Localization of connexin 30 in the luminal membrane of cells in the distal nephron

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Connexin (Cx) proteins are the building blocks of gap junction channels that allow electric coupling and the cell-to-cell diffusion of ions and signaling molecules between the cytoplasm of two adjacent cells. Gap junctions are common features in a variety of tissues and cell types and are essential components of intercellular communication in many physiological processes. Apart from the classic gap junction between two cells, Cx proteins can form large, nonselective ion channels on the surface of a single cell. The existence of single connexons (a hexamer of Cx proteins) in the nonjunctional plasma membrane raised the intriguing possibility that Cx proteins may function as transmembrane ion channels in addition to serving as precursors for the formation of gap junction channels (8, 11). It is now widely acknowledged that these so-called hemichannels indeed exist and are functional. They are permeable to a variety of small metabolites (including nucleotides, ATP, and NAD+) and function in paracrine signaling in many cell types (8).

In humans, there are at least 20 Cx genes. Some of the ubiquitous Cx isoforms (Cx37, Cx40, Cx43, and Cx45) have been identified in the kidney and localized to mainly vascular and glomerular components (2, 3). For example, intercellular communication between the cells of the juxtaglomerular apparatus involves an ATP release-mediated calcium wave that regulates contractile function as well as renin secretion (14, 33, 34). Although most cells of the juxtaglomerular apparatus are interconnected via gap junctions composed of Cx40, Cx43, and Cx45, the spreading of this calcium wave does not appear to require classic gap junction channels or physical contact between cells (33). This paracrine, ATP-dependent, and purinergic receptor-mediated intercellular calcium wave (34) may involve hemichannel function, because Cx hemichannels are top candidates for the plasma membrane ATP channel. However, the exact molecular identity of this channel is still to be determined (14). Also, the most ubiquitous Cx isoform, Cx43, has been localized to tubule segments, including the rat collecting duct system (3). However, the classic gap junction channels, in terms of electron microscopy, are reportedly absent (3). Activation of Cx43 hemichannels may cause cell damage in renal proximal tubule (PT) cells in culture (31).

Cx30 is a newly identified member of the Cx gene family. Cx30 was isolated by screening a mouse genomic library with a rat Cx26 probe (6). The Cx30 protein is highly homologous to Cx26 and has an additional 37 amino acids at its COOH terminus. Cx30 is considered the adult form of Cx26, because their expression patterns are clearly distinct. Specifically, Cx30 is highly expressed in adult skin and brain. However, it cannot be detected in the embryonic and fetal brain (6, 22). On the other hand, Cx26 is highly expressed in prenatal brain and decreases after birth. Importantly, the renal expression of Cx30 has been recently suggested (13).

This study provides evidence that the Cx30 protein, perhaps in the form of luminal hemichannels, is expressed in renal tubular epithelial cells and suggests that it may have a potential role in the regulation of salt reabsorption in the distal nephron.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats (200 g, Harlan, Madison, WI) were fed standard chow (0.3% NaCl), a high-salt diet (TD 92012: 8% NaCl, Harlan Teklad, Madison, WI), or a low-salt diet (TD 90228: 0.01% NaCl) for 2–3 days. The animals were killed by cervical dislocation, and the kidneys were excised and placed into ice-cold PBS.
NaCl, Harlan) for 1 wk. Rats fed the high-salt diet also received 0.45% NaCl (wt/vol)-containing drinking water. Swiss albino mice (20 g, in-house bred) and New Zealand White rabbits (500 g, Irish Farm, Norco, CA) were maintained on a standard diet with normal water. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Southern California and Institut National de la Santé et de la Recherche Médicale.

Antibodies. Mouse monoclonal anti-Cx30 and rabbit polyclonal anti-Cx30 antibodies were purchased from Zymed Laboratories (San Francisco, CA). The Cx30 blocking peptide was kindly provided by Zymed. These antibodies have been previously characterized (20–22, 24, 32), and the Cx30 antibody used in the present studies has been demonstrated to recognize Cx30 in the brain and cochlea using both immunoblot and immunohistochemistry of mouse and rat tissue (22, 24, 32). The mouse monoclonal anti-pendrin antibody was purchased from MBL International (Woburn, MA). Rabbit polyclonal anti-aquaporin-2 (AQP2) antibody (23) was a generous gift from Dr. Mark Knepper. Rabbit polyclonal antibody to the thiazide-sensitive NaCl cotransporter (NCC) was a gift from Dr. D. H. Ellison (Oregon Health and Science University, Portland, OR) and has also been previously extensively characterized (9, 26, 27). The mouse monoclonal antibody to rat anion exchanger AE1 (1) was kindly provided by Dr. Daniel Biemesderfer (Yale University, New Haven, CT). The NCC, pendrin, AE1, and AQP2 antibodies are from the original batch of immunization, which were initially used and characterized in previous publications (4, 17, 23).

Cell cultures. Renal medullary interstitial cells (RMIC) were a kind gift from Dr. Christine Marie (Georgetown University, Washington, DC) and have been previously characterized (19). The M1 cell line has a mixed phenotype, representative of both intercalated and principal cells of the collecting duct. These cells were originally purchased from the American Type Culture Collection and have been described by Fejes-Toth et al. (10).

Immunoblotting of rat tissue. Rats were anesthetized with 100 mg/ml Inactin, and kidneys were perfused retrograde with ice-cold PBS to remove blood and then removed. Slices of cortex and inner medulla were manually dissected, and tissue was homogenized with a rotor-stator homogenizer in a buffer containing 20 mM Tris–HCl, 1 mM EGTA, pH 7.0, and a protease inhibitor cocktail (BD Bioscience, San Jose, CA). Samples were centrifuged at low speed to pellet cellular debris, and the supernatant was collected and assayed. Forty micrograms of protein were run per lane, separated on a 4–20% SDS-polyacrylamide gel, and then transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After the membrane was blocked in 5% nonfat dry milk, immunoblotting was performed with a rabbit polyclonal antibody to Cx30 at a dilution of 1:250 (Zymed). Reactivity was detected by a horseradish peroxidase (HRP)-labeled goat anti-rabbit (1:1,000 dilution, BD Biosciences) or donkey anti-goat (1:1,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibody. An enhanced chemiluminescence kit (Amersham Biosciences, Little Chalfont, UK) was used to visualize the secondary antibody. The blot was stripped and reprobed with a goat polyclonal antibody to actin at a dilution of 1:1,000 (Santa Cruz Biotechnology) to test for protein loading and quality of transfer.

Densitometric analysis of blots was performed using ImageJ (National Institutes of Health) software. The data were then normalized against the control sample, and an average for each group (n = 4) was calculated. Statistical significance was tested using ANOVA, and data are shown as means ± SE.

Immunoblotting of cultured cells. RMIC and M1 cells were grown to confluence in plates as previously described (10, 19). Plates were then treated for 16 h with either 110 mM NaCl-supplemented media (high salt) or 220 mM-namnlitol supplemented media (to control for osmolality). Cells were lysed using CellLytic-M lysis buffer (Sigma) according to the manufacturer’s instructions and assayed for protein concentration by a modified Bradford method (Quick Start Bradford protein assay, Bio-Rad). Samples were blotted and analyzed for Cx30 and actin as described earlier.

Immunoperoxidase labeling of kidney tissue. Rat kidneys were fixed in situ by perfusion of 4% paraformaldehyde in Dulbecco’s Modified Eagle’s/F12 medium (Invitrogen, Carlsbad, CA). Coronal kidney sections containing all kidney zones were then postfixed for 4–6 h at 4°C in 4% paraformaldehyde and then embedded in paraffin. Subsequently, 4-μm sections of the paraffin block were deparaffinized in toluene and rehydrated through graded ethanol. Rehydration was completed in Tris-buffered saline (TBS), pH 7.6. Slides were then placed in a plastic tank filled with Target Retrieval Solution (Dako) and heated 3 x 5 min in a microwave at medium (450 W) heat. These steps unmasked antigens and allowed immunostaining of parafin-embedded fixed paraffin sections, as determined in preliminary experiments (not shown). To reduce nonspecific binding, sections were rinsed in TBS for 10 min and preincubated for 15 min with 20% normal goat serum, followed by treatment with background-reducing buffer (Dako) for 20 min. Rat kidney sections were then labeled with the rabbit polyclonal Cx30 antibody as follows. Anti-Cx30 was applied for 1 h at room temperature. After three washes, sections were incubated with a 1:600 dilution (in background-reducing buffer) of goat anti-rabbit IgG coupled to horseradish peroxidase (Vector Laboratories, Burlingame, CA) in TBS, 30 min at room temperature, followed by three TBS washes. Peroxidase activity was revealed with 3-aminoo-9-ethylcarbazole, which produces a red-brown precipitate.

To ascertain the presence of Cx30 in distal convoluted tubules (DCT), immunostainings of the same cells with antibodies to Cx30 and to the thiazide-sensitive NaCl cotransporter (NCC) were performed in two consecutive 4-μm-thick sections. Consecutive sections were also stained with an antibody to pendrin to identify connecting tubules (CNT) and cortical collecting ducts (CCD) and with an antibody to the chloride bicarbonate exchanger AE1 to identify the medullary collecting duct (MCD). Labeling for these three antibodies was performed using the same method as for Cx30 except that the antigen retrieval procedure consisted of a 40-min heating at 96–98°C in a water bath of the sections in 1 mM EDTA. Sections were then incubated with a 1:200 dilution of a rabbit anti-NCC, or 1:200 dilution of rabbit anti-AE1 antibody containing serum in place of the anti-Cx30 peptide antibody.

After staining, sections were counterstained with hematoxylin. Glass coverslips were mounted after liquid-phase Faramount solution (Dako) was applied to the tissue sections. Sections were examined with a Zeiss microscope.

Immunofluorescence labeling of kidney tissue. A method similar to the one described above was used to prepare animal tissue for immunofluorescence labeling. Blocking with goat anti-rabbit Fab IgG for rabbit tissue (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) for 40 min was used to reduce nonspecific binding with a rabbit polyclonal antibody. Sections were then incubated with Cx30 antibody at a 1:50 dilution overnight and washed in PBS. Sections were then incubated with HRP-conjugated goat anti-rabbit IgG and enhanced with Alexa Fluar 594-labeled tyramide signal amplification (TSA) according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). Some rabbit tissue sections were double-labeled with an anti-pendrin monoclonal antibody overnight at a 1:50 dilution, and the secondary and TSA steps were repeated as above, except using an HRP-conjugated goat anti-mouse IgG and Alexa Fluar 488 TSA (Molecular Probes). Following a wash step, sections were mounted with Vectashield mounting media containing the nuclear stain 4’,6’-diamindino-2-phenylindole (Vector Laboratories) and examined with a Leica TCS SP2 confocal microscope. All sections were labeled in parallel.

RT-PCR. Total RNA was purified from whole mouse kidney samples using a Total RNA Mini Kit in accordance with the manufacturer’s instructions (Bio-Rad). RNA was then quantified using spectrophotometry and reverse-transcribed to single-strand cDNA using avian reverse-transcriptase and random hexamers according to
RESULTS

Detection of Cx30 mRNA and protein in the kidney. RNA was isolated from whole mouse kidney, and the presence of Cx30 mRNA was detected using RT-PCR (Fig. 1A). A negative control (lack of template in the PCR reaction) and a positive control (β-actin) were also employed. Using ethidium bromide, we observed a band ~369 bp in size for the Cx30 sample, as expected. Negative control samples produced no visible bands, and β-actin samples produced bands of the expected size (400 bp). Western blotting (Fig. 1B) using a Cx30 antibody resulted in a single band around the expected molecular weight of 30 kDa, within the 25- to 62-kDa range of known Cx isoforms.

Immunolocalization of Cx30 in the rat kidney. Localization of Cx30 in the rat kidney was investigated by indirect immunoperoxidase on paraformaldehyde-fixed, paraffin-embedded kidney sections. Staining for Cx30 was restricted to some tubular structures and was observed in all major anatomic parts of the kidney: within the cortex, outer medulla, and inner medulla (Fig. 2, A–C). In the cortical labyrinth (Fig. 2A) and the medullary ray (Fig. 2, B and C), anti-Cx30 antibody strongly labeled the apical pole of tubules with additional, less intense staining of the cytosol (shown in insets). No basolateral staining was observed in any nephron segments. These Cx30-positive tubule segments were devoid of brush-border membranes and with morphological features of cortical thick ascending limb (cTAL), DCT, CNT, CCD, and MCD. Weak apical Cx30 staining was also observed in macula densa (MD) cells (Fig. 2A). Proximal tubules, the thin limbs of the loop of Henle, glomeruli, and the vasculature were devoid of staining.

Cx30-positive tubular segments were next identified in consecutive sections (Fig. 2, D–I) using well-known tubular markers. Staining consecutive sections with an anti-NCC antibody that identified the apical membrane of DCT (Fig. 2D) demonstrated that not only the DCT but also its downstream segment CNT both expressed Cx30 (Fig. 2E). Similarly, AE1 staining labeled the basolateral membrane of type A intercalated cells in the outer MCD (OMCD) (Fig. 2F). Staining of a consecutive section with the Cx30 antibody confirmed that the OMCD as well as the medullary TAL (MTAL) both expressed Cx30 (Fig. 2G). In the last pair of consecutive sections, pendrin, a marker of type B and non-A, non-B intercalated cells identified the CNT and CCD (Fig. 2H). These nephron segments also expressed Cx30 (Fig. 2I).

The same pattern of labeling was seen in rat sections using immunofluorescence techniques (not shown).

Immunolocalization of Cx30 in the rabbit kidney. Localization of Cx30 in the rabbit kidney was studied by immunofluorescence. Staining for Cx30 was restricted to certain tubular and interstitial structures in the cortex and outer medulla (Figs. 3, B–F). No staining was observed in the inner medulla (not shown), in vascular structures, and in the glomerulus. The most intense Cx30 labeling was observed at the apical plasma membrane of select cells in the CNT and CCD (Fig. 3B). These cells were identified as intercalated cells, because in some of these cells Cx30 was colocalized with pendrin (Fig. 3, A and C), an apical membrane anion exchanger of type B and non-A, non-B intercalated cells. Intercalated cells showed continuous luminal plasma membrane staining for both pendrin (Fig. 3A) and Cx30 (Fig. 3B).

Additional, weak Cx30 labeling was observed at the apical membrane of the cTAL; however, the MD was devoid of staining (Fig. 3D). In addition, renal medullary interstitial cells in the outer medulla showed expression of Cx30 (Fig. 3E). While most of these cells displayed a cytosolic staining pattern, some cells showed Cx30 localization at the end of the cell processes making contact with tubular cells. Weak cytosolic Cx30 staining was observed in the MTAL (Fig. 3E).

In addition to the luminal plasma membrane labeling, while very rare, we observed highly scattered and punctuate labeling in both the cortex and medulla between cells of the tubular epithelium, vasculature, and glomerulus, consistent with the antibody recognizing gap junction proteins (Fig. 3F). Preincubation of the Cx30 antibody with the blocking peptide resulted in absolutely no labeling (Fig. 4).

Using a Cx26-specific antibody, no labeling was found in the adult rabbit kidney, consistent with Cx26 being an embryonic isoform (not shown).

Immunolocalization of Cx30 in the mouse kidney. Localization of Cx30 in the mouse kidney was studied by immunofluorescence. Staining for Cx30 was present only in the cortex and restricted to the apical membrane of certain tubular cells of the CCD (Fig. 5). Double-labeling with an AQP2 antibody identified the apical membrane of principal cells in the CCD (Fig. 5). Principal cells were devoid of Cx30 staining, but all other cells of the CCD showed intense, apical Cx30 labeling.
Similar to the case in the rabbit (Fig. 4), preincubation of the Cx30 antibody with the blocking peptide resulted in absolutely no labeling in the mouse (not shown).

Regulation of Cx30 protein expression by dietary salt in rat kidney: an immunofluorescence approach. To help ascertain a functional role for Cx30 in the kidney, we tested whether changes in dietary salt content regulate the expression of Cx30 in the rat kidney. Using confocal microscopy and immunofluorescence, we observed a sharp upregulation of Cx30 expression in the rat inner medulla (IMCD) from high-salt-fed rats (Fig. 6B) compared with control (Fig. 6A) and low-salt-fed rats (Fig. 6C) under the same instrument imaging settings. Particularly in the high-salt kidney, Cx30 labeling was localized at the apical pole of cells in the IMCD (Fig. 6B).

Regulation of Cx30 protein expression by dietary salt in rat kidney: an immunoblotting approach. Using tissue samples from four rats per experimental group, we performed Western blotting using a Cx30 antibody, as well as an actin antibody as a loading control (Fig. 7, A and B). After densitometric analysis, a significant upregulation of Cx30 (approximate molecular weight of 30 kDa) was observed in samples from the inner medulla of high-salt-fed diet rats compared with control and low-salt groups (Fig. 7C). Inner medulla low-salt samples were not significantly different from control. While a difference between groups was occasionally seen in the cortical samples, it was not found to be statistically significant.

Regulation of Cx30 protein expression by salt in RMIC and M1 cell lines. To further investigate the role that dietary salt plays in the expression of Cx30, RMIC and M1 cell lines were treated with a control, high-salt-, or mannitol-supplemented media for 16 h before lysis and immunoblotted for Cx30 (Fig. 8, A and B). The mannitol-supplemented media served as a control for osmolality. RMIC cells showed no significant increase in Cx30 levels under high-salt conditions, and there
was no significant difference between control and mannitol treatment groups (Fig. 8C). In M1 cell lines, we observed increased expression of Cx30 due to increased salt levels and, again, the mannitol group showed no significant increase compared with control (Fig. 8C).

DISCUSSION

The present study describes the intrarenal localization of a novel gap junctional protein isoform, Cx30, in three species, with each species showing varying levels of expression (Table 1). The order of Cx30 expression level was rat > rabbit > mouse. In the rat, Cx30 was present continuously along the entire distal nephron from the MTAL to the IMCD, with the highest expression in the DCT. In the rabbit and mouse, Cx30 was restricted to cortical segments of the distal nephron, and the highest level of expression was observed in intercalated cells of the CCD. Particularly in the rabbit and mouse, Cx30 was localized in the apical cell membrane.

The recent cloning of Cx30 provided conflicting results regardless of whether it is expressed in the kidney (6, 13). Studies using Northern blot techniques suggested it is undetectable in the mouse kidney (6) or is present in the human kidney in very low amounts compared with the brain (13). This
information turns out to be misleading for at least two reasons. From the present work, we now know that Cx30 mRNA is indeed present in the mouse kidney and also that renal expression of Cx30 protein is regulated by certain factors. Thus it is possible that in the previous study, downregulation in the particular kidney tissue sample accounted for the low expression levels. Also, it is now apparent that other species express higher levels of Cx30 in the kidney than the mouse, which shows Cx30 labeling exclusively in intercalated cells of the collecting tubule (Fig. 5 and Table 1). The extremely tiny fraction of intercalated cells compared with the whole kidney mass can also explain why Cx30 was difficult to detect.

Because immunostaining of gap junction channels usually gives a punctuate pattern between cells, localization of Cx30 throughout the apical plasma membrane of distal tubular nephron segments is an unexpected finding. However, this work used specific, commercially available antibodies (Zymed) that have been well characterized by many other investigators (20–22, 24, 32). In our hands, immunoblotting using whole kidney homogenate and the Cx30 antibody produced an intense band around the expected molecular weight of 30 kDa. This single band was within the 25- to 62-kDa range of known Cx isoforms, indicating that the Cx30 antibody reacted with a single Cx species in the kidney. Further supporting specificity is that in addition to the apical label, although extremely rarely, we observed highly scattered and punctuate labeling between cells of the tubular epithelium, vasculature, and glomerulus, consistent with the antibody recognizing gap junction proteins.

Polarity, namely, the apical location of the continuous plasma membrane labeling, is even more unexpected. Colocalization of Cx30 with a well-known apical membrane transport protein, pendrin (25), further supports its presence in the

Fig. 5. Immunofluorescence labeling of Cx30 in the mouse kidney. Double-labeling of aquaporin-2 (AQP2) identified the apical membrane of principal cells (green) in the CCD (*). All AQP2-negative cells (intercalated cells) were stained for Cx30 (red) at the apical membrane. Bar = 20 μm.

Fig. 6. Regulation of Cx30 expression in the rat IMCD. Shown are kidney sections stained with Cx30 and imaged with confocal microscopy from rats on a control diet (A), high-salt (B), or low-salt diet (C). The expression of Cx30 was upregulated in rats fed a high-salt diet. High-salt rat tissue also exhibited an increase in the luminal membrane vs. cytosolic signal in the IMCD compared with low-salt and control experimental groups. All images were captured using the same instrument settings. Bars = 40 μm.

Fig. 7. Immunoblotting analysis of Cx30 in rat tissue under various dietary salt conditions. A: representative blot of rat tissue blotted with Cx30 (~30-kDa molecular wt). B: same blot was stripped and reprobed with β-actin to demonstrated even loading. C: densitometric analysis of immunoblots indicates that Cx30 was significantly upregulated in high-salt conditions compared with control (*P < 0.01). No significant difference between control and low-salt groups was observed. Values are means ± SE of 4 rats/experimental group.
Means significant difference between control and mannitol-treated groups. Values are P<0.01 compared with control cells, and there was no significant difference between the 3 groups of RMIC cells. High-salt-treated M1 cells showed a significant upregulation (*P<0.01) compared with control cells, and there was no significant difference between control and mannitol-treated groups. Values are means ± SE of 3 samples/experimental group.

Table 1. Summary of localization and expression of Cx30 in rat, rabbit, and mouse kidney tissue

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<th>Region of Kidney</th>
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<td>Distal convoluted tubule</td>
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<td>Connecting segment</td>
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<td>Cortical collecting duct</td>
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<tr>
<td>Inner medullary collecting duct</td>
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<td>Renal medullary interstitial cells</td>
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Cx30, Connexin 30. Intensity levels of Cx30 are as follows: −, absence of signal; +, low; ++, medium; +++, high.
further support that Cx30 may be involved in the regulation of renal salt reabsorption.

A possible association between Cx30 function and chloride secretion is evidenced by its colocalization with pendrin, an apical Cl/HCO₃⁻ anion exchanger in type B and non-A, non-B intercalated cells (Fig. 2, H and I, and Fig. 3, A–C). At present, there are no data that Cx hemichannels regulate ion transport in the major reabsorbing epithelia (i.e., gastrointestinal and renal epithelia). Interestingly, in the cochlea of the inner ear, Cx30 also colocalizes with pendrin in the epithelial marginal cells on the lateral wall (12). These cells also serve as a source of ATP for the endolymphatic fluid that regulates ion transport of the cochlea via purinergic receptors (12). Furthermore, deletion of pendrin (Pendred-syndrome) or Cx30 knockout is characterized by a similar phenotype, including nonsyndromic hearing loss or deafness (13, 25). The similarities in both Cx30 and pendrin localization and ion transport-regulatory function may indicate that ion transport in the renal collecting duct system is regulated similarly in the inner ear. This interesting association further necessitates the exploration of Cx30 function and its possible regulation of ion transport in more detail. Because of the lack of selective inhibitors, Cx30 hemichannel function could be best studied using the available Cx30 knockout mouse model (7).

In summary, we localized a novel Cx isoform, Cx30, in the luminal cell membrane of select cells in the distal nephron. The presence of this functional protein at the apical membrane in contact with only the tubular fluid suggests that it may function as a hemichannel, permeable to certain molecules that are yet to be identified. Cx30 may be involved in the regulation of distal tubular salt reabsorption, a function that needs to be further investigated. Emerging understanding of the gap junctional proteins and new tools for their investigation now offer the opportunity to explore the vital role that Cx molecules may play in the regulation of renal blood flow, the filtration process, renin release, and tubular reabsorption.

GRANTS

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