UROGUANYLIN (Ugn) is a short, cysteine-rich peptide that regulates ion transport in the intestine and the kidney (for a review, see Refs. 18 and 68). Ugn can thus control the movement of electrolytes both into and out of the body and has, accordingly, been proposed to play a homeostatic role in the management of dietary salt. In support of this hypothesis, Ugn knockout mice are hypertensive and excrete orally delivered sodium more slowly than do wild-type animals (10, 49).

The existence of an intrinsic Ugn-based signaling system within the gut is well established. The Ugn gene is strongly expressed in the intestine (2, 11, 45, 55, 56, 62, 88), as is the gene encoding a high affinity, Ugn-activated receptor-guanylate cyclase (rGC) known as GC-C (42, 77, 84). Furthermore, a wealth of evidence confirms that nearly all of the effects of Ugn on intestinal ion fluxes are mediated by GC-C via the downstream effects of cGMP on specific epithelial transporters and channels (6, 15, 22, 25, 29, 35, 51, 65, 71, 72).

In contrast, the renal actions of Ugn are poorly understood. In particular, two fundamentally important questions remain unanswered. The first concerns the tissue source of renally active Ugn. One popular model suggests that renal salt excretion is regulated by Ugn that is released from the intestine (18, 68). In support of this model, biochemical measurements show that the intestine secretes large amounts of prouroguanylin (proUgn) into the plasma (58), and infusion studies with radiolabeled recombinant proUgn demonstrate that the circulating propeptide can be processed within the kidney to Ugn, which is subsequently eliminated in the urine (68). On the other hand, animals consuming high-salt chow appear to maintain normal plasma concentrations of Ugn (24) and proUgn (10), while excreting significantly more Ugn in their urine than do control animals (24). This argues that salt ingestion may mobilize an intrarenal pool of Ugn, rather than a plasma pool supplied by the intestine. Consistent with this alternative hypothesis, several laboratories have detected Ugn-like polypeptides in kidney extracts (58, 62). However, the very low levels of preproUgn mRNA in the kidney (2, 11, 19, 45, 49, 50, 56, 67, 88) have led to the suggestion that Ugn is not synthesized by the kidney, but rather is taken up either from the circulation or from the glomerular filtrate (62), perhaps via receptor-mediated endocytosis (87). Thus the role (if any) of endogenously synthesized Ugn within the kidney remains an open question.

A second area of uncertainty concerns the identity of the receptor(s) and signal transduction mechanism(s) that mediate the natriuretic effects of Ugn in the kidney. The association of Ugn with GC-C and cGMP in the intestine has long been taken as implicit support for the hypothesis that Ugn acts via GC-C and cGMP in the kidney. Direct backing for this idea is provided by evidence that the opossum kidney expresses an mRNA transcript that encodes a GC-C-like receptor (48), as well as the observation that opossum renal and urinary cGMP levels increase after stimulation with Ugn and/or with heat-stable enterotoxin (ST), a bacterial toxin that acts as a high-affinity Ugn mimic (13, 20, 21). However, expression of GC-C in the kidneys of other mammals is controversial (4, 5, 9, 17, 38, 42, 49, 67, 77, 78, 84), and recent studies have shown that most (although perhaps not all) of the natriuretic actions of Ugn and ST are preserved when the GC-C gene is genetically ablated in mice (3, 4). Thus the roles played by GC-C and cGMP in renal responses to Ugn are not well defined.

Our understanding of Ugn and GC-C expression in the kidney is further complicated by the largely nonoverlapping nature of the experimental techniques and animal species that have been described in previous contributions to the literature.

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For this reason, we have focused our current studies on a single experimental preparation, the Sprague-Dawley rat, and have used multiple independent techniques (Northern blotting, real-time RT-PCR, quantitative immunoblotting, immunohistochemical analysis, radioreceptor binding assay, and guanylate cyclase activity measurements) to evaluate Ugn and GC-C expression in parallel. Our goal is to establish accurate renal mRNA and polypeptide expression profiles for Ugn and GC-C in the rat model system, where the physiological effects of Ugn on renal salt excretion have been best characterized (1, 16, 17, 58–60, 73, 74).

METHODS

Animals. Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) were maintained on a 12:12-h light-dark cycle with free access to water and standard rodent chow. All procedures were approved by the UNC Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Antibodies. Antibodies 6910, 6911, and 6912 were generated by our laboratory, as previously described (64), and antibody 1–11 was generated in the laboratory of Dr. Masamitsu Nakazato. Each antibody was raised against a small (–15 residue) peptide antigen and therefore targets a specific region of the preproteptide, as shown schematically in Fig. 2A. Note that antibodies 6910 and 6911 were raised against the same antigen (PALPLDLQPVCASTQEQ) in different rabbits, providing an internal check on each other’s validity. The two consistently target the same 9.4-kDa preUgn protein on Western blots (58) and produce comparable immunohistochemical staining patterns. Antibody 6912 (raised against the peptide sequence QQKSGLLP-DVCYNP) also recognizes preUgn robustly on Western blots (58) but is not suitable for immunohistochemistry, as it produces a weak signal that is difficult to distinguish from background labeling. Antibody 1–11 (raised against the peptide sequence TDECELCINVACTGC) has previously been shown to recognize preUgn on Western blots (58), and we show here that it is an effective immunohistochemical reagent. In addition, in contrast to the other antibodies, it recognizes both preUgn and free Ugn. Antibody 1–11 is available in limited supply and therefore used here exclusively for histological analyses.

Tissue isolation and extraction of protein and mRNA. Before tissue collection, animals were anesthetized either with urethane (1.6 g /kg body wt ip) or pentobarbital sodium (55 mg/kg body wt ip). For the Northern and Western blot data in Fig. 1, RNA and protein were isolated simultaneously from the same piece of tissue using the TRIzol method (Invitrogen, Carlsbad, CA) as specified by the manufacturer. For the Western blot data in Figs. 2 and 3, mucosal scrapings (obtained from a 2- to 4-cm-length of small bowel located 4 cm distal to the pyloric sphincter) or minced kidney tissues were homogenized at a ratio of 4 ml buffer/g tissue in 25 mM HEPES, pH 7.4, containing a protease inhibitor cocktail (Sigma, St. Louis, MO). Homogenates were then centrifuged at 60,000 g for 30 min at 4°C, and the supernatant fraction was collected and stored at –80°C before analysis. For the RT-PCR studies reported in Fig. 8, tissues were rapidly cut into small pieces (~1 mm3) and immersed overnight at room temperature in RNAlater (Qiagen, Valencia, CA). RNA was subsequently extracted and purified using an RNeasy Mini kit (Qiagen, Valencia, CA). For the Western blot data in Fig. 1B, tissues were treated with chelominescence reagent (Roche Diagnostics) and exposed to film. For quantitation, films were digitized at a resolution of 600 dpi (LaCie Silverscanner IV) and analyzed densitometrically using NIH Image software (available at http://rsb.info.nih.gov/nih-image/download.html). All other immunoblots were performed with an infrared-emitting secondary antibody (IRDye 800-coupled goat anti-rabbit from Rockland, Gilbertsville, PA, diluted 1:2,000) and analyzed with an Odyssey infrared gel imaging system (LI-COR Biosciences, Lincoln, NE). The quantitative Western blot-based assay is described in detail in a previous publication (58).

Northern blot procedures. For Northern blots, isolated RNA was denatured by glyoxal, size-fractionated by electrophoresis on 1% agarose gels, pressure-transferred to positively charged nylon membranes (ICN), and allowed to hybridize with an antisense GC-C probe, as previously described (43).

RT-PCR amplifications. Random hexamer-primed cDNA was prepared from isolated RNA, using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the supplier’s protocol. Subsequent RT-PCR analysis employed primer pairs and probes, as specified in Table 1.

Primer sets A and D were used for traditional endpoint RT-PCR. Reactions were performed in a DNA thermal cycler (Eicorp, San Diego, CA), using a GeneAmp DNA amplification kit (PerkinElmer Cetus) and standard ionic conditions. The cycler was programmed to heat to 94°C for 2 min, followed by up to 45 thermal cycles (94°C for 1 min, 60°C for 1 min, 72°C for 2 min), and a final incubation at 72°C for 10 min. PCR products were stained with ethidium bromide, electrophoresed on agarose gels, and visualized under UV light.

Primer sets B and C were used for real-time RT-PCR. Each sample included a fluorescent probe with a reporter dye (fluorescein) at the 5’ end and a quencher dye (tetramethylrhodamine) at the 3’ end, as indicated in Table 1. Amplifications were performed in duplicate in a 96-well plate in the ABI Prism 7700 sequence detector (PE Biosystems, Waltham, MA) for 40 thermal cycles (15 s at 94°C and 1 min at 60°C) as described by Kim et al. (37). Fluorescence was continuously measured in each well, and the fractional cycle at which each
sample crossed a fluorescence threshold, \( C_T \), was determined using software provided by the manufacturer. \( C_T \) values were not corrected for differences in amplification efficiency, because efficiencies were consistently very close to the theoretical maximum.

**Urinary cGMP measurements.** Rats were anesthetized (55 mg/kg body wt pentobarbital sodium ip) and prepared for renal clearance measurements as previously described (60). Urine was collected via ureteric cannulas, diluted 1:1,000, and assayed for cGMP content by standard RIA (Biomedical Technologies, Stoughton, MA). Samples and standards were acetylated to increase assay specificity and sensitivity.

**Guanylate cyclase assays.** Details of this assay are provided in a previous publication (27). In brief, individual kidney subregions (cortex, medulla, and papilla) were hand disected from tissue slices, using visual landmarks to identify each region. Membranes were isolated by ultracentrifugation, stored on ice, and assayed for activity within 15 min. Protein content was determined on a portion of the diluted membranes using a dye-binding assay (Bio-Rad Bradford Assay). Substrate, cofactors, and a phosphodiesterase inhibitor (IBMX) were added to initiate the reaction at 37°C, with or without a saturating dose of Ugn, ST, or atrial natriuretic peptide (ANP). Reactions were stopped with 6% TCA. Each sample was then extracted with diethyl ether (to remove TCA), and the cGMP content was determined by radioimmunoassay. Results are expressed as picomoles of cGMP synthesized per milligram of protein per minute.

**Quantitative ligand binding assay.** This assay is a modification of a previously described receptor binding assay (30). Our version of this assay is based on competitive binding between Ugn and \( 125^I \)-labeled \( N \)-tyrosyl Ugn (Y-Ugn) at GC-C receptors present in isolated T84 cell membranes. Y-Ugn (Bachem Americas, Torrance, CA) was iodinated at GC-C receptors present in isolated T84 cell membranes. Y-Ugn (Bachem Americas, Torrance, CA) was iodinated in 20 μl assay buffer [HBSS; 20 mM sodium citrate, 0.75% bovine serum albumin (Sigma) at pH 4.5], and incubated for 60 min with T84 cell membranes (1–10 μg protein) and 0.5 nM \( 125^I \)-Y-Ugn, then filtered through Whatman GF/C glass microfiber filters and rinsed twice with 5 ml of ice-cold buffer (50 mM Tris-HCl at pH 7.4). Bound radioactivity was quantified with a gamma counter (Packard Cobra, Perkin-Elmer Life and Analytical Sciences, Waltham, MA). Ugn concentrations in biological samples were calculated by interpolation on a standard curve, using the equation for a single-ligand displacement assay

\[
\text{cpm bound} = \text{NSB} + \frac{B_{\text{max}}}{1 + 10^{\log10(\text{IC50})}}
\]

where \( \text{NSB} \) = nonspecific ligand binding (measured in the presence of \( 10^{-5} \) M unlabeled Y-Ugn), \( B_{\text{max}} \) = maximal specific ligand binding, and IC\(_{50} \) = the concentration of competitor that results in 50% displacement of the bound ligand.

**Immunohistochemistry.** Animals were anesthetized with 9:1 ketamine/xylazine (1 ml ip), then perfused with phosphate-buffered saline by cardiac puncture, to clear the kidneys of blood and tubular fluid, followed by perfusion with fixative (4% paraformaldehyde/0.18% picric acid in 0.16 mol/l phosphate buffer, pH 7.2). Kidneys were then removed, postfixed for 2 h in the same fixative, and cryoprotected at 4°C in 0.1 M phosphate buffer, pH 7.2, containing 30% sucrose. Tissue was then embedded in Tissue-Tek Optimal Cutting Temperature Compound (Ted Pella, Redding, CA) and cryostat sectioned at 10 or 14 μm. Some sections were treated with 3% H\(_2\)O\(_2\) for 10 min before immunolabeling to quench endogenous peroxidase activity.

Peroxidase labeling was performed as previously described (47), using a biotinylated anti-rabbit IgG secondary antibody (ABC Elite Kit, Vector Laboratories, Burlingame, CA or Jackson ImmunoResearch Laboratories, West Grove, PA), a streptavidin-biotinylated horseradish peroxidase complex (ABC Elite Kit), and 0.02% DAB (Sigma). Preabsorption controls were carried out with 10 μM syn-
Antibody 6910. The black arrowheads. Kidney. Authenticated peptide standards elute at the points indicated by the left A: reverse-phase HPLC was used to fractionate a kidney extract (white symbols) or a kidney extract spiked with 10 nmol of synthetic rat Ugn (black symbols). After chromatography, individual HPLC fractions were dried, resuspended in assay buffer, and analyzed for Ugn-like binding activity as described in METHODS. White symbols plotted for fractions 39–45 represent mean results from 7 independent column runs, each based on an extract from an independent kidney. Authenticated peptide standards elute at the points indicated by the black arrowheads. B: quantitative Western blot assay for proUgn, using antibody 6910. The right half of the gel contains samples from 5 independent kidney extracts (A–E). The left half of the gel contains a standard curve constructed with known amounts of recombinant rat proUgn, as indicated.

Table 1. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (GC-C)</td>
<td>tccatasgctaggtctgg</td>
<td>caatctctcaggctgga</td>
<td>N/A</td>
</tr>
<tr>
<td>B (GC-C)</td>
<td>ac/tatctttctccctgaga</td>
<td>tggatgtatcggcctcct</td>
<td>fegagaccaccac/tgatgcacggga</td>
</tr>
<tr>
<td>C (β-actin)</td>
<td>tgcgctagctggctgca</td>
<td>caggagagctggag</td>
<td>N/A</td>
</tr>
<tr>
<td>D (cyclophilin A)</td>
<td>atccgacgatcag</td>
<td>atgtggagccaggttac</td>
<td>N/A</td>
</tr>
</tbody>
</table>

- Peptides. Rat and human Ugn were purchased from Anaspec (Fremont, CA) and Bachem Americas, respectively. Human Ugn exists in two relatively stable isomeric forms (53); we used both isomers for the current studies. Rat Ugn is available only as a fully equilibrated 50:50 mixture of A and B isomers. ST was purchased from Sigma. Rat ANP was purchased from Peninsula Laboratories (Belmont, CA).

**Results**

Expression of preproUgn mRNA and proUgn polypeptide in the rat kidney. Northern blot and RT-PCR studies have consistently shown that the kidney contains very small amounts of preproUgn mRNA, relative to expression levels observed in the intestine. It was therefore surprising when significant levels of proUgn were reported in rat kidney extracts (58, 62), suggesting that the preproUgn mRNA-to-protein ratio might vary significantly from tissue to tissue. To examine this quantitatively, we performed side-by-side Northern and Western blot measurements of steady-state preproUgn mRNA and proUgn levels in rat jejunum and kidney.

Relative mRNA expression was evaluated by hybridizing a 32P-labeled antisense Ugn probe against serial dilutions of RNA isolated from each tissue and quantifying probe binding in each sample with a phosphorimager. Regression lines were then fit to the initial linear portions of each dilution series (Fig. 1A). Based on the slopes of these lines, the 600-bp Ugn transcript is ~100-fold more abundant in jejunal RNA than in renal RNA.

Parallel measurements of polypeptide expression were then performed on Western blots of serially diluted protein extracts, using a propeptide-selective primary antibody (6910). The slopes of the lines fit to the Western blot data (Fig. 1B) show that the 9.4-kDa proUgn molecule is about six times more abundant in the intestine than in the kidney. Thus the data in Fig. 1 identify a substantial mismatch between protein and mRNA expression in the jejunum and kidney, with significantly more proUgn in the latter tissue than would be expected on the basis of its very low preproUgn mRNA levels.

The immunoreactive molecule in kidney is bona fide proUgn. The high polypeptide-to-RNA ratio in the kidney raises the possibility that the renal Western blot signal in Fig. 1B might represent artifactual antibody binding to a target other than authentic proUgn. To investigate this possibility, we performed side-by-side Western blots with three different preproUgn-specific antibodies (6910, 6911, and 6912), each of which was raised independently (see METHODS and Fig. 2A). In the resulting blots, the immunoreactive renal polypeptide was recognized by all three antibodies and had exactly the same molecular weight as proUgn extracted from the jejunum (Fig. 2B). We then dem-
onstrated that the immunoreactive kidney peptide migrated as a single peak on a C₁₈ reverse-phase HPLC column (Fig. 2C) with a retention time that was identical to that of enteric proUgn (Fig. 2D). Finally, we performed a Western blot with kidney extracts obtained from wild-type and Ugn knockout mice and verified that the putative proUgn polypeptide was observed in the former but not the latter (Fig. 2E). Thus, by multiple criteria, the immunoreactive peptide extracted from the rodent kidney appears to be authentic proUgn. Furthermore, previous studies with recombinant standards have shown that proUgn extracted from the rat small intestine is almost exclusively in full-length, unprocessed form (58). Thus the precise alignment of enteric and renal proUgn in Fig. 2A, and the absence of lower molecular weight processing fragments in the kidney extract, demonstrate that renal proUgn is also stored in fully intact form.

The kidney does not contain detectable levels of stored Ugn. To investigate whether the renal pool of proUgn is associated with a stored pool of processed Ugn, we used a competitive binding assay to measure the levels of Ugn-like binding activity in rat kidney extracts (see METHODS). Because the assay can also detect proUgn (very weakly), as well as the structurally related guanylin (Gn) peptide, we used a C₁₈ reverse-phase column to fractionate each extract before performing the assay. We first calibrated the column with synthetic and recombinant rat peptides. Rat Gn eluted in fractions 37 and 38, rat Ugn in fractions 40–43, and rat proUgn in fractions 47–49, as indicated by the black arrowheads in Fig. 3. We then ran a large volume of extract (equivalent to one-third of a kidney) across the column and analyzed fractions 30–65 with the binding assay. No Ugn-like binding activity was detected in fractions 30–65, although a very small peak of activity was present in fraction 38, possibly corresponding to Gn (Fig. 3, C). We then repeated the procedure with six additional extracts, each derived from an independent kidney. In these experiments we focused exclusively on fractions 39–45 (thus n = 7 for fractions 39–45 in Fig. 3A and n = 1 for all other data points in the figure). No peak corresponding to Ugn was observed in any of these individual column runs. To confirm that Ugn would have been detected if present in sufficient quantity, we repeated the procedure using a kidney extract that had been spiked with 10 nmol of Ugn standard. Approximately 70% of the added peptide was recovered (Fig. 3, black symbols). Based on this recovery, and the detection limit of the assay, we calculate that a single rat kidney must contain <100 fmol of free Ugn (n = 7).

For comparison, we then measured the propeptide content of the kidney, using a well-validated quantitative Western blot assay (58). After interpolating the observed kidney proUgn values into a standard curve generated with recombinant rat proUgn (Fig. 3B), we found that a single rat kidney contains 25.5 ± 1.4 pmol of propeptide (means ± SE, n = 12). Thus, as an upper limit, the free Ugn pool in the rat kidney cannot be >0.4% of the proUgn pool. This is comparable to the situation previously reported for the intestine, where the pool of stored Ugn is also drastically smaller than the pool of stored proUgn (58).

Localization of proUgn to distal tubules in rat kidney cortex. We then used immunohistological methods to define the cellular distribution of proUgn within the kidney. Because a previous study observed immunostaining in the kidney with an anti-Ugn antibody but not with an anti-proUgn antibody (62), our initial experiments compared antibody 1–11 (an antibody that recognizes both proUgn and free Ugn; see Fig. 2A) with antibody 6910 (a proUgn-selective antibody). Representative immunohistochemical results with 1–11 and 6910 are presented in Fig. 4, B and C (photomontages correspond approx-

1 Note that this value is lower than a previously published estimate of renal proUgn levels from our laboratory (58). We feel that the current determination is more accurate for two reasons: relative to the earlier study 1) the kidneys in the present study were more rigorously cleared of plasma (which contains very high levels of propeptide), and 2) the recombinant proUgn standard in current use is of higher purity, and its concentration was determined by a more accurate method.
immediately to the boxed region in Fig. 4A). Both antibodies labeled tubular structures that were scattered throughout the renal cortex (C), whereas labeling was not observed when preimmune serum was substituted for the primary antibody (Fig. 4D), or when the primary antibody was preadsorbed with its corresponding peptide antigen (data not shown).

A few tubular structures were also labeled in superficial regions of the outer medulla. However, medullary labeling dropped off rapidly and was not observed in deeper regions of the outer medulla. The inner medulla and papilla could not be evaluated by immuno histochemical methods, due to nonspecific labeling of most of the cellular structures present in these regions (observed even when sections were stained with preimmune serum or with secondary antibody only). However, Western blots performed on individually hand-dissected layers of the kidney provide direct evidence that these regions do not contain significant amounts of proUgn (Fig. 4E).

The cortical staining produced by antibodies 1–11 and 6910 is shown at higher magnification in Fig. 4, F and G, respectively. As in Fig. 4D, no immunoreactive profiles were observed in nearby sections stained with preimmune serum (Fig. 4H). The labeled structures were clearly identifiable as renal tubules (and distinguishable from glomeruli and blood vessels) on the basis of their sizes and characteristic morphologies.

Further analysis revealed that the populations of tubules labeled by each antibody were identical in terms of their mean diameters (Supplementary Fig. S1a; all supplementary material for this article is available on the journal web site), relative abundances (Supplementary Fig. S1b), and distributions within the cortex (Supplementary Fig. S1c), arguing strongly that both antibodies were targeting the same population of tubules. This, coupled with the failure to observe detectable levels of free Ugn in kidney extracts (Fig. 3), provides convincing evidence against the previously reported conclusion that the renal pool of proUgn does not represent filtered material that had adsorbed to luminal surfaces of the tubular lumen and resembles the brush-border staining that we had observed with the anti-CD26 antibody (2 examples are marked by yellow arrowheads). By comparing Fig. 5F to the corresponding autofluorescence image in Fig. 5G, it is evident that the former structures were only marginally autofluorescent (thus corresponding to distal tubules) while the latter structures were strongly autofluorescent (thus corresponding to proximal tubules). Figure 5H shows the distribution of proUgn-positive tubules in the same section. The PNA-positive profiles marked with white arrowheads (distal) were immunopositive for proUgn, whereas those marked with the yellow arrowheads (proximal) did not stain for proUgn. As seen in the merged image (Fig. 5I), every tubule in the section that can be identified as distal [i.e., PNA-positive (red) but not strongly autofluorescent (very faintly green)] was positive for proUgn whereas every tubule that can be identified as proximal (i.e., bright green, due to strong autofluorescence) was negative for proUgn. These correlations were confirmed in every double-labeled section that we examined. Thus proUgn is expressed only in distal tubules and appears to be present in all distal tubules.

The distal components of the nephron that reside in the cortex include the distal convoluted tubules, the connecting tubules, and the cortical collecting ducts. The connecting tubules and cortical collecting ducts express aquaporin-2 (AQP2), while the distal convoluted tubules do not (14). Figure 5J shows AQP2 staining in the kidney cortex (red profiles). Every single AQP2-positive tubule was also immunoreactive for proUgn (blue profiles), as can be seen by comparing Fig. 5J with Fig. 5L, or by examining the merged image in Fig. 5M (for reference, 2 AQP2-positive, proUgn-positive tubules are marked by white arrowheads). We consistently observed an identical colabeling pattern in numerous tissue sections. The distal identity of the AQP2-positive tubules was verified by their lack of autofluorescence (white arrowheads in Fig. 5, K and M). The observation that every AQP2-positive tubule also stained positively for proUgn indicates that the propeptide is expressed in both AQP2-positive nephron segments (the connecting tubules and the cortical collecting ducts). In addition, however, a number of proUgn-positive tubules were also AQP2 negative (2 examples are marked by the yellow arrowheads). These AQP2-negative tubules are also distal (because...
they were nonautofluorescent; Fig. 5, K and M) and therefore must correspond to the distal convoluted tubules.

Taken together, the immunohistochemical data presented in Fig. 5, C–M (and summarized in Table 2) indicate that proUgn is broadly expressed throughout the distal nephron, whereas it is completely absent from the proximal components of the nephron.

**GC-C (enteric Ugn receptor) is not appreciably expressed in the rat kidney.** Although it is universally acknowledged that GC-C mRNA is abundant in the intestine, only some studies have reported its presence in the kidney (4, 5, 17, 38, 67, 78), while other studies support the opposite conclusion (9, 42, 49, 77, 84). Interpretation of these published data is further complicated by differences in the probes, primers, techniques, and animal species that have been employed by different laboratories. Here, we have used two different primer pairs (Table 1) to evaluate GC-C expression in the Sprague-Dawley rat, using both traditional endpoint RT-PCR (primer set A) and quantitative real-time RT-PCR (primer set B).

Figure 6A shows endpoint RT-PCR results. When we analyzed RNA isolated from proximal jejunum, a well-defined amplicon of the appropriate size (340 bp) appeared at cycle 27 and increased in abundance during subsequent cycles until it reached a plateau level at around cycle 40. No such PCR product was evident if the RNA sample was not reverse transcribed to cDNA, even after 45 cycles (data not shown). By contrast, when we analyzed kidney RNA, the only observable amplification products were low-molecular-weight primer...
dimers and a diffuse background that increased over time but did not resolve into an individual amplicon. To assess the quality and integrity of the input RNA used for these experiments, the same intestinal and renal samples were tested with primers specific for rat cyclophilin A, a housekeeping gene that is consistently expressed in most tissues. In both samples, an appropriately sized amplicon appeared at cycle 17 and increased to a plateau by cycle 27, suggesting that mRNA degradation was not a significant problem in either case.

To measure GC-C expression more quantitatively, we then developed primers and fluorogenic probes for real-time RT-PCR analysis. Using this method, we found that cycle threshold (CT) values for β-actin, another commonly measured housekeeping gene, were very similar for RNA isolated from the jejunum and kidney (Fig. 6B, gray bars), while CT values for GC-C diverged widely between the two tissues (Fig. 6B, white bars). The average CT for GC-C was close to that of β-actin in the small intestine but very much higher than that of β-actin in the kidney. Indeed, the CT value plotted for the kidney is an underestimate, as 18 of 23 amplification reactions did not reach threshold within 40 cycles (when the PCR reaction was terminated) and were therefore assigned nominal CT values of 40. GC-C expression was then normalized to β-actin expression for each tissue by determining ΔCT (defined as CT of β-actin – CT of GC-C for each sample), and relative expression levels were calculated as 2−ΔCT (Fig. 6B, black bars). Consistent with the more qualitative data presented in Fig. 6A, rat intestine shows abundant GC-C expression, while rat kidney shows exceedingly low expression that, in most cases, fell below the limit of detection.

Does Ugn regulate cGMP metabolism in the rat kidney? The failure to detect significant renal expression of GC-C mRNA (Fig. 6) raises the possibility that the natriuretic actions of Ugn in the rat kidney are cGMP independent. However, it is also possible that Ugn does affect cGMP metabolism but does so by acting through a signal-transducing guanylate cyclase that is distinct from GC-C. Therefore, to directly investigate the effect of Ugn on renal cGMP metabolism, we measured urinary excretion of cGMP before, during, and after intravascular infusion of Ugn. This well-established procedure has been used as a sensitive way to detect coupling of other natriuretic agents, such as ANP or nitric oxide, to the cGMP second messenger cascade (28). Indeed, our initial control experiments demonstrated a rapid and robust increase in urinary cGMP levels during intravascular infusion of ANP at a dose of 7 nmol·h−1·kg body wt−1 (Fig. 7A, white symbols, n = 2).

We then examined the effect of Ugn in the same experimental setting. For these experiments, we used human Ugn, which is available in two isomeric forms. The A isomer is a potent ligand for GC-C and produces large cGMP increases in GC-C-expressing cells, whereas the B isomer has no known effect on cGMP metabolism in any target cell, although it is a very effective natriuretic agent in the rat kidney (60). As might be expected from its lack of effect on cGMP metabolism in other systems, Ugn B did not stimulate urinary cGMP excretion by the rat kidney when infused at 50 nmol·h−1·kg body wt−1 (Fig. 7A, gray symbols, n = 3), a dose that evokes maximal natriuretic activity in rats. Perhaps less predictably, Ugn A also did not stimulate urinary cGMP excretion when delivered at 25 (Fig. 7A, black symbols, n = 3), 50 (data not shown, n = 5), or 200 nmol·h−1·kg body wt−1 (data not shown, n = 1). These doses span the range over which infused human Ugn A produces maximal natriuretic responses in rats (60). The physiological efficacy of the peptides thus argues that their failure to evoke cGMP production does not reflect a failure to reach appropriate receptors. Furthermore, previous studies have documented substantial recovery of human UgnA and B in the urine after intravascular infusion (60), confirming that both peptides would have the opportunity to interact with luminally oriented receptors at any point along the nephron.

It has also been reported that infused proUgn is natriuretic (58, 59), presumably because the propeptide is converted to Ugn and other metabolites within the renal tubules (68). Interestingly, a recent study (59) has shown that, mole-for-mole, proUgn is a more potent natriuretic agent than Ugn, suggesting that intrarenal processing of proUgn may produce an alternate metabolite that is more active than Ugn. To test whether proUgn and/or any of its metabolites might activate cGMP synthesis, we measured urinary cGMP levels before, during, and after infusion of proUgn at a natriuretic dose of 10 nmol·h−1·kg body wt−1. No change in cGMP excretion was evoked by the infusion (Fig. 7B, black symbols). We also measured Ugn levels in the same urine samples and observed

Table 2. Histological characteristics of tubules in the rat kidney cortex

<table>
<thead>
<tr>
<th>CD26</th>
<th>Autofluorescence</th>
<th>PNA</th>
<th>AQP2</th>
<th>proUgn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early proximal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Late proximal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Distal convoluted tubule</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Connecting tubule</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Initial collecting tubule</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cortical collecting duct</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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</table>

Histological properties (described in the literature) define distinct types of cortical tubules. Based on these criteria, prouroguanylin (proUgn)-positive tubules correspond to distal components of the nephron (distal convoluted tubules, connecting tubules, initial collecting tubules, and cortical collecting ducts) but not to proximal components of the nephron (early and late proximal tubules). PNA, peanut agglutinin; AQP2, aquaporin-2.
a large increase in Ugn excretion during and after proUgn infusion (Fig. 7B, white symbols), confirming that processing of the propeptide had occurred in these experiments.

Thus the data in Fig. 7, A and B, are consistent with the idea that, unlike ANP, neither Ugn nor proUgn regulates renal sodium excretion in a cGMP-dependent manner. One caveat to this interpretation, however, is that cGMP might have been produced in response to peptide infusions but was retained in an impermeable intracellular compartment and/or broken down by a phosphodiesterase before it could reach the urine. To test this, we performed standard ligand-activated guanylate cyclase assays with broken cell preparations, in the presence of IBMX, a broad-spectrum phosphodiesterase inhibitor. We prepared membranes for these assays from three different regions of the rat kidney (cortex, medulla, or papilla) to increase the likelihood that we would observe a response even if it was restricted to a specific anatomic region. We also used rat Ugn (a spontaneously equilibrating 50:50 mixture of A and B isomers) to avoid possible complications that might arise from applying the human peptide to rat receptors. Under these conditions (Fig. 7C), we could still observe a prominent stimulatory effect of ANP (10^{-6} M), which was most evident with membranes prepared from isolated papillae. However, neither rat Ugn (10^{-6} M), nor its more potent analog ST (100 U/ml), had any effect on cyclase activity in membranes derived from any region of the kidney.

**DISCUSSION**

Our studies provide a comprehensive description of Ugn and GC-C expression in the kidney of the Sprague-Dawley rat. The principal conclusions are as follows. 1) The rat kidney contains significant amounts of authentic proUgn (16% of the level found in the intestine; Fig. 1B), despite expressing only very low amounts of preproUgn mRNA (1% of the level found in the intestine; Fig. 1A). 2) Using a sensitive and selective binding assay, we failed to detect Ugn itself in extracts of rat kidney (Fig. 3). Based on the limit of detection of the assay, the pool of free Ugn maintained by kidney (if any) is <0.4% of the pool of proUgn. Thus very little of the proUgn stored within the kidney is processed intracellularly to mature Ugn. 3) The relatively abundant intrarenal proUgn pool is localized primarily to the kidney cortex (Fig. 4 and Supplementary Fig. S1c), where it is restricted to distal convoluted tubules, connecting tubules, and cortical collecting ducts (Fig. 5 and Table 2). 4) GC-C, the previously identified Ugn receptor, is abundant in the intestine, but nearly undetectable in the kidney at either the mRNA level (based on RT-PCR analysis; Fig. 6) or the functional level (based on measurements of Ugn-evoked cGMP synthesis; Fig. 7). Thus, consistent with many previous studies, these observations support the well-established hypothesis that Ugn signaling in the kidney involves a receptor that is distinct from GC-C (3, 4, 60, 79, 80, 81).

**Renal expression of proUgn.** Ugn was originally discovered in urine (32, 40). A potential tissue source was subsequently defined when Ugn was purified and sequenced from intestinal extracts (31, 33, 45). This biochemical evidence for enteric production of Ugn has since been corroborated and extended by numerous Northern blot studies showing high and relatively selective expression of preproUgn mRNA in the intestine (2, 11, 19, 45, 49, 55–57, 67, 88). However, several of these Northern blot studies have also noted the presence of very low levels of preproUgn mRNA in the kidney, an observation that has been consistently confirmed by more sensitive RT-PCR assays (5, 13, 36, 50). Here, we show that these low levels of renal mRNA are associated with an unexpectedly high abundance of preproUgn polypeptide.

The reason for the divergent polypeptide-to-mRNA ratios in the kidney and intestine is unclear. Possibilities include 1) an inherently high rate of proUgn synthesis in the kidney, relative to that of the intestine; 2) comparable transcription and translation rates in the two tissues, but a higher rate of propeptide processing, secretion, and/or degradation in the intestine; or...
3) a renal transport mechanism that translocates previously synthesized propeptide from the plasma (where it is plentiful) into the kidney, with no need for significant local transcription. Convincing evidence against the latter of these hypotheses has been provided by a study that traced the metabolic fate of radiolabeled recombinant proUgn after intravascular infusion (68). Although these experiments did reveal significant accumulation of radioactivity in the kidney, the retained material was almost entirely in the form of free amino acids, rather than intact proUgn, and was associated with proximal tubules, rather than with the distal tubular profiles that we have currently identified as the site of endogenous renal proUgn expression (Fig. 5). Thus circulating propeptide does not appear to contribute to the pool of proUgn that resides in the distal tubules.

**ProUgn localization.** Our identification of the distal tubule as the site of proUgn expression in the rat kidney can be compared with previous renal localization studies, in which Ugn-like immunoreactivity has been reported in proximal tubules (23), distal tubules (62), and an unidentified tubular compartment that is restricted to the corticomedullary border (7, 85). Our results are in best agreement with the analysis of Nakazato et al. (62), who identified the distal convoluted tubule as the site of expression of Ugn. However, the immunostaining observed in the Nakazato study was obtained with an antibody directed against the mature Ugn peptide, whereas the propeptide-selective antibody 6912 failed to produce a detectable signal in their experiments. For this reason, the authors concluded that the kidney contains free Ugn, but little or no proUgn, a conclusion that contrasts strikingly with the data that we present in Figs. 1–5.

There are several likely explanations for the immunohistochemical discrepancy between our results and those of Nakazato et al. (62). First, we fixed our kidneys with Zamboni’s fixative, which includes both paraformaldehyde and picric acid, whereas the Nakazato study employed a formaldehyde/phosphate buffer-based fixative. In previous analyses of enteric proGn and proUgn expression, we have found that the antigenicity of the propeptides was much better preserved with Zamboni’s fixative than with fixatives that lack picric acid (46, 64). Second, our experiments focused on anti-proUgn antibodies 6910 and 6911, whereas the Nakazato experiments focused on anti-proUgn antibody 6912. In our hands, the latter is a relatively ineffective histochemical reagent, producing weak signals in the intestine and nearly undetectable signals in the kidney (data not shown).

The localization of authentic proUgn to distal tubules raises intriguing issues about Ugn signaling. 1) Most importantly, the kidney must now be recognized as a second major tissue reservoir for the propeptide, presumably operating in parallel with the small intestine. This introduces the possibility that the abundant urinary pool of Ugn (12, 40) could be derived from renal proUgn, from enteric proUgn, or from propeptide supplied by both tissues. Indeed, each tissue source could play a distinct role in regulating whole body sodium balance, as is the case for other endocrine agents that are generated both extra- and intrarenally [such as angiotensin (41) and ANP/urodilatin (34)].

2) The kidney does not secrete proUgn into the circulation (58). Thus any proUgn released from cells in the distal nephron would likely be delivered directly to the tubular lumen. Furthermore, since proUgn cannot be detected in normal rat urine (62) (59), any such luminal propeptide must be converted to Ugn before excretion. Most of the currently recognized luminal active proteases in the kidney are restricted to the proximal tubule (26) and thus upstream from the site of renal proUgn expression. Nevertheless, the distal nephron contains several membrane-associated ectoproteases that could potentially serve as luminal processing enzymes (63, 66, 83). In addition, a broad spectrum of soluble proteases has been identified in urine (69), implying that these enzymes are also present in the luminal fluid within the distal nephron. Thus mechanisms are available that could potentially generate Ugn from distally secreted proUgn.

3) A distal site of proUgn expression also implies a distal site of physiological action. Natriuretic agents that inhibit sodium reabsorption late in the nephron (such as ANP) typically evoke a natriuresis without a significant kaliuresis (70). This is consistent with the previously described saluretic effects of infused proUgn in the anesthetized rat, where prominent increases in total and fractional sodium excretion were observed with either marginal increases (58) or net decreases (59) in total and fractional excretion of potassium. However, unlike ANP, which acts in the collecting duct exclusively through a cGMP-dependent mechanism (39), our current experiments reveal that the effects of Ugn are strikingly independent of cGMP (Fig. 7).

**GC-C expression in the rat kidney.** The failure to implicate cGMP in the renal actions of Ugn is somewhat surprising, given that Ugn has long been linked to intestinal cGMP metabolism through the catalytic activity of its well-known enteric receptor, GC-C (18). However, the mouse kidney responds to Ugn after genetic ablation of the GC-C gene (3, 4, 80), and the rat kidney displays robust responses to Ugn (4, 16, 17, 59, 60, 74) despite its failure to express detectable levels of GC-C mRNA or protein in RT-PCR assays (Fig. 6), functional assays (Fig. 7), or Western blot assays (67). Thus it appears that the rodent kidney contains an alternate Ugn receptor that mediates the effects of the peptide in the absence of GC-C. Further corroboration of this hypothesis is provided by reports of GC-C-independent responses to Ugn in rat kidneys (60), isolated mouse cortical collecting ducts (80), isolated human cortical collecting ducts (78), and a human kidney epithelial cell line (81), as well as by the observation that GC-C knockout mice excrete orally delivered sodium normally, in contrast to the delayed sodium excretion and hypertension that characterize Ugn knockout mice, presumably because the kidneys of the GC-C-deficient animals respond to Ugn via this alternate receptor (10).

Despite this abundant evidence that GC-C is not required to support renal responses to Ugn, it must be acknowledged that several previous RT-PCR studies have reported GC-C expression in the rat kidney (5, 17). We are not sure why these results differ from ours. However, we do note that GC-C expression has proven to be relatively malleable. Thus GC-C mRNA is ectopically expressed in the rat liver during fetal development (42), as well as during postinjury hepatic regeneration (52, 76). Expression is also observed in esophageal adenocarcinoma cells (but not normal esophageal epithelium) and is further upregulated in these neoplastic cells after exposure to bile acids (8). In addition, GC-C expression shows a pronounced diurnal rhythm in the rat intestine (75) and is affected by oral salt...
intake in both the kidney (17) and intestine (44). Thus some of the variability in renal GC-C expression that has been reported in the literature may reflect differences in the handling, feeding, or harvesting of experimental animals. Indeed, even in our own study, 5 of the 24 rats included in our real-time RT-PCR analysis showed presumptive evidence for a small amount of renal GC-C expression (although the levels in these animals were still exceedingly low relative to the levels in the intestine). Thus, although GC-C is undoubtedly not the main receptor that mediates the natriuretic effects of Ugn in the rat kidney, it may regulate an as-yet undefined component of renal function in some animals under some conditions.

Perspectives. We have presented evidence that the rat kidney contains unexpectedly large amounts of proUgn, relative to its strikingly low levels of preproUgn mRNA. Thus the propeptide is relatively abundant, and apparently selectively expressed, in the tissues that control the flow of sodium into and out of the body (the intestine and the kidney, respectively). Like the intestine, the kidney does not process its intracellularly stored pool of propeptide before secretion but presumably releases it intact for subsequent extracellular processing. Given the restricted expression of proUgn in the distal nephron, and the previous observation that the kidney makes little or no contribution to the circulating plasma pool of propeptide (58), we believe that the most likely processing site for renal proUgn is within the tubular lumen at a distal site. However, this hypothesis has not been confirmed, and the protease(s) that would act at this location (and the processing products that they might generate) have not yet been identified. The receptor that mediates the natriuretic effects of Ugn or proUgn within the kidney is also not known, although our data strongly support the idea that it is not GC-C, and does not employ a cGMP-dependent signaling mechanism. Intriguingly, this raises the likelihood that such a novel receptor would display a ligand specificity that is distinct from that of GC-C. In this regard, we have recently found that intravascularly infused proUgn actually evokes a more effective natriuresis than an equivalent dose of intravascularly infused Ugn (59), arguing that proUgn is converted within the kidney to an as-yet unidentified natriuretic peptide that is distinct from, and more potent than, Ugn. Thus the Ugn signaling pathways in the gut and kidney appear to have diverged significantly with respect to the receptors, the ligands, and the propeptide processing mechanisms that are active in each tissue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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