Proximal tubular epithelial cells possess a novel 42-kilodalton guanine nucleotide-binding regulatory protein

(adenylate cyclase/ADP-ribosylation/immunoblotting)

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ABSTRACT   The proximal tubule of the kidney represents an important location where adenylate cyclase regulates salt and water transport; yet a detailed characterization of the distribution and classification of guanine nucleotide-binding protein (G protein) and adenylate cyclase is lacking. We used purified brush border (20-fold) and basolateral membranes (14-fold) to characterize parathyroid hormone- and G protein-regulated adenylate cyclase and G-protein distribution. Adenylate cyclase was predominantly localized to basolateral membranes, while the 46-kDa α subunit of the stimulatory G protein (G_s) was 2-fold higher in brush border membranes than in basolateral membranes. The α subunit of the inhibitory G protein (G_i; 41 kDa) was equally distributed on immunoblotting but was 2-fold higher in brush border membranes than in basolateral membranes on radiolabeling with pertussis toxin. A 42-kDa cholera toxin substrate that cross-reacted with antisera to the common α subunit of G proteins and to G_s on immunoblotting and that was not immunoprecipitated with two G_i antisera was the most abundant α subunit and comprised $\approx 1\%$ of the total membrane proteins. These observations suggest that G proteins are important regulators of proximal tubular transport independent of adenylate cyclase.

Guanine nucleotide-binding regulatory proteins (G proteins) are a family of proteins that transduce signals between a variety of cell surface receptors and effectors (1). In most tissues there is a close association between the distribution of the α subunit of the heterotrimeric G proteins (G_s and G_i, the stimulatory and inhibitory G proteins) and hormonal stimulation and inhibition of adenylate cyclase. Hormonal modulation of cAMP through interactions with G proteins represents an important regulator of kidney epithelial water and electrolyte transport (2–4). As examples, vasopressin and parathyroid hormone stimulate cAMP and angiotensin inhibits cAMP, thereby influencing transport responses (2–5). In addition, ion-transporting epithelial cells represent an important potential site for regulation of ion transport directly by G proteins, as they have been described to regulate channel activity (calcium and potassium) independent of adenylate cyclase in several tissues (1, 6, 7). G proteins have also been implicated in the regulation of phospholipase C and Na^+/K^+-ATPase activity in kidney epithelial cells (8–10).

Recent observations in gastrointestinal epithelium suggest that adenylate cyclase may be asymmetrically distributed with the α subunit of G_s ($G_{s\alpha}$; cholera toxin substrate), which regulates ligand-mediated adenylate cyclase stimulation, on apical and basolateral membranes (11–13). Adenylate cyclase was exclusively localized to basolateral membranes, whereas cholera toxin substrate was predominantly on the apical

surface of the epithelial cell. The kidney proximal tubule represents a site that transports 75–80% of salt and water load, yet a detailed characterization of G proteins and adenylate cyclase distribution in this tissue is lacking. Therefore, the present report provides an in-depth characterization of the distribution of G proteins and G protein-regulated adenylate cyclase activity of purified apical and basolateral membranes of the proximal tubule. We demonstrate that the predominant G protein of apical and basolateral membranes is $G_{s\alpha}$, a 42-kDa cholera toxin substrate. In apical membranes this G protein and G_i were associated with minimal adenylate cyclase activity, suggesting an important role for these proteins in direct regulation of ion transport or other functions.

MATERIALS AND METHODS

Materials. [^{32}P]NAD$^+$ (10–50 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear, and guanosine 5'-[γ-thio]triphosphate (GTP[γ-S]) was from Boehringer Mannheim. Pertussis toxin was obtained from List Biological Laboratory, and ATP, phosphocreatine, creatine phosphokinase, and cholera toxin A subunit and other chemicals and hormones were obtained from Sigma Chemical Co. Antisera to $G_{s\alpha}$ ("2347"), $G_{\alpha \text{ common}}$ [an identical amino acid sequence found in α subunits of G_s, G_i (1,2,3), G_o ("other" G protein; an inhibitory modulator of neuronal Ca^{2+} channels and an activator of K^+ channels), and transducin (T; a G protein coupling light-activated rhodopsin to the activation of retinal cGMP phosphodiesterase)] ("1398"), and G_i ("1521" and "8730") were provided by David R. Manning (Department of Pharmacology, University of Pennsylvania, Philadelphia; ref. 14). $G_{s\alpha}$ standard (45 kDa, purified from turkey erythrocytes) was provided by Robert Graham (Cleveland Clinic Foundation, Cleveland, Ohio).

Preparation of Membranes and Marker Enzyme Assays. Male New Zealand White rabbits (1.5–2 kg) were employed for preparation of brush border and basolateral membranes as described (ref. 16; R. Singh, J.D. and U.H., unpublished results). Purified membranes were stored in aliquots in 20% (vol/vol) glycerol at −70°C. Adenylate cyclase activity was assessed by measuring the formation of cyclic AMP by radioimmunoassay after acetylation (17). Leucine aminopeptidase was determined by using L-leucine-p-nitroanilide as substrate in 0.2 M phosphate buffer (pH 7.1) as described by Szasz (18). Maltase was determined by the method of Berger and Sacktor (19). Na^+/K^+-ATPase was assayed by its K^+-

Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; G_s and G_i, stimulatory and inhibitory G proteins; $G_{s\alpha}$ and $G_{i\alpha}$, α subunits of G_s and G_i that regulate ligand-mediated adenylate cyclase stimulation and inhibition, respectively; G_o, "other" G protein; T, transducin; $G_{\alpha \text{ common}}$, sequence common to α subunits of G_s, G_i, G_o, and T; GTP[γ-S], guanosine 5'-[γ-thio]triphosphate; PTH, parathyroid hormone.
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dependent hydrolysis of *p*-nitrophenyl phosphate as described by Murer *et al.* (20). Protein concentration was determined by the method of Lowry *et al.* (21).

ADP-Ribosylation of Membranes. ADP-ribosylation was carried out as described by Bokoch *et al.* (22). Membranes (25–50 μg) were incubated in a medium containing 1 mM ATP, 100 μM GTP[γ-S], 5 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 mM thymidine, 10 μM [^{32}P]NAD$^+$ (7–13 Ci/mmol), and 100 mM potassium phosphate (pH 7.6) with or without cholera toxin A subunit (20 μg/ml) in a final volume of 0.1 ml. A similar reaction mixture was used for incubation with activated pertussis toxin (20 μg/ml), except that 100 μM GTP and 100 mM Tris·HCl (pH 8.0) were substituted for GTP[γ-S] and potassium phosphate, respectively. The reaction was carried out at 30°C for 60 min and stopped by addition of 4× Laemmli sample buffer (23) (4% NaDodSO$_4$/ 4% 2-mercaptoethanol/40% glycerol/0.04% bromophenol blue/80 mM Tris·HCl, pH 6.8). After boiling at 95°C for 5 min, the membranes were analyzed by NaDodSO$_4$/PAGE on 11% polyacrylamide gels by the method of Laemmli (23). The samples were stained with Coomassie blue, destained, and dried. Radioactivity on the dried gels was detected by autoradiography using Kodak X-Omat film. The quantity of [^{32}P]ADP-ribose incorporated into membrane proteins was estimated by analyzing the autoradiograms via densitometry. Absorbance signals corresponding to radiolabeled proteins on autoradiograms were converted to arbitrary units through the use of chart recording and automatic digital printout of integrated values.

Blotting and Immunostaining. Membranes (50–300 μg of protein) or purified G$_{s\alpha}$ subunits were transferred from gel to nitrocellulose paper with constant current (50 mA) for 2 hr in a Bio-Rad Trans-Blot apparatus. Immunostaining was performed basically by the method of Woolkalis *et al.* (24). After transfer, the paper was incubated in PBS (25 mM Na$_2$HPO$_4$, pH 7.4/150 mM NaCl) with 3% bovine serum albumin to block nonspecific protein binding. The paper was then incubated with a 1:100 dilution of antiserum in 0.1% bovine serum albumin in PBS for 2 hr at room temperature. After being washed in PBS with 0.1% Tween-20, the paper was incubated with a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase for 1 hr at room temperature. After repeated washings, the nitrocellulose paper was stained in PBS containing 0.6 mg of 4-chloro-naphthol per ml and 0.015% H$_2$O$_2$ for 10–20 min at room temperature, and the reaction was terminated by washing in distilled water. The intensity of staining was quantitated by laser densitometry.

Immunoprecipitation. G proteins were immunoprecipitated by a modification of the procedure of Carlson *et al.* (14). Membranes (100–150 μg) were [^{32}P]ADP-ribosylated as described (except in a final reaction volume of 0.05 ml), and the reaction was terminated with 17 μl of 1 M sodium phosphate (pH 8.0) containing 10% NaDodSO$_4$, 1 M dithiothreitol, and 100 mM EDTA (5× denaturing buffer). Immediately after, 17 μl of denaturing inhibitor cocktail was added to yield final

concentrations of 5 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 200 μg of leupeptin. Samples were then boiled for 3 min and diluted with a concentrated stock of solubilization buffer to yield 50 mM sodium phosphate (pH 7.2) containing 1% sodium deoxycholate, 1% Triton X-100, 0.15 M NaCl, 0.5% NaDodSO$_4$, 2 mM EDTA, 1% aprotinin, and 1 mM dithiothreitol. This was followed by the addition of a 7× stock inhibitor cocktail containing 5 mM NaF, 2 mM Na$_4$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, and 200 μg of leupeptin per ml in a final volume of 0.139 ml. After incubation at 4°C for 30 min, antiserum (or preimmune serum) was added at dilutions of 1:8 for 1521 and 1:20 for 1398 and 8730. Incubations continued overnight at 4°C and were followed by the addition of 200 μl of 20% protein A-Sepharose (preincubated with 4 mg of bovine serum albumin per ml for 30 min). After a 2-hr incubation, samples were centrifuged, and precipitates were washed three times with 50 mM sodium phosphate (pH 8.0) containing 0.15 M NaCl, 0.5% Triton X-100, and 2 mM EDTA. The precipitates were then diluted with 4× Laemmli sample buffer and boiled for 5 min; the products were analyzed by NaDodSo$_4$/PAGE and autoradiography. Supernatants saved from the initial centrifugation were also processed for the purpose of determining the percentage of total radiolabeled G protein precipitated by antisera.

RESULTS

Membrane Characterization. The procedures for the preparation of brush border and basolateral membranes are very different so that different cortical homogenates served as sources for the isolated membranes. The purity of brush border and basolateral membranes was assessed by determining enrichment of the marker enzyme leucine aminopeptidase and maltase (brush border) and K-stimulated phosphatase (basolateral). The specific activity of brush border membranes for leucine aminopeptidase as compared with homogenate was 20-fold higher (Table 1). The specific activity of basolateral membrane for K-stimulated phosphatase was 14-fold enriched over the homogenate. There was no enrichment of maltase in basolateral membranes.

Adenylate Cyclase. Basal adenylate cyclase activity was 3.5-fold higher in basolateral (23 ± 2, fmol·min^{-1}·μg^{-1}, n = 6) than brush border membranes (7 ± 2, fmol·min^{-1}·μg^{-1}, n = 6). Adenylate cyclase activity was linear with protein concentration (3–50 μg) and time (3–30 min) in both membranes (data not shown). To further assess the distribution of adenylate cyclase, receptor activation with parathyroid hormone (PTH), direct activation of the G protein with AlF$_4^-$ and GTP[γ-S], and activation of the catalytic subunit with forskolin were determined. Under all conditions, basolateral membrane demonstrated significantly greater activation of adenylate cyclase as compared with brush border membranes (Fig. 1). GTP[γ-S] stimulated adenylate cyclase activity at concentrations between 0.1 and 20 μM, with a maximal

Table 1. Marker enzyme activity in homogenate and plasma membrane fractions of rabbit kidney proximal tubule

| | | BBM | | BLM | | |
| | | Leucine aminopeptidase, μmol·min^{-1}·mg^{-1} | Maltase, μmol·hr^{-1}·μg^{-1} | K$^+$-stimulated phosphatase, μmol·hr^{-1}·μg^{-1} | Fold enrichment | Method ref. |
Fraction	n					
Homogenate	5	17 ± 4	ND	0.11 ± 0.07	1	—*
BBM	5	333 ± 62	ND	0.22 ± 0.05	20 ± 3	—*
Homogenate	6	ND	7 ± 6	0.04 ± 0.02	1	16
BLM	6	ND	6 ± 3	0.51 ± 0.3	14 ± 6	16

BBM, brush border membrane; BLM, basolateral membrane; ND, not determined.
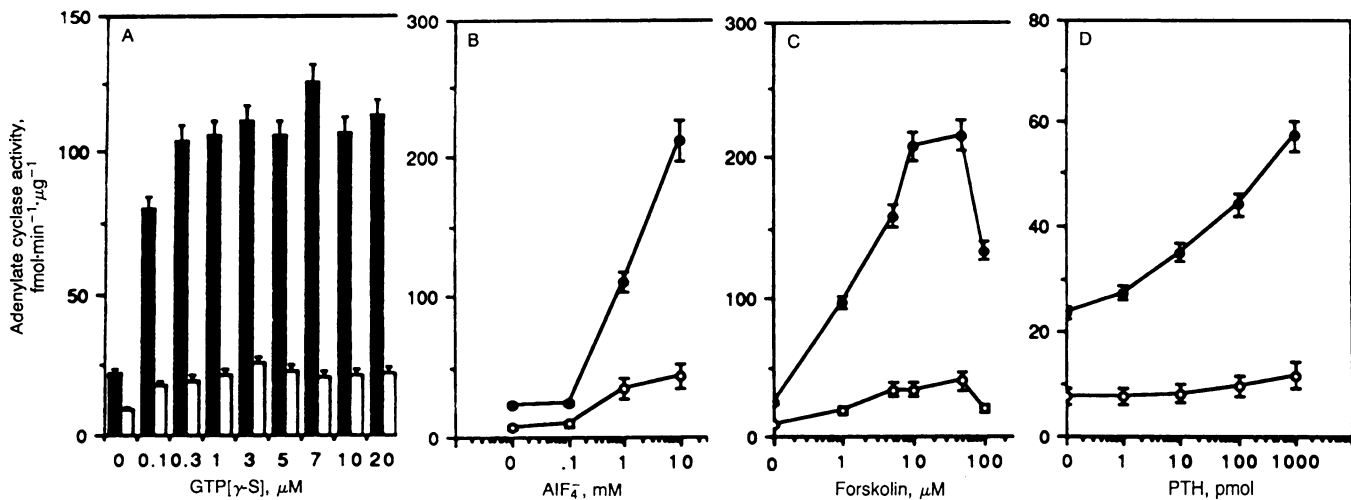*R. Singh, J.D., and U.H., unpublished results.

FIG. 1.   Stimulation of adenylate cyclase by GTP[γ-S], AlF$_4^-$, forskolin, and PTH. Brush border membrane (open bars and symbols) and basolateral membrane (black bars and symbols) of the kidney proximal tubule were incubated with the indicated concentrations of GTP[γ-S], (*A*), AlF$_4^-$ (*B*), forskolin (*C*), or PTH (*D*). The membranes were incubated in parallel, and the effectors were added and maintained for 10 min at 37°C. Each value represents the mean of two or three experiments conducted in duplicate.

increase of 6-fold over basal activity (Fig. 1*A*). A small but significant increase over the basal level was observed in brush border membranes that peaked at 3 μM GTP[γ-S]. The maximum response was 6.2-fold higher in basolateral than brush border membranes. AlF$_4^-$, which has been postulated to activate G proteins by mimicking the terminal phosphate of GTP, was the most effective stimulator of adenylate cyclase (1). A 9.3-fold maximal increase in activity over basal was observed at 10 mM AlF$_4^-$ (10 mM NaF/10 μM AlCl$_3$) in basolateral membranes (Fig. 1*B*). By contrast, brush border membranes showed only a small increase that peaked at 1 mM AlF$_4^-$ (1 mM NaF/1 μM AlCl$_3$). The response to 10 mM AlF$_4^-$ in basolateral membranes was 6.4-fold higher than the maximum response of brush border membranes. Forskolin was an effective activator of adenylate cyclase, causing an 8.6-fold increase in basolateral membrane adenylate cyclase activity at 10 μM (Fig. 1*C*). However, at concentrations >10 μM, adenylate cyclase activity was reduced. The response in brush border was significantly less at 2.2-fold higher than the basal level, yet the same pattern of supramaximal inhibition was observed (Fig. 1*C*). PTH-(1–34) fragment at concentrations ranging from 1 nM to 1 μM stimulated a 2.4-fold maximal response over basal (Fig. 1*D*). No significant stimulation was observed with brush border membranes.

ADP-Ribosylation of Membranes. Pertussis toxin [^{32}P]ADP-ribosylated a single protein band in brush border and basolateral membranes with an apparent molecular mass of 41 kDa (Fig. 2, lane C). Radiolabeling was 2-fold greater in brush border than in basolateral membranes. Cholera toxin ADP-ribosylated three proteins with apparent molecular masses of 42, 46, and 48 kDa in brush border and basolateral membranes. (Fig. 2, lane F). The 42-kDa protein was the most abundant cholera toxin substrate. In addition, cholera toxin labeled brush border 1.9 ± 0.1 (*n* = 3)-fold greater than basolateral membranes. Thus, in striking contrast to the relatively low activity of basal and G protein-stimulated adenylate cyclase in brush border, G proteins showed a higher level of bacterial toxin-catalyzed ADP-ribosylation in brush border as compared with basolateral membranes.

Immunoblotting. We used immunoblotting with a variety of antisera to further characterize the α subunits of the G proteins and their distribution. Antiserum 1398 (against G$_{\alpha\,common}$), which recognizes an identical amino acid sequence (Cys-Gly-Ala-Gly-Glu-Ser-Gly-Lys-Ser-Thr-Ile-Val-Lys-Gln-Met-Lys) of the α subunits of G$_s$, G$_{i(1,2,3)}$, G$_o$, and T (14), revealed that the most abundant protein(s) comigrated as a doublet at 41/42 kDa (Fig. 2, lane A). The relative abundance of protein(s) observed with the antiserum against G$_{\alpha\,common}$ was 41/42 kDa >> 46 kDa ≅ 48 kDa. The doublet of 41/42 kDa could be
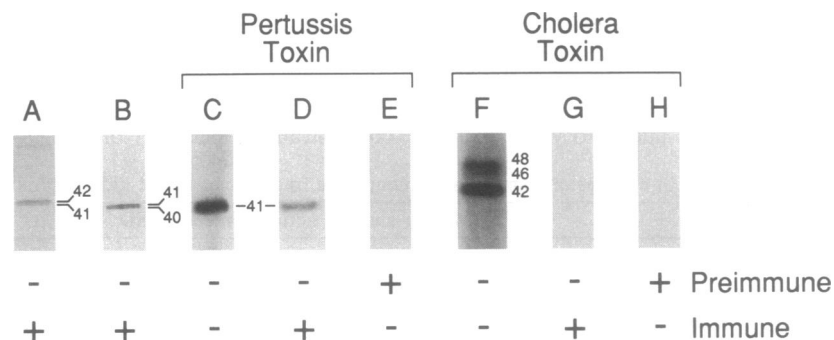

FIG. 2.   Detection of G proteins in basolateral membranes by immunoblotting, immunoprecipitation, and ADP-ribosylation with cholera and pertussis toxins. Immunoreactive proteins were identified with G$_{\alpha\,common}$ antiserum (lane A) and G$_{i\alpha}$ antiserum (lane B) on immunotransfer blots following NaDodSO$_4$/PAGE. Membranes ADP-ribosylated with pertussis and cholera toxins were immunoprecipitated with G protein α subunit-generated antisera. Shown are autoradiograms representing total membrane pertussis and cholera toxin substrates (lanes C and F, respectively) and the precipitation of toxin substrates with G$_i$ antiserum 8730 (lanes D and G) or preimmune serum (lanes E and H). Mobility of G protein α subunits on NaDodSO$_4$/11% PAGE gels is indicated, distinguishing the 41-kDa and 42-kDa forms associated with G$_i$ and G$_s$, respectively.

resolved into two proteins G_i (41 kDa) and G_s (42 kDa). The 42-kDa protein was the most abundant and represented $63 \pm 2\%$ ($n = 3$) of the total in brush border and $58 \pm 0.7\%$ ($n = 3$) in basolateral membranes as determined by laser densitometry. The 42-kDa protein was $\approx 1\%$ of membrane protein in these epithelial cells as determined by comparison with the 46-kDa $G_{s\alpha}$ standard. These results differ from most other tissues (bovine brain, adrenal gland, heart, retinal rod outer segments, tracheal smooth muscle, and human platelets) where the 41-kDa protein (G_i) was the most abundant α subunit detected with the $G_{\alpha\,common}$ antiserum (25). Use of $G_{s\alpha}$ antiserum 2347 verified the identity of the 42-kDa protein as $G_{s\alpha}$. A second protein was identified at 46 kDa that was significantly less than the 42-kDa protein as quantitated by laser densitometry. G_i antiserum 8730, which recognized the carboxyl-terminal amino acid sequence (Lys-Asn-Asn-Leu-Lys-Asp-Cys-Gly-Leu-Phe) but did not distinguish the G_i α_1, α_2, and α_3 subunit isoforms, identified a doublet consistent with the presence of at least two isoforms (Fig. 2, lane B).

The 42-kDa protein was 1.7 ± 0.05 ($n = 2$)-fold more abundant with $G_{\alpha\,common}$ antiserum and 1.5-fold more abundant with $G_{s\alpha}$ antiserum in brush border as compared with basolateral membranes, which is consistent with greater labeling observed with cholera toxin in brush border. Use of G_i antiserum revealed no difference in the amount of 41-kDa protein between brush border and basolateral membrane despite 2-fold greater labeling of brush border with pertussis toxin.

Immunoprecipitation. We used immunoprecipitation with two G_i antisera to further validate that the 42-kDa cholera toxin substrate was immunologically distinct from the 40/41-kDa pertussis toxin substrate. Antiserum 8730 immunoprecipitated 21% and 30% of pertussis toxin substrate in two experiments (Fig. 2, lane D). By comparison, this same antiserum immunoprecipitated 38% of G_i in platelet membranes (14). A second G_i antiserum, 1521, immunoprecipitated 4.4% (data not shown) in epithelial cell membranes. The efficiency of immunoprecipitation by 1521 also is quite similar to that in platelet membranes (6%) (14). By contrast, none of the 42-kDa cholera toxin substrate was immunoprecipitated with either G_i antiserum. Lane G in Fig. 2 illustrates the lack of efficacy of antiserum 8730 to immunoprecipitate the cholera toxin substrate.

DISCUSSION

Renal proximal tubular epithelial cells possess hormonal and neurotransmitter receptors for a variety of agents that play integral roles in kidney function. The biochemical mechanisms subserving these effects are incompletely understood. The aim of this study was to characterize the adenylate cyclase system in membranes purified from rabbit renal proximal tubule, and to identify the relationship of G proteins and this system. We have demonstrated that the predominant G protein α subunits were a 42-kDa cholera toxin substrate (G_s) and a 41-kDa pertussis toxin substrate (G_i). A traditional, higher molecular mass $G_{s\alpha}$ (46 kDa) was also identified in both membranes. This latter $G_{s\alpha}$ is likely the relevant G protein involved in adenylate cyclase stimulation (1), whereas the function of the 42-kDa protein is unclear. Toxin-catalyzed ribosylation of G_s and G_i was more abundant in brush border membranes. These differences were confirmed by immunoblotting for G_s but not G_i. G protein-stimulated adenylate cyclase was not absent in brush border membranes as observed previously in gastrointestinal membranes (11); however, basal and stimulated adenylate cyclase activity ranged from one-third to one-seventh that in basolateral

membranes. We cannot rule out the possibility that the small amount of G protein-stimulated adenylate cyclase activity may have resulted from residual basolateral contamination of the brush border membrane preparation. Thus, the abundance of G_s and G_i of brush border membranes that was unassociated with adenylate cyclase raises the possibility that these G proteins regulate other functions of these transporting epithelial cells (e.g., channels, phospholipases, and other enzymes) (6–9).

We identified a 42-kDa α subunit of a G protein that is most appropriately classified as $G_{s\alpha}$. The 42-kDa protein was a substrate for cholera toxin induced-ribosylation. Additionally, the protein bound $G_{s\alpha}$ antiserum (2374, Manning) and $G_{\alpha\,common}$ antiserum (1398, Manning) and was not immunoprecipitated by two $G_{i\alpha}$ antisera (8730 and 1521, Manning). Molina y Vedia *et al.* (15) described a 42-kDa cholera toxin substrate in platelets that was recognized by anti-$G_{s\alpha}$ but not anti-$G_{\alpha\,common}$ or anti-G_i. Thus, the kidney epithelial cell 42-kDa protein is probably structurally dissimilar as it contained the amino acid sequence common to G_s, G_i, G_o, and T recognized by $G_{\alpha\,common}$ antisera (14). The potential importance of the 42-kDa $G_{s\alpha}$ is emphasized by the observation that the protein comprised $\approx 1\%$ of the epithelial cell membrane protein and was more abundant than G_i.

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