Localization of the high-affinity glutamate transporter EAAC1 in rat kidney

CHAIRAT SHAYAKUL, YOSHIKATSU KANAI, WEN-SEN LEE, DENNIS BROWN, JEFFREY D. ROTHSTEIN, AND MATTHIAS A. HEDIGER

1Renal Division, Department of Medicine, Brigham & Women’s Hospital, Harvard Medical School, Boston 02115; 2Renal Unit, Massachusetts General Hospital, Boston, Massachusetts 02129; and 3Department of Neurology, Johns Hopkins University, Baltimore, Maryland 21287

Shayakul, Chairat, Yoshikatsu Kanai, Wen-Sen Lee, Dennis Brown, Jeffrey D. Rothstein, and Matthias A. Hediger. Localization of the high-affinity glutamate transporter EAAC1 in rat kidney. Am. J. Physiol. 273 (Renal Physiol. 42): F1023–F1029, 1997.—Most amino acids filtered by the glomerulus are reabsorbed in the kidney via specialized transport systems. Recently, the cDNA encoding a high-affinity glutamate transporter, EAAC1, has been isolated and shown to be expressed at high levels in the kidney. To determine the potential role of EAAC1 in renal acidic amino acid reabsorption, the distribution of EAAC1 mRNA and protein in rat kidney was examined. In situ hybridization revealed that EAAC1 mRNA is expressed predominantly in S2 and S3 segments of the proximal tubules and at low levels in the inner stripe of outer medulla and inner medulla. Polyclonal antibodies raised against the carboxy terminus of EAAC1 recognized a single band of ~70 kDa on Western blots of membrane protein from kidney cortex and medulla. Immunofluorescence microscopy revealed intense signals in the luminal membrane of S2 and S3 segments and weaker signals in S1 segments, descending thin limbs of long-loop nephrons, medullary thick ascending limbs, and distal convoluted tubules. These results are consistent with EAAC1 encoding the previously described apical high-affinity glutamate transporter in the kidney that mediates reabsorption of acidic amino acids in tubules beyond early proximal tubule S1 segments. Potential additional roles of EAAC1 in acid/base balance, cell volume regulation, and amino acid metabolism are discussed.

acetic acid; amino acid; proximal tubule; loop of Henle; distal tubule; metabolic acidosis; glutamine; ammonia; volume regulation

Transport of amino acids across cell membranes is mediated by different transport systems with overlapping substrate specificities (5). The importance of amino acid transporters has been defined in several aspects such as protein synthesis, regulation of cellular metabolism, production of metabolic energy, cell growth, cell volume regulation, nerve transmission, and absorption of amino acids from the lumen in polarized epithelia. In the kidney, these transporters are important in reabsorption of most amino acids that are filtered at the glomerulus, thus leaving only small amounts excreted in the final urine (33, 35). Reabsorption of amino acids occurs by transport systems that are selective in terms of charge- and stereoselectivity. As for acidic amino acids, glutamate and aspartate are nearly completely reabsorbed in the kidney, which results in fractional urinary excretions of 0.2–0.5% for glutamate and 0.4–2.5% for aspartate (34). Early studies showed that more than 90% of the filtered glutamate is reabsorbed in the first third of the proximal tubule (34). This process is mediated by a transport system that is electrogenic and accepts both aspartate and L-glutamate (24, 36). On the basis of rat renal brush-border membrane vesicle studies, Na+- and K+-coupled glutamate uptake occurred via two distinct saturable processes, i.e., a high- and a low-affinity transport system, with Michaelis constant (Km) values of 0.016 and 3.6 mM, respectively (39). Interestingly, Na+-coupled glutamate transport has also been shown in a vesicle preparation enriched in rat renal basolateral membranes (31).

The nephron segments distal to the proximal tubule were thought to handle acidic amino acids differently. On the basis of in vivo and in situ micropuncture studies, significant reabsorption of glutamate between the proximal tubules and collecting ducts was found to occur in deep cortical and juxtamedullary nephrons (7) but not in superficial cortical nephrons (14). It was demonstrated that absorption at this site lowers fractional amino acid excretion 2- to 40-fold when there are increased filtered loads. Furthermore, these studies also suggested that the acidic and neutral amino acids might be recycled between the vasa recta and the loops of Henle in the inner medulla (7, 8), but the exact pathway is not clearly defined.

During the past few years, four mammalian high-affinity Na+-dependent glutamate transporters have been identified at the molecular level. These are termed EAAC1 (16), GLT-1 (26), GLAST-1 (37), and EAAT4 (10). In the central nervous system, these high-affinity glutamate transporters are essential to prevent glutamate from reaching neurotoxic levels during synaptic transmission. A study based on Northern analysis revealed that EAAC1 is the only glutamate transporter that is widely expressed in various tissues outside the nervous system including kidney and intestine (16). This suggested that EAAC1 is involved in acidic amino acid reabsorption in the kidney.

This study was undertaken to investigate the nephron distribution and cellular localization of EAAC1 in rat kidney both at the mRNA and protein levels. The results indicate that the distribution of EAAC1 mRNA and protein correlates with the functionally defined distribution of the previously described apical acidic amino acid transporter. Our data provide a fundamental basis for understanding the mechanism of acidic amino acid reabsorption in different nephron segments and the potential role of glutamate in renal amino acid metabolism and other cell functions.

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METHODS

Northern analysis. Poly(A)+ RNA was prepared from different regions of male Sprague-Dawley rat kidney, i.e., superficial cortex, deep cortex, outer inner stripe of outer medulla, inner medulla, and papilla. About 3 μg of each sample was separated on a 1% agarose gel in the presence of 2.2 M formaldehyde and blotted onto a nitrocellulose filter. The filter was hybridized at 42°C with a32P-labeled probe synthesized from nucleotides 500–1751 of rat EAAC1 cDNA. After washing at 50°C the air-dried membrane was hybridized at 42°C with 35S-labeled sense or antisense RNA probes synthesized from the full-length rat EAAC1 cDNA (15) and washed in 0.1× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) + 0.1% sodium dodecyl sulfate (SDS) at 65°C.

In situ hybridization. The basic procedure for in situ hybridization was as previously described (16). Briefly, rat kidneys were perfusion fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), postfixed in the same solution at 4°C, and immersed in 30% ice-cold sucrose for 48 h. Two serial cryosections of ~4 μm were prepared and hybridized at 42°C with 39S-labeled sense or antisense RNA probes synthesized from nucleotides 500–1751 of rat EAAC1 cDNA. The probe was degraded by partial hydrolysis to nucleotides in length. After washing at 50°C the air-dried slides were dipped into Kodak NTB2 emulsion and developed 2–3 wk later.

To localize EAAC1 mRNA in different segments of the kidney proximal tubules, adjacent sections were used for immunocytochemistry using the antibodies which recognize specific antigens in each segment and the immunoperoxidase staining method. S1 segments were identified using antibody against the facilitated glucose transporter GLUT-2 (38), S2 segments by an anti-carbonic anhydrase IV antibody (1), and S3 segments by anti-ectoadenosinotriphosphatase (anti-ecto-ATPase) antibody (30).

Immunoblotting. The monospecific polyclonal anti-EAAC1 antibodies used in the immunoblot and immunocytochemistry experiments were raised against a peptide corresponding to amino acids 511–524 of the carboxy terminus of EAAC1 from rat, as previously described (27). For immunoblot, the protein was extracted from the kidney cortex and medulla of male Sprague-Dawley rats (200–250 g). The samples were homogenized in a solution containing 250 mM sucrose, 10 mM triethanolamine, 1 μM leupeptin, and 0.1 mg/ml phenylmethylsulfonyl fluoride (pH 7.6) and centrifuged at 1,000 g for 10 min. The supernatant was saved and recentrifuged at 17,000 g for 20 min. The pelleted membrane fractions were suspended in the same solution, measured for protein concentrations, and solubilized at 60°C for 15 min in Laemmlı sample buffer. SDS-polyacrylamide gel electrophoresis was performed by minigel electrophoresis using a 10% polyacrylamide gel, and the gel was transferred to a nitrocellulose membrane by electrobblotting. The membrane was blocked for 1 h with 5% nonfat dried milk powder + PBS-T (0.1% Tween 20 in PBS) and exposed to primary antibody diluted in 0.1% milk powder + PBS-T for 1 h. The secondary antibody was a donkey anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (Amersham Life Science, Arlington Heights, IL). The antigen-antibody complexes were detected by autoradiography using the enhanced chemiluminescence method (ECL Western blotting analysis system, Amersham Life Science).

Immunocytochemistry. Immunocytochemistry was performed as previously described (27) with some modifications. Briefly, the rat kidneys were perfusion fixed with 4% paraformaldehyde in PBS, postfixed in the same solution at 4°C overnight, and infiltrated with 30% sucrose in PBS. Thin 4-μm cryosections were treated with 1% SDS for 5 min to expose antigenic sites (2), rinsed three times with PBS, and preincubated with 5% normal goat serum for 15 min. Sections were incubated overnight at 4°C with anti-EAAC1 antibody (0.2 μg/ml IgG), washed three times with PBS, and then incubated for 1 h with goat anti-rabbit indocarbocyanine Cy3 (1:100; Jackson ImmunoResearch, West Grove, PA). Specificity of the staining was tested by using anti-EAAC1 antibody preadsorbed with immunizing peptide at 50 μg/ml. Sections were examined and photographed on a Nikon FXA photomicroscope, using Kodak Ektachrome 400 film. The possible autofluorescence was determined by switching filters and was negligible.

RESULTS

Localization of EAAC1 mRNA in rat kidney. Northern analysis using full-length EAAC1 as the probe revealed strong hybridization to a 4.2-kb band for almost every region of the rat kidney except the renal papilla (Fig. 1). The signals in the superficial and deep cortex and in the outer stripe of outer medulla were stronger than those in the inner stripe of outer medulla and inner medulla. The weaker 2.7-kb band, which is
likely the result of the use of an internal polyadenylation site (15), exhibited the same distribution in the kidney.

In situ hybridization was initially used to investigate the expression of EAAC1 mRNA in different nephron segments in the rat kidney. Figure 2 shows a low-magnification view and demonstrates dense hybridization of the antisense EAAC1 cRNA probe to tubules of both cortex and outer stripe of outer medulla. Consistent with Northern analysis, a weak signal was also evident in inner stripe of outer medulla and in inner medulla. The pattern of hybridization in these regions suggest that EAAC1 is expressed in thick ascending limbs and/or thin descending limbs of loops of Henle.

Figure 3A shows a low-power micrograph of the hybridization pattern of EAAC1 antisense cRNA probe in cortex and outer stripe of outer medulla. Figure 3, B and C, shows the adjacent sections stained with the S2-specific carbonic anhydrase IV antibody and the S3-specific anti-ecto-ATPase antibody, respectively. The strong in situ hybridization signal in the cortex was present both in tubules that were immunopositive and immunonegative for the S2-specific antibody. The signal in the medullary rays was present in tubules that were immunopositive for the S3 antibody, consistent with a previous study of rabbit kidney (22).

Localization of EAAC1 protein in rat kidney. To establish that the antibody used in this study specifically recognizes EAAC1 protein, we first performed Western blot analysis of crude membrane fractions from cortex and whole medulla of rat kidney. Figure 4 shows that the antibody recognized a single band of ~70 kDa in both regions, with stronger signal detected in cortex than in medulla. The apparent molecular mass of labeled protein is consistent with the molecular mass of the glycosylated protein predicted from the cDNA clone (16) and the molecular mass previously reported for total rat brain homogenates (27).

The cellular localization of EAAC1 protein was further investigated by indirect immunofluorescence using affinity-purified antibodies applied to rat kidney frozen sections. Staining for this transporter was detected in different nephron segments with different intensities, whereas no significant staining was observed using antibody preadsorbed with peptide antigen (not shown). The results are shown in Fig. 5.

Cortex. EAAC1 was detected in the apical membrane of cells from the proximal tubule. However, the different proximal tubule segments were labeled with different intensities. Staining for EAAC1 was strongest in S2 segments, whereas S1 segments, identified by connec-

![Fig. 3. Sequential 5-µm sections of the corticomedullary junction of paraformaldehyde-fixed rat kidney hybridized with [35S]-labeled EAAC1 antisense probe (A) or immunostained with an anti-carbonic anhydrase type IV antibody (S2 marker) (B) or anti-ecto-ATPase antibody (S3 marker) (C). *Blood vessel. G, glomerulus. Bar = 100 µm.](image)

![Fig. 4. Immunoblot of membranes prepared from rat kidney cortex and medulla. Five micrograms of total protein were loaded in each lane. Migration of molecular mass markers (Bio-Rad, Hercules, CA) is indicated on right.](image)
Fig. 5. Immunolocalization of EAAC1 in different nephron segments of rat kidney. A: immunostaining in apical membranes of cells in proximal convoluted tubule with lesser intensity in the S1 segment (connected to glomerulus, G) than in S2 segment. B: immunostaining is on the apical membrane of S2 segments; no staining is detectable on the basolateral membrane. C: apical staining of cells in a distal convoluted tubule (DCT), which is less intense than in the S2 segments. D: bright apical staining in S3 segments. No staining is observed in the collecting duct (CD). E: transitional zone between S3 segments of proximal tubules and descending thin limbs of short loops of Henle (DTL-S). No significant staining is detected in epithelial cells of DTL-S. F: transitional zone between S3 segments of proximal tubules and the descending thin limbs of long loops of Henle (DTL-L). Immunostaining is observed in epithelial cells of DTL-L and also in thick ascending limbs (TAL). G: inner stripe of outer medulla shows immunostaining in DTL-L (asterisks), whereas there is no staining in DTL-S and vasa recta in the vascular bundles (VB). H: inner stripe of outer medulla/inner medulla border shows a portion of a long-looped nephron where immunostaining is positive in DTL-L and a transition from an unstained ascending thin limb (ATL) to a medullary thick ascending limb (mTAL) that is weakly positive.
tion to a glomeruli, showed weaker staining (Fig. 5, A and B). Moderate labeling was also detected in distal convoluted tubules (Fig. 5, A and C).

Outer stripe of outer medulla. EAAC1 was detected in the apical membrane of the straight (S3) part of proximal tubules with similar intensity as in S2 segments (Fig. 5D). No staining was detected in collecting ducts (Fig. 5D).

Inner stripe of outer medulla. Figure 5, E and F, shows the transitional zone between S3 segments and thin descending limbs of short-loop and long-loop nephrons, respectively. The staining shows evidence for internephron heterogeneity with respect to the expression pattern of EAAC1, because a significant labeling was evident only in thin descending limbs of long-loop but not short-loop nephrons. Figure 5G shows vascular bundles that contain descending limbs of short loops of Henle surrounded by vasa recta, as well as adjacent thin descending limbs of long loops of Henle. Labeling was evident only in thin descending limbs of long loops of Henle but not in vascular bundles. Another segment that expresses EAAC1 at an intermediate level in this region is the medullary thick ascending limbs (Fig. 5F).

Inner medulla. Figure 5H shows the frontier between inner stripe of outer medulla and inner medulla. EAAC1 labeling was identified only in thin descending limbs of long loops of Henle and medullary thick ascending limb, whereas the ascending thin limb is negative. The staining was not evident along the inner medullary collecting duct.

**DISCUSSION**

The evidence presented in this report and in our previous studies (15, 16) indicates that EAAC1 encodes the apical, high-affinity glutamate transporter in the kidney. We showed that expression of rat EAAC1 in Xenopus oocytes induces a high-affinity uptake of DL-aspartate and L-glutamate with an apparent K_m of 14 ± 2 μM and that transport is coupled to the cotransport of Na^+ and the countertransport of K^+ (16, 18, 25). These data are in accordance with the high-affinity transport system reported from rat renal proximal tubular brush-border membrane vesicles (24, 39). In the present study, we show that expression of EAAC1 in the proximal tubule is much higher in the brush-border membranes of S2 and S3 segments than in S1 segments, thus indicating that acidic amino acid reabsorption in the S2 and S3 segments is mediated by the high-affinity transporter EAAC1.

The localization of EAAC1 in the late portion of proximal tubules indicates that the absorptive mechanism for acidic amino acids is similar to that for other solutes. For example, the low-affinity, high-capacity glucose transporter SGLT2 is located in the S1 segment to reabsorb the majority of filtered D-glucose (17), whereas the remaining glucose is further reabsorbed by the high-affinity, low-capacity glucose transporter SGLT1 in the S3 segment (20). In the case of acidic amino acid reabsorption, the first third of the rat proximal convoluted tubule is responsible for reabsorption of >90% of filtered glutamate under normal conditions (34). This process is likely mediated by a low-affinity, high-capacity transport system (39) that has not yet been cloned. The high-affinity, low-capacity transporter EAAC1 will participate in final reabsorption of acidic amino acids that may escape the early part of the proximal tubule, especially when the filtered load of glutamate is increased (3, 34).

As stated above, there is internephron heterogeneity for amino acid reabsorption distal to the proximal tubules. The localization of EAAC1 expression distal to the proximal tubules reveals additional nephron segments where acidic amino acids are further reabsorbed, i.e., along the entire length of the thin descending limbs of long-looped nephrons, the thick ascending limbs, and distal convoluted tubules. The absence of EAAC1 expression in thin descending limbs of short-looped nephrons and collecting ducts is in agreement with previous data showing that there is no amino acid reabsorption in these segments of the kidney (14).

Regulation of acidic amino acid reabsorption in the kidney has not been clearly demonstrated. An early microperfusion study showed that reabsorption of glutamate in the proximal tubule is influenced only to a minor or negligible extent by acute alteration in cellular metabolism (36). However, it is well known that acute metabolic acidosis results in a fall in cellular glutamate content both in vivo (6, 13) and in LLC-PK_1 cells (32). This results in increased mitochondrial phosphate-dependent glutaminase activity and increased ammoniagenesis (12, 40). Recent studies showed that reduced cellular glutamate in LLC-PK_1-F^- cells during metabolic acidosis is caused by at least two pathways: 1) enhanced intracellular glutamate removal due to acidosis-induced activation of glutamate dehydrogenase, and 2) reduced cellular glutamate influx due to inhibition of γ-glutamyl transpeptidase-phosphate-independent glutaminase in the proximal tubule (9, 29) by the reduced extracellular bicarbonate concentration (23, 40). However, metabolic acidosis increased glutamate uptake in LLC-PK_1-F^- cells (23). This might be explained by the direct effect of lower extracellular pH on transport activity, consistent with a previous study that showed that glutamate is transported by EAAC1 in its protonated form (42). Additional in vivo studies to investigate the effects of metabolic acidosis on both EAAC1 and the low-affinity glutamate transporter in the proximal tubule will be necessary to understand the role of glutamate transport in renal ammoniagenesis and its effect on intracellular acidification.

Expression of EAAC1 in segments other than proximal tubules raises the question of whether glutamate might be used as a metabolic fuel in these segments. A previous study showed that glutamate can be metabolized and oxidized to produce CO_2 in thick ascending limbs (19). However, it has been shown that glutamate is not the preferred substrate to maintain cellular ATP content in thick ascending limbs and to energize transepithelial Na^+ transport (4, 41). This can be attributed to the high metabolic expenditure required...
Recently, it has been shown that hypertonic stress activates the high-affinity glutamate transport system X_Ac in the bovine renal epithelial cell line NBL-1 (11). In contrast to the neutral amino acids transport system A, which appears to be activated through a regulatory protein (28), upregulation of glutamate transporters in hypertonic medium is accompanied by an increase in EAAC1 mRNA levels and is dependent on protein synthesis. Expression of EAAC1 in thin descending limbs of long-looped nephrons suggests a potential role for acidic amino acids in cell volume regulation in the medullary interstitium. An immunohistochemical study using monospecific antibodies for free glutamate and aspartate also showed that these amino acids are accumulated in the medullary interstitium (21). Although there is no evidence that the concentration of glutamate or aspartate alone is sufficiently high to account for the increased osmolality inside the cell, the sum of these acidic amino acids and others may allow their contribution as significant organic osmotanes in cells of the renal medulla.

In conclusion, we have examined the tubular distribution and cellular localization of the high-affinity glutamate transporter EAAC1 in the rat kidney at both the mRNA and protein levels. The predominant localization of EAAC1 in the apical membrane of S2 and S3 segments of the proximal tubule supports the view that EAAC1 is responsible for glutamate reabsorption in these segments. In addition, the expression of EAAC1 in thin descending limbs of long-looped nephrons, medullary thick ascending limbs, and distal convoluted tubules is in agreement with previous physiological studies showing significant glutamate reabsorption distal to the proximal tubules and indicates that EAAC1 is possibly involved in glutamate recycling and cell volume regulation in the inner medulla.

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Present addresses: Y. Kanai, Department of Pharmacology and Toxicology, Kyorin University School of Medicine, 6–20–2 Shinkawa, Mitaka, Tokyo 181, Japan; and W.-S. Lee, Graduate Institute of Medical Sciences, Taipeh Medical College, 250 Wu-Hsiin Street, Taipei 110, Taiwan, ROC.

Address for reprint requests: M. A. Hediger, Harvard Institutes of Medicine, 77 Ave. Louis Pasteur, Boston, MA 02115.

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