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Research Article

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A Molecular Map of G Protein α Chains in Microdissected Rat Nephron Segments

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Abstract

Membrane-associated guanine nucleotide binding proteins regulate many receptor-mediated signals. Heterogeneity of biochemical and functional properties in nephron segments could be due to differences in G protein expression. To ascertain whether such heterogeneity of G proteins is present in various nephron segments, this study examines the distribution and relative abundance of G protein α chains in microdissected medullary thick ascending limb, cortical collecting tubules, outer medullary collecting tubules, proximal inner medullary tubules, and distal inner medullary tubules. Reverse transcription and polymerase chain reactions were employed using oligonucleotides encoding highly conserved regions of all known α chains. The cDNA was sequenced for α chain identification. The α_{i2} versus α_s distribution was different in the outer medullary collecting tubules, when compared with the medullary thick ascending limb ($P < 0.001$) or the cortical collecting tubule, the proximal inner medullary tubules, and the distal inner medullary tubules ($P < 0.05$). These latter four segments did not significantly differ from each other. A similar analysis was applied to the frequently used line of kidney cells, LLC-PK1, whose exact cellular origin remains unclear. Interestingly, we detected both α_{i2} and α_{i3}, while only α_{i2} was detected in the rat distal nephron. No α_o or α_z reverse transcription PCR products were detected. In contrast α_{11} and α_{14} members of the more recently described α_q family were detected in the outer medullary collecting tubules and the proximal inner medullary tubules, respectively. We conclude that the majority of nephron segments have a relatively constant distribution of G protein α chains. (*J. Clin. Invest.* 1993. 92:786–790.) Key words: (5)G proteins • microdissected • rat • nephron • PCR

Introduction

Membrane-associated guanine nucleotide binding proteins (G proteins) act as regulatory elements for many receptor mediated signals (1, 2). The α component of the heterotrimeric protein appears to convey specificity for enzymatic and ion transport processes. A role for G proteins in physiologic events and pathologic states is being increasingly recognized (1, 3).

The various segments of the mammalian nephron have a multitude of receptors and respond to a large number of effectors, thereby allowing for diverse and complex responses that subserve its many functions (4). Of great interest, however, is the demonstration that even a single hormone can bring about a variety of effects in any given nephron segment (5–7). For example, in the inner medullary collecting tubules, vasopressin by its V2 receptor stimulates adenylyl cyclase. This results in increased water permeability, and in its most terminal segment, also in increased urea permeability. Likewise, in this same segment the hormone also increases cell Ca^{2+} by V2 receptor-mediated pathways (8). A possible explanation for this observation is the presence of V2 receptor subtypes for which evidence is as yet not forthcoming but could well emerge when the newly cloned vasopressin receptor is investigated further. An alternative explanation of the various responses to a single hormone is divergent coupling of a single receptor to various effectors. Such a diversity of responses could be in part attributed to G protein α chain heterogeneity. Such a mechanism may well be operant in the response of LLC-PK1 cells to calcitonin where, in a cell cycle–dependent fashion, the hormone stimulates cyclase via Gs or activates the protein kinase C pathway via Gi (9). The heterogeneity in functional and biochemical properties on the various regions of the nephron could thus relate to differences in the α chain subunits of the guanine nucleotide binding proteins. In this regard, recent attempts have been undertaken to localize G proteins along various nephron segments using immunocytochemical methods with variable results (10, 11). In contrast, we have chosen to identify the specific α chain mRNA expressed in vivo using a reverse transcription PCR protocol in vasopressin-sensitive regions of the nephron.

Methods

Preparation of tissue. Studies were performed on male Sprague-Dawley rats (Sasco Inc., Omaha, NE) weighing between 250 and 300 g. The animals were fed a commercially available diet (ICN Nutritional Biochemicals, Cleveland, OH) and unrestricted water. Microdissection was performed as previously described (12), modified by the addition of 10 mM Vanadyl ribonucleoside complex to the dissection bath to inhibit RNAase. The following segments were dissected: medullary thick ascending limb of the loop of Henle (MTAL),[1] cortical collecting tubule (CCT), outer medullary collecting tubule (OMCT), proximal inner medullary collecting tubule (PIM), and distal inner medullary collecting tubule (DIM).

Preparation of RNA extraction in cultured cells. LLC-PK1 purchased from American Type Culture Collection, Rockville, MD, were grown to confluence in Dulbecco's modified Eagle's media supplemented with newborn calf and bovine calf serum. RNA was extracted using 1 ml of a 6 M urea and 3 M LiCl solution followed by sonication for 30 s. After 2 d of 4°C refrigeration, the sample was centrifuged at 10,000 rpm for 15 min and the RNA pellet resuspended in 500 μl of a

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1. *Abbreviations used in this paper:* CCT, cortical collecting tubule; DIM, distal inner medullary collecting tubule; MTAL, medullary thick ascending limb; OMCT, outer medullary collecting tubule; PIM, proximal inner medullary collecting tubule.

buffer containing 10 mM Tris (pH 7.5), 5 mM EDTA, and 0.1% wt/vol SDS. The mixture was extracted with phenol/chloroform (1:1) and chloroform followed by ethanol extraction. RNA quality was confirmed by agarose gel electrophoresis in the presence of formaldehyde.

Reverse transcription. The dissected tubules as well as the RNA extracted from the cultured cells were subjected to reverse transcription using a synthetic antisense oligonucleotide (29 mer) (vide infra as primer 1). Specifically, the microdissected tubules were centrifuged for a few seconds (10,000 rpm) to pellet the sample. To this was added 9 μl of a mixture containing 2% Triton X-100, 1 U/μl placental RNAase inhibitor and 5 mM DTT. After addition of this mixture, 11 μl of a second mixture containing 2 μl of 10× amplification buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM MgCl2, and 0.1% wt/vol gelatin), 1 μl 25 U/μl placental RNAase inhibitor, 2 μl of 10 mM deoxynucleotides (dNTP's), 2 μl of 50 μM primer 1, 1 μl of 50 mM MgCl2, 0.5 μl of avian myeloblastoma virus reverse transcriptase (Boehringer Mannheim, Corp., Indianapolis, IN) and 2.5 μl of doubly deionized water was added. The samples and appropriate controls were incubated at 37°C for 1 h followed by 95°C for 5 min.

For reverse transcription of the RNA obtained from cultured cells, the RNA was combined with 10 μl of a mixture containing 2 μl of deoxynucleotides (10 mM each dNTP), 1 μl of placental RNAase inhibitor, 1 μl of 50 mM MgCl2, 2 μl of 50 μM primer 1, 0.5 μl of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Corp.) plus doubly deionized water to a final volume of 20 μl. Samples were incubated as above.

Polymerase chain reaction. PCR reaction was performed using the DNA Thermal Cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT). The antisense oligonucleotide was designed from a highly conserved region near the COOH terminus. Inosine was used at points of high divergence. The sequence of primer 1 was as follows: 5′-CCAGCAAGCTTIGTRTCIRYIGCRCAIGT-3′. Primer 2 was designed from a second highly conserved region roughly 150 amino acids toward the NH_2 terminus (α_s) and was as follows: 5′-CCAGCGGTACCGAYGTIGGIGSICARBG-3′. In these primers, R = A or G, Y = C or T, S = G or C, B = A or C, H = A, C, or T.

After reverse transcription and heat inactivation, the 20-μl reaction mixture was combined with 80 μl of the PCR reaction mixture. This included 10 μl of 10× reaction buffer (100 mM Tris-Hcl, pH 8.3, 500 mM KCl, 0.01% [wt/vol] gelatin), 16 μl of (1.25 mM each) deoxynucleotides (dNTP's), 10 μl of 10 μM primer 1, 10 μl of 10 μM primer 2, 0.5 μl of Amplitaq DNA polymerase (5 U/μl; Perkin-Elmer Cetus) and 33.5 μl of doubly distilled, sterile water. Lastly, 100 μl of mineral oil was added to prevent evaporation. The first setting was 94°C for 2 min, followed by 72°C for 2 min, and annealing temp of 60°C for 2 min. The total number of cycles was 30. Upon completion of the reaction, 50 μl of the PCR product was placed in an agarose gel along with appropriate standards. The area of product, based on expected size, was extracted and DNA recovered using glass milk silica matrix technique provided by GeneClean II Kit (BIO 101, Inc., La Jolla, CA). The DNA was recovered in 20 μl doubly deionized water and subjected to a second round of PCR using the same PCR mixture and reaction conditions. The final product was subjected to phenol chloroform and chloroform extraction followed by ethanol precipitation.

Restriction digestion. The oligonucleotides were designed with HindIII and KpnI sites on the 5′ end of primer 1 and primer 2, respectively. Therefore, the final PCR products were subjected to restriction digestion with HindIII and KpnI (New England Biolabs Inc., Beverly, MA) as well as the plasmid vector PUC 18. After digestion, the products were run on an agarose gel and the areas of interest were extracted as described above.

Ligation. Ligation was carried out using 3 μl of doubly digested PUC 18 and 5 μl of similarly digested PCR product. The mixture was incubated at 68°C for 10 min, placed on ice, and 2 μl of 10× ligation buffer (500 mM Tris pH 7.4, 100 mM MgCl2, 100 mM DTT, 10 mM spermidine, 10 mM ATP, and 1 mg/ml BSA), 2 μl of 1:10 diluted T4 polynucleotide ligase, and 8 μl of doubly deionized water were added. The samples were incubated at 12°C for 12 h.

Bacterial transformation. Competent HB101 *Escherichia coli* were transformed with the ligation product. 5-ml cultures were grown in Luria Bertan medium plus 50 μg/ml ampicillin overnight. Next, the bacteria was pelleted by a 2-min, 3,000 rpm centrifugation, and the supernatant discarded. The pellet was resuspended in 0.3 ml of 400 μg/ml RNAase; 50 mM Tris-HCl; 10 mM EDTA. Next, 0.3 ml of 200 mM NaOH; 1% SDS was added and incubated at room temperature for 5 min. Lastly, 0.3 ml of 2.55 KAc (pH 4.8) was added and the entire mixture was centrifuged for 20 min at 10,000*g*. The supernatant was recovered and 0.7 vol of isopropanol added. The sample was recentrifuged at 10,000*g* and the pellet washed with 70% ethanol, dried, and resuspended in 25 μl of water. A small sample was removed for restriction digest with KpnI and HindIII to confirm the presence of an appropriate size insert.

Sequencing. Sequencing of the double stranded PUC 18 plus insert was performed using the Sequenase version 2.0 system supplied by United States Biochemical Corp. (Cleveland, OH) and the M13 or Reverse M13 primer. Sequences were analyzed using the computer program Macvector.

Statistical analyses. The data were subjected to chi square analysis with statistical significance defined as $P < 0.05$ (13).

Results

To ensure that the above procedures in general and the PCR, in particular, did not preferentially amplify one cDNA α chain subunit over another, known amounts of α_s and α_{i2} cDNAs at ratios of 1:1, 1:10, and 10:1 were subjected to PCR and agarose gel analysis. As shown in Fig. 1, PCR successfully amplified the α chain cDNA subunit without distortion of their relative amounts of the two α chain cDNAs.

G proteins α chain in LLC-PK1 cells. LLC-PK1 cells of porcine renal origin have been extensively studied yet their precise nephronal origin remains unclear. We decided to identify the pattern of α chain subunit mRNA in this cell line to potentially identify its cellular origin. Reverse transcription

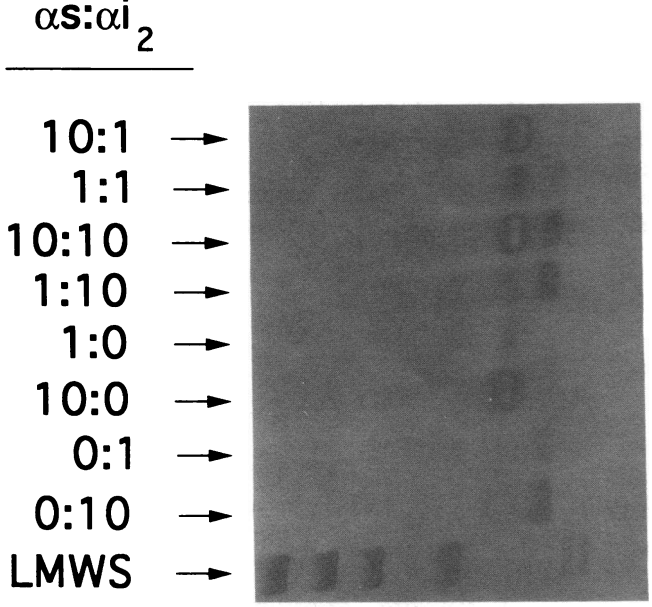

Figure 1. PCR amplification of various ratios of α_s and αi_2. On a 1.5% agarose gel, it is easy to identify the heavier α_s cDNA. Stock solutions were not exactly the same concentration but the relative ratios of α_s and αi_2 were preserved. (LMWS, low molecular weight standard).

and PCR amplification of the LLC-PK1 cells provided a high yield of cDNA. RNA was prepared on two separate occasions from confluent 10-cm dishes. No PCR contamination was observed in the control samples. 36 PCR products were sequenced for identification. As is depicted in Fig. 2, α_{i2} was the predominant α chain cDNA, comprising 53% of all samples sequenced. The rest of the cDNAs were approximately equally divided between α_s, α_{i3}, and α_q. α_{i3} contributed 19% and α_q added 17%. α_s was present in 11% of the PCR products. No other forms of α chains were identified.

G protein α chains in dissected nephron segments. Fig. 3 demonstrates the final PCR product from the proximal inner medullary collecting duct tubule and the distal inner medullary collecting duct tubule. Note the paucity of signal at the region which the heavier α_s control runs. Sequencing confirmed the low percentage of α_s in these two segments. The relative abundance of α chains in the five dissected segments of the nephron is shown in Fig. 4. In all instances, sequenced samples were obtained from tissues from at least two different rats. In microdissected MTAL, 41 samples were sequenced and 34 identified (83%). The great majority of these were α_{i2} (94%) while the remaining were α_s (6%). No other forms of α chains were detected. A similar pattern was obtained in dissected CCT. 41 samples were sequenced and only 14 were identifiable (33%). Of these, 13 were α_{i2} (93%) and 1 sample was α_s (7%). Whereas in all other segments examined no more than 22% of inserts proved to be nonsense sequence, 67% of those in the CCT segment proved unidentifiable.

The pattern of α chains prevalent in the OMCT proved to be different. In this segment, 33 products were sequenced and 32 identified (97%). Of these the predominant α chain was α_s, 16 samples accounting for 50% while only 12 were α_{i2} (37%). Of particular interest is the finding of four cDNAs of an α chain that is identical to the one designated as α_{11} (13%) by Strathman et al. (14), an α chain in the recently described q family.

Because the PIM and DIM appear to have distinct functional and structural characteristics, we microdissected these two areas and studied them separately. The G protein α chain distribution was, however, not markedly different. In the PIM, 55 samples were sequenced and 43 were identified (78%) compared with 41 out of 49 in the DIM (84%). Specifically, both had a predominance of α_{i2}, 33 of 43 products (77%) in the PIM and 36 of 41 products (88%) in the distal or terminal inner medulla. In the former, 9 of 43 samples were α_s (21%) and in the latter 5 of 41 samples were of this nature (12%). One cDNA

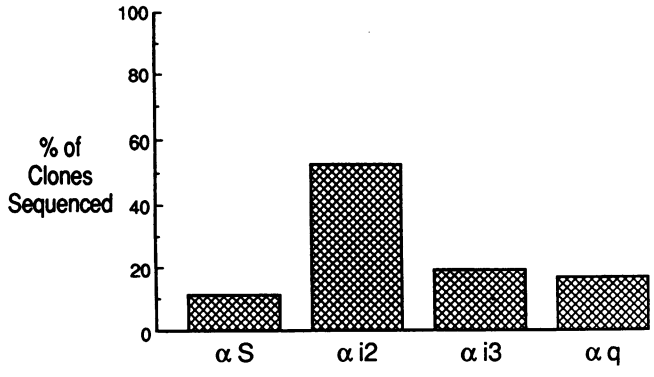

Figure 2. Distribution of G protein α chains mRNA in cultured LLC-PK1 cells.

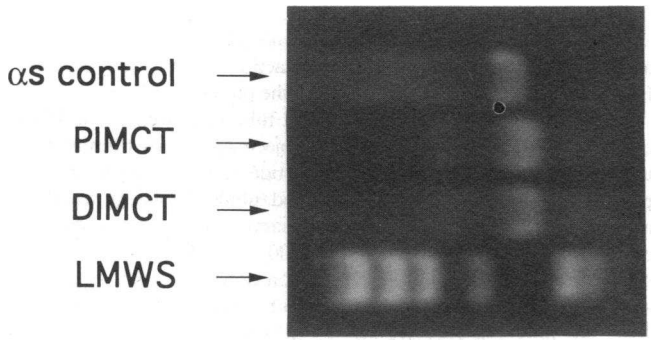

Figure 3. Agarose gel electrophoresis (1%) of the final PCR product from two regions of the nephron. A known α_s control was used for PCR control and size identification. The predominant band is slightly lighter and represents αi_2. Sequencing data confirmed the agarose gel observation. (LMWS, low molecular weight standard; *DIMCT*, distal inner medullary collecting tubule; and *PIMCT*, proximal inner medullary collecting tubule).

of a G protein identical in sequence to α_{14} (15) was found in the PIM, another member of the recently described q family.

A statistical comparison of the ratio of α_{i2} and α_s in the various segments is depicted in Fig. 5. By chi square analysis the OMCT stands out as being different ($P < 0.01$) from all other segments. However, the other regions (MTAL, CCT, PIM, and DIM) do not statistically differ from each other.

Discussion

G proteins play a pivotal role in a number of cellular processes including hormonal responses, transport of ions, and cell division. It has been well recognized that the protein is markedly heterogeneous, particularly in the structure of its α chain as an increasing number of such chains are being described and cloned (14–17). While G proteins are rather ubiquitous there appears to be differences in the tissue distribution. For example, α_o is more prevalent in the brain and α_{16} in hematopoietic tissue (18). Microdissection and PCR analysis has been applied to the detection of aldose reductase (19) and more recently to localize the atrial natriuretic peptide (20) and endo-

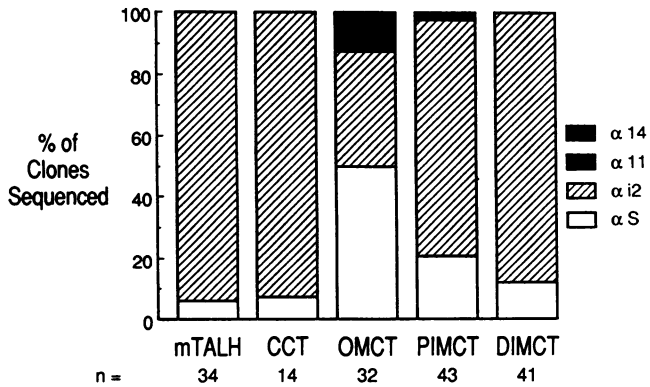

Figure 4. Distribution of the G protein α chain mRNA in the five microdissected regions of the nephron. Nonsense sequences were excluded from the analysis. (*mTALH*, medullary thick ascending limb of the loop of Henle, *CCT*, cortical collecting tubule; *OMCT*, outer medullary collecting tubule; *PIMCT*, proximal inner medullary collecting tubule; and *DIMCT*, distal inner medullary collecting tubule).

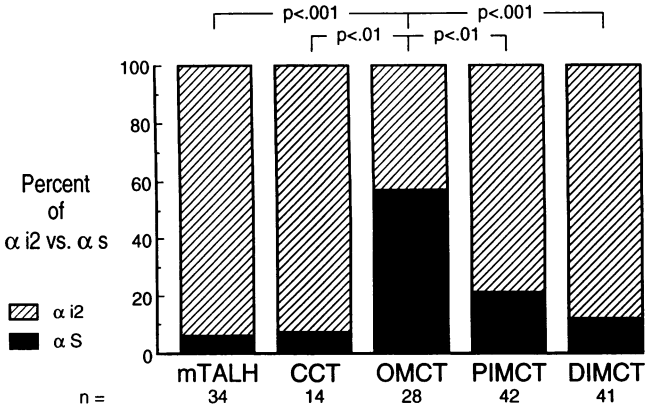

Figure 5. Percentage of α_s versus αi_2 mRNA in the five microdissected regions of the nephron. (*mTALH*, medullary thick ascending limb of the loop of Henle; *CCT*, cortical collecting tubule; *OMCT*, outer medullary collecting tubule; *PIMCT*, proximal inner medullary collecting tubule; and *DIMCT*, distal inner medullary collecting tubule).

thelin (21) receptor. Our study is the first to analyze the distribution of G protein α chains in the mammalian nephron by analyzing the mRNA content of the dissected segments. However, we took this approach further to analyze not only the presence of a single protein but rather a family of proteins. In contrast to analysis using PCR alone, we could exactly identify the subtype of α chain and exclude nonsense sequences. Fig. 3 represents the PCR signal but does not exclude the possible contribution made by nonsense sequences. Yet, it does readily show the paucity of α_s in the PIMCT and DIMCT. After ligation, bacterial transformation, and, ultimately, sequencing, this observation remains true; α_s represents only a small portion of α chains in these two segment. Another example of the virtue of this method is the design of the oligonucleotides which allowed the detection of the previously described G protein α chains (1) plus the more recently reported α chains (14–17). Furthermore, we could compare the relative abundance of the mRNA in the various segments. This pattern is also likely to represent the prevalence of the various proteins, as Watkins et al. found a strong correlation between changes in α chain mRNA levels and changes in protein expression in 3T3-L cells (22). Using a single antibody and single Northern probe while altering the pathologic state of an animal, the literature has numerous other examples of mRNA levels of G proteins correlating with protein levels in such tissues as cardiac, arterial, cerebral, and testicular membrane preparations (23–26). Our results reveal a high prevalence of α_{i2} mRNA in the MTAL, CCT, and in both the PIM and DIM as α_{i2} is four to five times as abundant as α_s in these segments. Control experiments confirm that the oligonucleotide primers and the PCR reaction preserve the relative ratio of α_s and α_{i2}. Although α_s is 18 amino acids larger in the region between our two primers this technique maintained the α_s to α_{i2} ratio. Furthermore, using the same techniques the pattern was entirely different in the OMCT, where α_s was the predominant α chain. In this regard, the OMCT was the only segment different from the other four that were analyzed. The significance of this difference vis-à-vis the function of this segment remains to be determined.

Attempts to localize the various α chains in renal tissue have been recently undertaken primarily by using the histocytochemical technique (10, 11). While considerable information can be obtained by this approach, particularly in regard to subcellular localization, the sensitivity and specificity of this technique is difficult to assess. For example, in Brunskill's study the antibody directed to α_{i2} cross-reacts with α_{i1}, a fact that is not surprising considering the great homology between α_{i1}, α_{i2}, and α_{i3}. It is also of interest that the above mentioned studies do not have uniform results. Thus, while Stow et al. find a heavy concentration of α_s in basolateral membranes of collecting tubule cells and less intense staining in apical membranes of proximal tubules, the opposite pattern is described by Brunskill et al. Likewise, while the former finds α_{i2} in the basolateral membrane of collecting duct cells, the latter study finds no such α chains in this location. In this study, we employed a different approach using in situ reverse transcription and PCR amplification to study the mRNA content of tubules. As in our study, α_s was found in all segments studied by immunocytochemistry in Stow's and Brunskill's papers (10, 11). In the study by Stow et al. (11), α_{i1}, was prominent in the cortical thick ascending limb, less so in the outer medullary stripe, and absent in the inner medullary stripe of the TAL. α_{i1} was also noted in the epithelial cells covering the surface of the papilla. In contrast, we did not detect any α_{i1} in the segments analyzed in our study. In examining α_{i2}, Stow noted staining throughout the collecting duct but limited to the principal cells. They did not observe any α_{i2} protein in the thick ascending limb of the loop of Henle. Brunskill noted less α_{i2} protein staining in the outer medulla than the distal tubule and inner medulla. Interestingly, in our study, α_{i2} was the predominant mRNA in all segments except in the outer medullary collecting ducts. In contrast to the study by Stow et al., we found α_{i2} in the thick ascending limb of the loop of Henle. In the study by Stow et al., α_{i3} was weakly staining in the thick ascending limb while weakly staining and variable staining in the cortex and tip of the collecting tubule. Brunskill found faint staining in the distal tubules of the cortex, no staining in the outer medulla, but did observe prominent staining in the inner medulla. In our study of mRNA by PCR analysis, we did not detect α_{i3} in any segments studied.

It is also of interest that the only α_i cDNA we identified in the tubules was α_{i2}. This is clearly not a result of our inability to detect other α chains and α_{i3} in particular. In this regard, using the same oligonucleotides and reaction conditions, we found that LLC-PK1 cells had both α_{i2} and α_{i3} but not the α_{i1} mRNA. In concurrence, Ercolani et al. (27), using immunocytochemistry analysis of LLC-PK1 cells, detected the presence of both α_{i2} and α_{i3} proteins. In addition, they were unable to detect any α_{i1} in these cells. These similar observations also calls into question the exact cellular origin of LLC-PK_1 cells given its very different pattern of G protein α chain mRNA.

We also were able to detect, in low abundance, some members of the more recently described α_q family in renal tubules, a protein felt to be involved in pertussis toxin insensitive phospholipase C signaling (28). Using Northern blots for this family of G proteins, Strathman et al. (17) found some localization to the kidney. They observed a relatively high amount for α_{11}, a moderate amount of α_{14}, and a small amount for α_{10}, α_{12}, and α_{13}. We found α_{11} in the OMCT and one clone of α_{14} in the PIM. Since these α chains are present in low abundance, our failure to encounter them in the other segments does not entirely rule out their presence. Nonetheless, we can unequivocally state that these two members of the family are present in the distal nephron and α_q itself is preva-

lent in LLC-PK1 cells. Lastly, failure to detect other alpha chains does not rule out their existence but rather suggests their potential abundance compared to the observed G protein alpha chain mRNA's.

Taken together, our data reflect a high degree of homology in the pattern of G protein α chains in the distal nephron. It would not seem very plausible, therefore, that the different cellular responses to a given hormone or ligand is a consequence of divergent coupling of a single receptor to different G proteins. However, such a system has been shown by others (9, 29, 30). Cell cycle–dependent coupling of the calcitonin receptor to different G proteins was recently demonstrated. When LLC-PK1 cells were stimulated with calcitonin in G2 phase, there was activation of a cholera toxin-sensitive G protein resulting in an increase in cAMP. In contrast, when the cells were stimulated in S phase, there was a pertussis toxin–sensitive inhibition of adenylate cyclase and stimulation on protein kinase C (9). Another example of heterogenous hormonal response to a single receptor is the expression cloning of the parathyroid hormone receptor in COS cells. These investigators demonstrated stimulation of both the adenylate cyclase and phospholipase C pathways via the expressed parathyroid hormone receptor (30). Interpreting our data, it seems more likely, however, that the specificity for the heterogeneous response to a single hormone lies elsewhere, in the form of receptor subtypes or distal to G protein coupling.

In summary, we report herein a molecular map of G protein α chain mRNA in the distal segments of the rat nephron. This is the first known attempt to identify specifically which mRNA is present in which segment rather than the Northern blot or immunocytochemical analysis. With the exception of the OMCT, α_{i2} is the most prevalent G protein alpha chain mRNA moiety, detected four to five times as frequently as α_s. Members of the α_q family are present in low abundance. The pattern of α chains in LLC-PK1 cells is clearly different from any of the studied segments and again calls into question the precise nephronal origin of this often used cell line. Given the high degree of G protein homology in all the nephron segments studied, G protein distribution is an unlikely avenue for the final regulation of hormonal response. Regulation most likely resides elsewhere, such as at the level of receptor subtypes or post receptor events.

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