry. In the renal cortex, strong staining was seen in the proximal tubules (Fig. 1A), but the distal tubules were scarcely stained. In the glomeruli and Bowman's capsules, no p33/41 was detected. Examination of urinary tubules at higher resolution is shown in Fig. 1C. A striking feature of the immunostaining of renal proximal tubules is that p33/41 is highly concentrated along the apical domain of the plasma membrane, in which the brush border membrane is well-developed. The staining was observed in neither basal nor lateral membranes. In renal medulla, the epithelial cells of the papillary ducts, the terminal portion of collecting ducts, and the epithelium of renal papilla were stained throughout the cytoplasm (Fig. 2). The transitional epithelium of the renal calyx was not stained. The distribution of p33/41, i.e. the distribution throughout the cytoplasm of the papillary ducts and the epithelium of renal papilla, and the highly polarized localization in the brush border membrane of the proximal tubules, are interesting in view of the cell specificity of expression sites and the possible biological roles of p33/41, suggesting a distinct function of each expression site in vivo.

Our previous studies showed that most partial amino acid sequences determined for p33/41 correspond to those of annexin IV, indicating that p33/41 is identical or closely related to annexin IV. Therefore, we cloned a cDNA for p33/41, which was identical with that for bovine annexin IV except for minor substitution of three nucleotides, and produced recombinant p33/41. Analysis of the immunoreactivity and Ca^{2+}-dependent carbohydrate-binding activity of the recombinant p33/41 demonstrated that p33/41 is identical with annexin IV (in preparation for publication). Annexin IV is mainly localized along the basolateral plasma membrane in highly polarized epithelial cells, intestinal absorbing cells, hepatocytes, and pancreatic acinar cells [15]. However, we showed here that p33/41 is strictly localized in the apical plasma membrane in renal proximal tubules. Further investigation is required to elucidate whether or not the apical sorting of p33/41 is a specific phenomenon occurring only in the epithelial cells of the proximal tubules. The difference in the sorting sites might be due to the functional differences between epithelial cells.

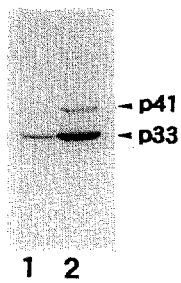

Fig. 3. Detection of p33/41 in a kidney homogenate (lane 1) and renal brush border membrane vesicles (lane 2) on immunoblotting. Extracts were prepared in the presence of 2 mM EDTA and 1% Triton X-100. For SDS-PAGE, 50 μg of proteins was applied to each lane for electrophoresis and electroblotting.

Fig. 4. Sequential extraction from brush border membrane vesicles. Renal brush border membrane vesicles were extracted with or TN buffer (lane 1), TN buffer containing 2 mM EDTA (lane 2), 1% (v/v) Triton X-100 at 4°C (lane 3) or 37°C (lane 4), 1% (v/v) Triton X-114 at 4°C (lane 5) or 37°C (lane 6), or 45 mM *n*-octyl β-D-glucoside (lane 7), and each supernatant (sup.) was saved. The pellets (ppt.) were further extracted with TN buffer containing 2% SDS, and p33/41 was detected by immunoblotting.

3.2. Detection of p33/41 in the renal brush border membrane fraction and its solubility

To further confirm the concentration of p33/41 in the apical plasma membrane, a brush border membrane fraction was prepared from a bovine kidney homogenate and associated p33/41 was detected by immunoblotting. When equivalent amounts of the proteins in the crude

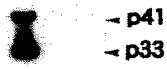

Fig. 5. Partition experiment with Triton X-114. Renal brush border membrane vesicles were extracted with TN buffer containing 2 mM EDTA and 1% Triton X-114, and the extracts were partitioned, and then p33/41 in the aqueous phase (lane 1) and detergent-rich phase (lane 2) was detected by immunoblotting.

extract and brush border membrane fraction were analyzed, p33/41 was found to be enriched in the brush border membrane fraction, as shown in Fig. 3.

Renal brush border membrane-associated p33/41 was sequentially extracted with EDTA, Triton X-100, Triton X-114 and *n*-octyl β-D-glucoside to study its membrane association mode, dependency on calcium ions and detergent solubility. When the brush border membrane-associated p33/41 was partly extracted by chelation with TN buffer containing 2 mM EDTA, a significant amount of p33/41 remained in the membrane pellet (Fig. 4). By use of 1% (v/v) Triton X-100, 1% (v/v) Triton X-114 or 45 mM *n*-octyl β-D-glucoside, p33/41 was completely extracted. When the brush border membrane-associated proteins solubilized with 1% Triton X-114 were partitioned, p33/41 was found in the aqueous phase (Fig. 5). In summary, p33/41 associates with the renal brush border membrane in two modes; a part is Ca^{2+}-dependent, while most of the protein is Ca^{2+}-independent.

Annexins can bind Ca^{2+}-dependently to membranes, and phospholipid vesicles composed of phosphatidylserine, phosphatidylethanolamine, and/or phosphatidylinositol in in vitro assay systems [16]. Direct amino acid sequencing or cDNA sequencing showed that annexins, including annexin IV, have no hydrophobic membrane-spanning region in their primary structure. These proteins were considered to be located and concentrated on the surfaces of membranes according to the concentration of Ca^{2+}. Hence, it is plausible that a decrease in the level of Ca^{2+} results in the release of annexins from functional sites on membranes. However, many annexins actually exhibit two modes of membrane association, Ca^{2+}-dependent and Ca^{2+}-independent, as supported by several reports [17,18]. Similar results were obtained for p33/41 in this investigation. It is important to elucidate the mechanism by which annexins are associated with the plasma membrane and integrated into it.

Since annexin amino acid sequences deduced from cDNA lack a hydrophobic signal peptide, these proteins have been considered not to be secreted from cells nor expressed on the cell surface. Unexpectedly, recent studies demonstrated that annexins are present extracellularly, not due to cell decomposition [19], but due to expression on the external surfaces of human and rodent tumor cells as cell adhesive molecules [20]. Whether p33/41 is located on the outside of the brush border membrane or not is the next problem to be solved.

3.3. Possible interaction of p33/41 with carbohydrate ligands in the kidney

Other questions to be answered are, which kind of carbohydrate ligands p33/41 has and what biological functions it has concerning their specific binding. Some glycoproteins located in the kidneys possibly to interact with p33/41.

Tamm–Horsfall glycoprotein (THG) and podocalyxin are sialoglycoproteins which are produced and expressed specifically in the kidneys. Our unpublished data showed that THG is a weak inhibitor of p33/41 in an in vitro assay system. However, the expression site of THG is the thick ascending limb of Henle's loop [21], where p33/41 is scarcely present. Podocalyxin is mainly present in the glomerular capillary wall and forms the permselective barrier in coordination with heparan sulfate in the glomerular basement membrane [22]. Since these results show that they never coexist histochemically, the possibility of the interaction of p33/41 with these glycoconjugates seems to be low. However, it cannot be completely ruled out, e.g. it is possible when one of them is released from its site through unknown mechanisms. In renal proximal tubules, several reports showed that membrane glycoproteins gp330 and gp160 are localized in the brush border membrane [23,24]. Since their carbohydrate structures have not yet been determined, the possibility of specific interaction between p33/41 and these glycoproteins is unclear. Whether or not p33/41 interacts with glycoproteins on the renal brush border membrane, such as gp330 and/or gp160, through carbohydrate specific affinity is an interesting question to be answered.

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