Identification and Localization of Pertussis Toxin-Sensitive GTP-Binding Proteins in Bovine Kidney Glomeruli

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ABSTRACT
The vascular tree and the mesangium in the glomerulus respond to various hormones, growth factors, and autonomic signals, leading to generation of second messengers and regulation of ion channels. Guanine nucleotide regulatory proteins (G proteins) mediate these effects in other systems. Glomerular G proteins were studied by immunoblotting and immunohistochemical techniques. Glomeruli were isolated from bovine kidney cortex by differential sieving. Glomerular proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and nitrocellulose transfers were immunoblotted with antibodies to G proteins. Gα, common antisera (P-960) recognized proteins with a molecular mass of 41 to 45 kDa. Antibodies against peptide sequences specific to Gα1 and Gαi demonstrated Gα1,2 (molecular mass, 39 to 41 kDa), Gα2 (molecular mass, 40 kDa), and Gαi (molecular mass, 39 kDa). Presence of these proteins was further confirmed by pertussis toxin-catalyzed ADP ribosylation of protein(s) with a molecular mass of 39 to 41 kDa in the glomeruli. Immunohistochemical staining of frozen sections from bovine kidney cortex revealed the presence of Gα1,2 in capillary loop distribution in glomeruli and interstitium, but Gα1,3 or Gαi could not be demonstrated. The pattern of immunofluorescence with Gα2 antisera suggested localization of Gα1,2 to the endothelium in glomerular and interstitial vasculature. The novel finding of Gαi in glomeruli requires localization of Gαi to specific cells and determination of its role in glomerular physiology.

In conclusion, these studies demonstrate that bovine kidney glomeruli express α subunits of pertussis toxin-sensitive GTP-binding proteins Gα1,2, Gα2, and Gαi. Gα1,2 is localized to the vascular endothelium in glomeruli and interstitium, but Gα1,3 could not be localized. Further work is required to determine the role of these proteins in mediating effects of neuronal stimuli, hormones, and cytokines on glomerular blood flow.

Key Words: Immunoblotting, immunohistochemistry, ADP ribosylation, endothelium, Gαi

The glomerulus is a dynamic component of the nephron, actively involved in the filtration process, regulated by endocrine, paracrine, and autonomic neuronal stimuli. The translation of these stimuli into their ultimate effects is a complex process requiring multiple steps for signal transduction. Heterotrimeric guanine nucleotide regulatory proteins (G proteins), consisting of α, β, and γ subunits, are sequentially involved at several steps of hormone action and signal transduction. These include three pertussis toxin (PTX)-sensitive inhibitory G proteins (Gα1, Gα2, and Gα3), which are distinguished by unique α subunits (1–3). A survey of the literature revealed that research on GTP-binding proteins present in the glomerulus has been confined to its mesangial component. It has been suggested that Gα1,2 is the major PTX-sensitive G protein in cultured rat mesangial cells and couples angiotensin II and platelet-activating factor receptors to phospholipase C or A2 (4) and also lipopolysaccharide receptors to phospholipase A2 (5). However, G proteins associated with other types of cells in the glomerulus, i.e., epithelial cells and endothelial cells, have not been examined. Also, in vitro studies on cultured mesangial cells may not truly reflect the properties of mesangial cells in vivo. Our interest in signal transduction events and physiological processes mediated by PTX-sensitive G proteins and the convenience of studying these processes by using PTX to uncouple the receptor from the G proteins have led us to examine the PTX-sensitive G proteins present in whole glomeruli. The α subunits of G proteins have been identified by immunoblotting glomerular proteins with specific antibodies, and these subunits have been localized to various cellular components of the glomerulus by immunohistochemical techniques.
We have found that bovine kidney glomeruli have PTX-sensitive G protein α subunits G\(_{\alpha12}\), G\(_{\alpha2}\), and G\(_{\alpha1\alpha2}\) was demonstrated by indirect immunofluorescence to be localized to a capillary distribution in the glomerulus and the interstitium, suggesting that it is associated with the endothelium. However, G\(_{\alpha11}\) could not be localized topographically in the glomerulus.

**METHODS**

Bovine kidneys provided immediately upon slaughter were packed in ice and transported to the laboratory within 20 min. Cortical sections were excised, weighed, and mixed with an equivalent weight of buffer S (0.25 M sucrose, 10 mM Tris (pH 7.5), 1 mM EDTA).

**Isolation of Bovine Kidney Glomeruli**

Glomeruli were isolated by a slight modification of the method of Schreiner et al. (6). Bovine kidney cortical slices were dissected on ice, and glomeruli were obtained by slices of renal cortex being pressed through three consecutive sieves (mesh size, 250, 150, and 75 μm). Glomeruli collected on the 150-μm-mesh-size sieve. This sieve was washed three times with phosphate-buffered saline to wash away any debris or tubule fragments, and glomeruli were suspended in buffer A. Purity of the glomeruli was assessed by light microscopy. Preparations were found to contain more than 95% glomeruli. Glomeruli were homogenized in a Potter-Elvehjem homogenizer with five strokes. The glomerular homogenate was diluted with buffer S to a protein concentration of 5 mg/mL, divided into samples, and frozen at -70°C for future use.

**Immunoblotting**

For western blotting, glomerular proteins were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were transferred to nitrocellulose in a solution containing 25 mM Tris, 0.19 M glycine, and 20% methanol. Blots were blocked with buffer A consisting of 5% nonfat dry milk, 0.2% Nonidet P-40, 0.02% Na\(_2\)PO\(_4\), 50 mM Tris-HCl (pH 8), 2 mM CaCl\(_2\), and 80 mM NaCl. A 1-h incubation with antipeptide antiserum in buffer A supplemented with 2% Nonidet P-40 and 0.2% SDS (high-detergent buffer A) was followed by three washes with high-detergent buffer A. Antibody binding was detected by incubation of the blots with goat antibody to rabbit immunoglobulin G (IgG) labeled with \(^{125}\)I (DuPont, NEN Research Products, Boston, MA) at 3.5 × 10\(^5\) cpm/mL. After the blots were washed and dried, the autoradiographic images of the blots were obtained with Kodak XAR-5 film after exposure with an intensifying screen.

**PTX ADP Ribosylation**

G-protein substrates of PTX were assayed by using PTX-catalyzed incorporation of \(^{32}\)P]ADP-ribose from \(^{32}\)P]NAD (7). Glomerular homogenate (100 μg of protein) was centrifuged (15,000 × g for 5 min) and resuspended in 200 μL of incubation buffer containing 100 mM Tris-Cl (pH 8), 6 mM MgCl\(_2\), 2 mM GTP, 10 mM thymidine, and 2.5 mM ATP, 10 μM \(^{32}\)P]NAD (10 Ci/mmol). PTX was activated by incubating in 10 mM dithiothreitol for 30 min at room temperature. Toxin or control buffer was added to the suspensions of glomerular homogenate, and the reaction was continued for 90 min at 37°C.

The ADP ribosylation reaction was stopped by addition of 1 mL of ice-cold trichloroacetic acid and centrifugation at 15,000 × g for 5 min. The pellet was washed with ice-cold ether five times and then solubilized in NaDodSO\(_4\) sample buffer. Proteins were separated by electrophoresis in the presence of 0.1% SDS on 12.5% polyacrylamide slab gels with 4% stacking gels as described by Laemmli (8). Gels were stained with Coomassie brilliant blue, dried under vacuum, and exposed to Kodak XAR-5 film for 1 to 2 days at -80°C without intensifying screen, and autoradiograms were developed.

**Immunohistochemistry**

Cortical sections (1 mm thick) from kidneys of freshly slaughtered cows were transported to the laboratory in B-5 (0.22 M HgCl\(_2\), 90 mM sodium acetate). Tissue blocks were prepared in O.C.T. compound 4583 (Miles Inc., Diagnostic Division, Elkhart, IN) on dry ice and stored at -70°C. Sections were mounted on glass slides immediately before being immunostained and placed in 100% methanol at -20°C for 3 min. Sections were subsequently washed with PBS and incubated with blocking solution (1% polyethylene glycol [mol wt, 20,000] and 20% goat serum in PBS) for 45 min. Sections were then incubated with appropriate dilutions of primary antibody for 1 h at room temperature. Sections were washed in PBS for 15 min and then incubated for 20 min with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC). Sections were again washed in PBS for 15 min and mounted in freshly prepared 50% glycerol in PBS and para-phenylene diamine (2 mg/mL). Sections were visualized with a confocal microscope (Carl Zeiss Inc., Thornwood, NY) and photographed. Incubation of antiserum with excess peptide was able to block staining, thus verifying specificity.

**Materials**

PTX was obtained from Sigma Chemical Company (St. Louis, MO) and \(^{32}\)P]NAD was obtained from Du Pont, NEN Research Products. The antibodies and respective peptides were a generous gift of Dr. Suz...
Amino acid sequence is given in one-letter code. Amino-terminal cysteine of each peptide was used to couple the peptide to hemocyanin and is not part of the G protein sequence.

Disclosure

anne Mumby and Dr. Janet Robishaw. All other chemicals were of the highest purity available. FITC-labeled goat anti-rabbit IgG was obtained from Fisher Bio sciences, Inc. (Plano, TX).

RESULTS

Immunoblotting with Antibodies to G Proteins

The antisera used in this study were raised in rabbits by immunizing the animals with peptide sequences specific for different G proteins (Table 1). Antiserum P-960 (9) was raised against the Gα.common peptide sequence found in Gαa, Ta, and Gαb (but not Gαi); it is similar to antiserum A 569 (10) and was a generous gift from Dr. S. Mumby. Antiserum for Gαa (U46) was also provided by Dr. S. Mumby (10). Antisera for Gαc (584), Gαa1/3 (A56), and Gαa2 (A54) were generously provided by Dr. J. Robishaw and have been described previously (11). The specificity of antisera 584, U46, A56, and A54 has been previously determined by immunoblotting with recombinant Gα subunits expressed in Escherichia coli. The Gαa39 antibody (U46) recognizes only Gαa39 and Gαa2 antibody (A-54) detects only Gαa2. However, Gαa2 antibody (A-56) detects both Gαa1 and Gαa3 because of the close amino acid identity between Gαa1 and Gαa3 (11). Therefore, the protein(s) recognized by A-56 on immunoblots will be referred to as Gαa1/3. The antisera were incubated overnight at 4°C with the specific peptide or control buffer before being immunoblotted to verify specificity.

Immunoblotting of bovine glomerular proteins on nitrocellulose transfers was done as described in Methods with the Gα.common antiserum, P960. After incubation with 125I-labeled secondary goat anti-rabbit IgG antibody, Western blots were dried and autoradiograms were developed after 24 h. Protein(s) (39 to 41 kDa) were labeled in the glomeruli (Figure 1, lane B), and binding of Gα.common antibody to this protein(s) was prevented by overnight preincubation of the antisera with the Gα.common peptide (Figure 1, lane A). When the primary antibody was omitted, no band was seen (data not shown). The results demonstrate the presence of G proteins with a molecular mass 39 to 41 kDa in the glomeruli.

The known Gα proteins found in tissues other than retina with molecular masses of 39 to 41 kDa are Gαa1 (41 kDa), Gαa2 (40 kDa), Gαa3 (41 kDa), Gαa (39 kDa), and Gαs (41 kDa). All of the above Gα subunits, except Gαs, are substrates for ADP ribosylation by PTX. Having identified the G proteins with molecular masses 39 to 41 kDa in the glomeruli, we used PTX ADP ribosylation and immunoblotting with antibodies to different Gα subunits and Gαa to further confirm and establish the identity of these G proteins. Antiserum A54 (recognizes Gαa2) identified a ~40-kDa protein (Figure 1, lane H). Antiserum A56 (recognizes both Gαa1 and Gαa3) identified a broad band with a molecular mass of ~41 kDa, suggesting the presence of Gαa1, Gαa3, or both (Figure 1, lane F). Antiserum A56 also recognized a protein with a molecular mass of 36 kDa. The identity of this protein is not clear. It

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a Amino acid sequence is given in one-letter code. Amino-terminal cysteine of each peptide was used to couple the peptide to hemocyanin and is not part of the G protein sequence.
b Reference 9.
c Reference 11.
d Reference 10.

Figure 1. Immunoblotting of glomerular proteins with antisera to different Gα subunits. Bovine kidney glomerular homogenate (50 µg) was applied to each lane of a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Different antisera (2 µL) were incubated overnight at 4°C with 10 µL of specific peptides (100 µg/mL) or control buffer. Dilutions (500x) of the above were used for Western blotting (except for Gα.common antisem which was used in 1,000x dilution). 125I-labeled goat anti-rabbit IgG was used as secondary antibody. Western blots were dried, and autoradiograms were developed after 24 h. Ab, antibody.
may be a proteolytic product of \( G_{i,1,0} \) or the 36-kDa protein could represent another \( \alpha \) subunit of the \( G \) proteins that is closely related to \( G_{i,1,0} \). However, antibody P-960 made to a region common to known \( \alpha \) subunits (except \( G_i \)) did not recognize this protein. Surprisingly, \( G_{i,1} \) could be identified (molecular mass, 39 kDa) in bovine kidney glomeruli by using antiserum U46 which is raised against a peptide sequence specific for \( G_{i,1} \) (Figure 1, lane J). The above results establish the presence of \( G_{i,2}, G_{i,1,0}, \) and \( G_{i,1} \) in bovine kidney glomeruli. Immunoblotting of \( G_i \) subunits present in glomeruli by various antibodies was blocked by overnight incubation of the antibodies with the specific peptides (lanes A, C, E, G, and I).

The presence of inhibitory \( G \) protein (\( G_i \)) led us to look for the stimulatory \( G \) protein (\( G_s \)). Antisera for \( G_{i,1} \) (7) identified two different \( G_{i,1} \) subunits with molecular masses of 45 and 52 kDa (lane D), even though the \( G_{i,1,0} \) antiserum (P-960) failed to identify any proteins with molecular masses >41 kDa. However, \( G_{i,1,0} \) antiserum has less affinity for \( G_{i,1} \) compared with \( G_{i,1,0} \) or \( G_{i,2} \) (10).

PTX ADP Ribosylation

To further confirm the presence of known PTX substrates, \( G_{i,2} \) and \( G_{i,1,0} \), in bovine kidney glomeruli, we performed ADP ribosylation of glomerular proteins in the presence and absence of PTX. Protein(s) with molecular masses of 39 to 41 kDa were labeled with \( ^{32}P \)ADP, only in the presence of PTX (Figure 2). This further confirmed the presence of PTX-sensitive \( G \) proteins, identified by immunoblotting, in bovine kidney glomeruli. Only a single band of ADP ribosylated protein(s) was visualized, even though at least three PTX substrates (\( G_{i,2}, G_{i,1,0}, \) and \( G_{i,1,0} \)) were identified by immunoblotting. However, it has been previously reported that PTX-catalyzed ADP ribosylation and one-dimensional SDS-polyacrylamide gel electrophoresis may not discriminate several structurally, and possibly functionally, distinct \( G \) proteins (12).

Immunohistochemistry

We attempted to localize the various PTX-sensitive \( G \) proteins to specific cells present in the glomerulus by indirect immunofluorescence technique with the various antibodies as described in Methods. Preliminary experiments showed that the immunostaining was absent when paraffin sections were used. This was thought to be due, possibly, to the use of paraformaldehyde as a fixative. Therefore, 4-\( \mu \)m frozen sections of superficial layers from bovine kidney cortex were used for further studies. Immunostaining was performed with antibodies against \( G_{i,2}, G_{i,1,0}, \) \( G_{i,1} \), and \( G_{i,1,0} \). Only the antibody to \( G_{i,2} \) (A54)
Immunostained the glomeruli. Immunofluorescence was observed in a capillary distribution throughout the glomerulus (Figure 3B) as shown by linear fluorescence of capillary loops. Some capillaries do not show circular immunofluorescence, as these have been sectioned tangentially. All of the glomeruli present in the section showed similar immunostaining. Immunofluorescence extended out from the glomeruli into the interstitium where capillaries show linear immunofluorescence (Figure 3C). Immunohistochemical staining of the glomerular and interstitial components was absent when antiserum A54 was incubated overnight with excess G1a2 peptide before being immunostained, thus verifying specificity (Figure 3A). A more detailed view of the interstitium is seen in Figure 4A, showing immunofluorescence of circular capillary loops. Luminal surfaces of small arteries and arterioles also showed immunofluorescence (Figure 4B). The pattern of immunofluorescence in the glomeruli and the interstitium suggests localization of G1a2 to the endothelium.

DISCUSSION

The critical role of heterotrimeric G proteins as intermediaries in signal transduction has been established for a variety of effector systems, including adenylyl cyclase and ion channel regulation (see References 1, 13, and 14 for reviews). It is now evident from molecular cloning of the subunits of G proteins that they are each distinct members of a large supergene family (3). All subunits are susceptible to ADP ribosylation by cholera and/or PTX, except Gs (15). Gs and G1a3 are ADP ribosylated exclusively by PTX, a process that uncouples the receptor from these subunits and thereby provides a method for studying the function regulated by these G proteins when activated. All G proteins are known to regulate adenylyl cyclase, but specific subunits may be involved in the regulation of phospholipase C and ion channels. Also, the different subunits may have distinct cellular locations consistent with their diverse functional roles. For instance, in the renal epithelial cell line LLC-PK, G1a2 is localized to the basolateral membrane and regulates the adenylyl cyclase system; G1a3 is present in the apical membrane and regulates the activity of Na+ channels (16). Gs, on the other hand, is localized primarily to the brain and also to some endocrine cells and mediates hormone-induced inhibition of Ca2+ channels (17). As to the kidney, glomerular G proteins have only been studied in rat glomerular mesangial cells. These cells contain G1a2 which regulates phospholipase C and A2 in vitro (4). We have examined PTX-sensitive G proteins present in the bovine kidney glomeruli as a whole. Multiple subtypes of Gn have been previously demonstrated in bovine kidney cortex (18) by immunoblotting. We have extended these observations by studying the G proteins localized to the glomeruli in bovine kidney cortex. In addition to finding multiple Gn subtypes, we have also detected Gs in bovine glomeruli. Immunofluorescence pattern suggests that G1a2 is localized primarily to the endothelial cells in the glomeruli and vessels of the interstitium. However, the other G proteins could not be localized to specific glomerular structures. Reasons for the failure of various antibodies to detect Gn subunits (other than G1a2) with immunohistochemistry are not clear. Failure to demonstrate Gs may be because of its low abundance in the kidney glomeruli. However, this explanation does...
not seem likely for $G_{10}$ or $G_{10,1/2}$ (Figure 1). Sequestration of the antigens should not be a factor, as 4-μm frozen sections would be expected to slice the cells open, thus exposing the antigens located on the cytoplasmic side of the cell to the antibodies. The possibility that the epitopes are not exposed unless the proteins are denatured (as by SDS) cannot be ruled out. It is also possible that the antibodies used in this study (except $G_{10a2}$) are good for immunoblotting but not for immunohistochemistry. Higher concentrations of the antisera (up to 100× dilution) were equally unsuccessful, suggesting that adequacy of antibody titer is unlikely to be a determinant. Our findings, however, do not rule out the presence of $G_{10a2}$ in other glomerular cells, e.g., mesangial cells or epithelial cells. Failure to localize $G_{10a2}$ to mesangial cells by immunohistochemistry may also be because of low abundance of $G_{10a2}$ in other glomerular cells, e.g., mesangial cells or epithelial cells. Failure to localize $G_{10a2}$ to mesangial cells by immunohistochemistry may also be because of low abundance of $G_{10a2}$ in mesangial cells, compared with endothelial cells. On the other hand, bovine mesangial cells in situ may differ from rat mesangial cells in vitro (4), as regards expression of $G_{10a2}$.

Lee et al. have shown that human endothelial cells express mRNA for all three $G_{10}$ subtypes, although the level of $G_{10a1}$ mRNA is very low (19). These workers have also shown that interleukin-1 beta, an inflammatory mediator which stimulates a complex series of responses in endothelial cells leading to increased coagulation and platelet adhesion, increases expression of only one subtype of $G_{10}$ ($G_{10a2}$) mRNA in human endothelial cells. In view of the above, it is conceivable that $G_{10a2}$ present in glomerular endothelium may mediate glomerular inflammation in response to cytokines, such as interleukins.

PTX-sensitive G proteins present in endothelial monolayers have also been shown to mediate signal transduction in response to inflammatory mediators like histamine (20) and tumor necrosis factor (TNF) (21). Interestingly, these workers found that TNF induced increased passage of solutes, and cell shape/cytoskeletal perturbation was sensitive to PTX. However, TNF-induced modulation of endothelial cell coagulant properties was not altered by PTX. The identity of the PTX-sensitive G protein(s) that mediate some, but not all, of the effects of TNF is not known (21). The involvement of $G_{10a2}$ present in glomerular and interstitial vascular endothelium in inflammatory processes affecting the kidney remains to be studied.

We have also demonstrated that bovine glomeruli, like most other tissues, contain two species of $G_{10}$ protein with molecular masses of 45 and 52 kDa (22). These two forms are encoded by mRNA generated by alternative splicing of exon 3: inclusion of this exon results in the insertion of 15-amino-acid residues (23). Further diversity results from alternative splicing at exon 4, which allows for the generation of two short and two long forms of $G_{10}$ that differ only by the addition of a serine residue (24). The relative amounts of the two $M_r$ species may vary depending on the tissue examined and stage of development (11). Both the 45- and 52-kDa forms of $G_{10}$ stimulate adenyl cyclase, although the two forms show differences in their rates of GDP dissociation (25). Both forms of $G_{10}$ can also directly activate cardiac calcium channels (26).

Recently, it has been reported that there are two $a_0$ variants that result as a consequence of alternative splicing of a single $a_0$ transcript (27). Both $a_{01}$ and $a_{02}$ are substrates for ADP ribosylation by PTX, and both are found in brain (27). The $a_{02}$ sequence with 354 amino acids (like the previously described $a_{01}$ sequence) is identical to $a_{01}$, up to and including amino acid 248, and differs thereafter in 26 amino acids. Antibody U-46 is made against a peptide sequence common to both $a_{01}$ and $a_{02}$ (amino acids 22 through 35). Therefore, the protein detected in bovine glomeruli by $a_0$ antibody U-46 may be $a_{01}$ or $a_{02}$, or both. In this regard, it should be noted that Murakami and co-workers (18) were unable to detect $G_{10}$ in bovine kidney cortex. However, they used a different antibody which reacted with carboxy-terminal sequence of $G_{10a}$.

The finding of $G_{10}$ in bovine kidney glomeruli raises speculations about its functional role. It has been shown in reconstitution experiments that the inhibition of $Ca^{2+}$ channels by enkephalin in neuroblastoma-glioma hybrid cells (17), or by neuropeptide Y in rat dorsal root ganglion neurons (28), is mediated by $G_{10}$. Also, dopamine-induced inhibition of $Ca^{2+}$ current in HeLa neurons (29), and noradrenaline-induced inhibition of $Ca^{2+}$ current in NG 108-15 hybrid cells (30) are blocked by antibodies to $G_{10}$, but not those to $G_{10}$, Taken together, these data from other systems implicate a role for $G_{10}$ in coupling neurotransmitter receptors to alterations in voltage-dependent $Ca^{2+}$ channel function. Moreover, $G_{10}$ has been recently shown to be directly involved in regulation of phospholipase C (31). Therefore, the novel finding of $G_{10}$ in glomeruli requires localization of $G_{10}$ to specific cells and determination of its role in glomerular physiology. In conclusion, these studies demonstrate that bovine kidney glomeruli express $a_0$ subunits of PTX-sensitive GTP-binding proteins $G_{10,1/2}$, $G_{10}$ and $G_{0}$. By immunohistochemical techniques, $G_{10a2}$ was localized to the vascular endothelium in glomeruli and interstitium, but $G_{10a1}$ or $G_{10w}$ could not be localized. Further work is required to determine the role of these proteins in mediating effects of neuronal stimuli, hormones, and cytokines on glomerular blood flow and glomerular inflammation.
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