Postnatal development of carbonic anhydrase IV expression in rabbit kidney

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Schwartz, George J., Jennifer Olson, Ann M. Kittelberger, Tohru Matsumoto, Abdul Waheed, and William S. Sly. Postnatal development of carbonic anhydrase IV expression in rabbit kidney. Am. J. Physiol. 276 (Renal Physiol. 45): F510–F520, 1999.—Carbonic anhydrase (CA) IV activity facilitates renal acidification by catalyzing the dehydration of luminal carbonic acid. CA IV has been localized to the proximal tubules and medullary collecting ducts. Maturation of CA IV expression has been considered to be important in the development of renal acid excretion. The purpose of the present study was to determine the maturational expression of CA IV in rabbit kidney. A guinea pig polyclonal antibody to purified rabbit lung microsomal membrane CA IV was generated. Immunoblotting of membrane proteins after peptide-N-glycosidase F treatment revealed two N-glycosylation sites and reduction in size from ~52 to 35 kDa; there appeared to be heavier glycosylation in the medulla. In membrane and total proteins from the kidney cortex, CA IV was 15–30% of the adult level during the first 2 wk of life but increased to mature levels by 5 wk of age. The maturational pattern in the cortex was confirmed by measuring SDS-resistant CA hydratase activity. In the medulla, both membrane and total proteins were generally less than one-fourth of the adult level of CA IV during the first 2 wk of life before reaching mature levels by 5 wk of age. Immunohistochemistry showed staining in proximal tubules (apical > basolateral), with maximal label in the S2 segment. CA IV also appeared on the apical membranes of a minority cell type of the cortical collecting duct, presumably the α-intercalated cell. Several labeled cells also appeared to be the process of being extruded from medullary collecting ducts of 1- to 2-wk rabbits. The antibody did not reliably detect medullary CA IV expression in sections from mature rabbits. These studies indicate that there is a substantial postnatal increase in expression of CA IV in the maturing kidney in both the cortex and medulla. The disappearance of intercalated cells in the maturing rabbit medullary collecting duct may be part of a normal renal developmental program as previously reported [J. Kim, J.-H. Cha, C. C. Tisher, and K. M. Madsen. Am. J. Physiol. 270 (Renal Fluid Electrolyte Physiol. 39): F575–F592, 1996]. It is likely that the maturational pattern of CA IV expression contributes to the increase in renal acidification observed early in postnatal life.

Kidney cortex; kidney medulla; hydratase activity; Western blot; deglycosylation; immunohistochemistry

DURING EARLY POSTNATAL LIFE, there is a maturational increase in the renal threshold for HCO\(_3^-\) and in the ability of the kidney to excrete an acid load (13, 37, 41). Microperefusion studies have previously shown that the rate of bicarbonate absorption in neonatal proximal tubules is approximately one-third of that observed in mature segments (38). Bicarbonate absorption remains relatively constant through the first 3–4 wk of age and then surges between 4 and 6 wk of age (38). A maturational increase in the renal HCO\(_3^-\) threshold and in net acid excretion (13, 37, 41).

Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyzes the hydration of CO\(_2\) and the dehydration of carbonic acid. More than 95% of CA activity is located in the cytosol as CA II, whereas up to 5% is membrane bound and corresponds to CA IV (8, 28, 51). CA IV facilitates acidification by catalyzing the dehydration of intraluminal carbonic acid that results from the secretion of protons into the lumen (12). Membrane-bound CA activity has been detected in the brush border and basolateral membranes of proximal tubules (28, 33, 51) and in the apical membranes of intercalated cells and medullary collecting duct cells (22, 32). Functional studies have identified luminal membrane CA activity in rat proximal convoluted tubules (23), along the inner stripe of the rat outer medullary collecting duct (42), and in the initial segment of the rat inner medullary collecting duct (49). In CA II-deficient patients and mice, inhibition of CA IV activity diminishes renal acid excretion (5, 40). Furthermore, in microperefusion experiments, inhibition of luminal CA markedly reduces HCO\(_3^-\) reabsorption in the proximal tubule (23) and outer medullary collecting duct (46). Thus CA IV appears to be a critical enzyme that mediates HCO\(_3^-\) absorption in both the proximal and distal nephrons.

Studies addressing the maturation of renal CA IV expression in the kidney are limited. CA IV mRNA is expressed in the 20-day rat fetal kidney, and there is a major postnatal increase by 17 days of age (16). CA IV mRNA is also expressed in the rabbit mesonephric kidney, and sodium dodecyl sulfate (SDS)-resistant hydratase activity [presumably CA IV activity (28, 35)]...
is expressed by rabbit mesonephric proximal and collecting tubules (27).

Despite the vast amount of acid-base physiology known in the rabbit, the maturation pattern of CA IV protein and its relationship to HCO3− secretion in the early postnatal rabbit metanephric kidney are presently unknown. We studied the developmental expression of CA IV in the rabbit kidney to determine whether the maturation of the enzyme is correlated with the postnatal increase in H+ secretion. To obtain a probe, we purified CA IV from the lungs of rabbits and prepared a polyclonal antibody in guinea pigs.

Methods

Animals. New Zealand White rabbits were purchased from Hazleton-Dutchland Farms (Denver, PA). Pregnant dams were allowed to deliver in our animal quarters to provide newborns (1–7 days of age). For older postnatal babies, litters of 1–2 wk-old pups were purchased with their mothers and allowed to grow up in our facility. Adult females (1.5–2.5 kg) and pregnant dams were fed standard laboratory chow (Purina Mills, Richmond, IN) and allowed free access to tap water. The pups were fed by and raised with their mothers. At least three different litters were used at each age group for these studies.

Each rabbit was anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg). Adult rabbits were first sedated with intramuscular xylazine (5 mg/kg) and ketamine (44 mg/kg). The kidneys were rapidly removed and cut into coronal slices of 1–2-mm thickness. Because the outer medulla cannot be readily distinguished from the inner medulla during the first 3 wk of life (47; G. J. Schwartz, personal observations), we performed two comparisons of the maturing medulla. In the first series, whole medullas were obtained from the younger animals and compared with the inner medulla of adult rabbits. Specifically, the cortex and medulla were isolated from animals 3 wk old and younger, and the cortex and inner medulla were isolated from the older animals. In a second series of experiments, the medulla was cut 1 mm below the cortex and ~1 mm of the papillary tip was removed from animals of all ages. Therefore, whole medullas were compared maturationally. The tissues were coded and snap-frozen.

Purification of CA IV from rabbit lung. The microsomal membrane preparations from rabbit lung and the extraction and affinity purification of CA IV were carried out as described by Zhu and Sly (52). The enzyme activity was determined according to Maren (24) as previously described (43). When 0.2% SDS was present during the enzyme assay, the enzyme was pretreated for 30 min at room temperature before the CA assay was performed. The protein concentration was determined with the micro-Lowry procedure (30). Homogeneity of the enzyme was checked by SDS-polycrylamide gel electrophoresis (PAGE) under reducing conditions as previously described (21).

Generation of polyclonal antiserum. The antisera against rabbit lung CA IV were raised in guinea pigs with an injection schedule as previously described (52). Antibody titers and specificity were checked by immunoblotting (48) with goat anti-guinea pig IgG-peroxidase as a secondary antibody. Peroxidase activity was demonstrated with 4-chloro-1-phenol and hydrogen peroxide.

Preparation of kidney membrane proteins. Membrane proteins were prepared from 40 to 200 mg of each dissected zone of frozen kidney tissue by homogenization on ice for three 30-s bursts with a Tissuemizer (Ultra-Turrax, J anke-Kunkel, T ekm ar, Cincinnati, OH) with an S25N 10-gauge probe at 24,000 rpm in 7 ml of Tris sulfate buffer, pH 7.5 (25 mM Tris sulfate and 0.9% NaCl). This buffer also contained protease inhibitors including 1 mM EDTA, 1 mM iodoacetate, 0.1 mg/ml of 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc, Boehringer Mannheim, Indianapolis, IN), 0.1 mg/ml of 1,10-phenanthroline, 2 µg/ml of pepstatin A, 5 µg/ml of chymostatin, 10 µg/ml of leupeptin, and 10 µg/ml of aprotinin (9). Samples were stored on ice for 30 min and centrifuged at 1,000 g for 10 min at 4°C. The supernatant was centrifuged at 110,000 g for 60 min at 4°C, and the pellet was solubilized in Sato's buffer (25 mM triethanolamine, pH 8.1, 59 mM Na2SO4, and 1 mM benzamidine chloride) (35) containing 5% SDS, 0.1% saponin, and the same protease inhibitors as used in the homogenization buffer. Solubilization was performed by breaking up the membrane pellet with a pipette tip, agitating at room temperature, and passing the material 10 times through a series of decreasing needle sizes (smallest 21 gauge). Protein concentration was measured with bicinchoninic acid (micro BCA protein assay, Pierce Biotech, Rockford, IL), with bovine serum albumin (BSA) as a standard. Ten to twenty-five micrograms of membrane protein were size fractionated on reducing SDS-PAGE through a 10% separating and a 4% stacking polyacrylamide gel.

Preparation of total cellular proteins. We were concerned that the effort devoted to isolating membrane proteins could hasten degradation despite large amounts of protease inhibitors (9). Therefore, we also isolated total cellular cortical proteins in a simpler procedure: −250 mg of dissected zone of frozen rabbit kidney tissue was homogenized on ice in 3 ml of 50 mM Tris, pH 8.0, 10 mM EGTA, 1 mM benzamidine chloride, 150 mM NaCl, and 0.2 mg/ml of Pefabloc with three 30-s bursts of the Tissuemizer with the S25N probe. Samples were stored on ice for 30 min, frozen at −70°C, and subsequently thawed. Cellular membranes were disrupted by the addition of 3 ml of 10% SDS, rocking for 30 min at 20°C, and passing through 18- and 21-gauge needles. After centrifugation at 1,000 g for 10 min at 4°C, the supernatant was assayed for protein by micro BCA assay. Fifteen micrograms of total protein were size fractionated on reducing SDS-PAGE as noted in Preparation of kidney membrane proteins.

Immunoblot analysis. Fractionated proteins were transferred to nitrocellulose membranes with a transblot electrophoretic transfer cell (Bio-Rad, Hercules, CA). After transfer, the nitrocellulose membranes were blocked for 90 min at 20°C in Tris-buffered saline-Tween 20–5% milk and probed with a dilution of 1:1,000 guinea pig anti-rabbit CA IV serum that was preabsorbed with 5% BSA in 5% milk overnight at 4°C. Then the filter was probed with 1:20,000 goat anti-guinea pig antibody conjugated to horseradish peroxidase (Cappel, Organon, Durham, NC) that had been preabsorbed with 1% normal rabbit serum and 2% BSA in 5% milk. Signals were visualized with enhanced chemoluminescence (Amersham, Arlington Heights, IL) and Kodak XAR film (Eastman Kodak, Rochester, NY).

The intensity of the signals in the film was quantitated by scanning densitometry (SigmaGel, J andel, San Rafael, CA). To allow for differences in intensities among the various gels, membrane or total protein from the same adult kidney cortex or medulla sample was run with each maturational study and was considered as 100% (50).
Densitometric data are presented as means ± SE. Data were grouped by postnatal age (1, 2, 3–4, and 5 wk and adult), and comparisons were analyzed by one-way ANOVA plus the Tukey-Kramer test for multiple comparisons (NCSS, Kaysville, UT). Significance was asserted when P < 0.05.

Deglycosylation with peptide-N-glycosidase F. Ten micrograms of protein were denatured by boiling for 3 min in 1% mercaptoethanol and placed on ice. The deglycosylation was carried out in buffer (containing 45 mM EDTA, pH 7.4, 45 mM sodium phosphate, pH 7.4, and a protease inhibitor cocktail) plus 1 µl of peptide-N-glycosidase F. Control incubations substituted water for peptide-N-glycosidase F. To determine the number of N-glycosylation sites, we used 10 µl of peptide-N-glycosidase F and incubated the samples for 5 and 30 min at 37°C, whereas for analysis of multiple samples of fully deglycosylated kidney tissue, we used 100 µl of peptide-N-glycosidase F and incubated the samples for 1 h at 37°C. The reaction was stopped by boiling for 3 min. Samples were fractionated on SDS-PAGE and examined for CA IV by immunoblot. When total lung microsomes were used, a single broad polypeptide band of 45–50 kDa on immunoblot was observed. Antibodies raised against rabbit lung CA IV in guinea pigs is a monospecific polyclonal antibody.

Expression of CA IV protein in adult rabbit kidney. CA IV was expressed in rabbit kidney as a single product with an approximate molecular mass of 46–50 kDa (Fig. 1). Densitometric analysis of the signals derived from the kidney cortex of rabbits ranging in size from 1.7 to 2.7 kg showed relatively little variability (coefficient of variation = 10%). Thereafter, we generally used one sample of membrane protein or total cellular protein from the cortex or medulla of a kidney from a mature animal of 1.5–1.9 kg as a reference (set equal to 100%) for each of the gels showing maturing rabbit kidney CA IV expression.

Surprisingly, the expression of CA IV in the inner medulla appeared at a slightly higher molecular mass than that in the cortex and was more diffuse, suggesting substantially more posttranslational modification in the medullary CA IV (Fig. 2, lanes 0').

Deglycosylation of rabbit kidney CA IV. To determine whether rabbit CA IV contained N-linked oligosaccharide (48), because two sites are predicted from the nucleotide sequence (44, 50), we treated 10 µg of kidney membranes with 10 µl of peptide-N-glycosidase F for 5 and 30 min at 37°C before size fractionation and immunoblotting. There were two deglycosylation sites observed in both the cortical and medullary membranes (Fig. 2), with the second resulting in a product of 34–35 kDa, similar to the size predicted previously from the nucleotide sequence (44, 50). In the inner medulla, there appeared to be more glycosylation per site and a larger molecular mass of the fully processed.

RESULTS

Purification of CA IV from rabbit lung membranes. Affinity-purified rabbit lung CA IV showed a specific activity of 3,053 EU/mg protein. The enzyme activity was resistant to 0.2% SDS, which is a characteristic feature of the CA IV isozyme (44). The homogeneous enzyme migrated as a single polypeptide band of 45–46 kDa on SDS-PAGE. In a few preparations, proteolytically clipped polypeptides of 18–20 kDa were also observed under reducing conditions of the electrophoresis.

Antibodies raised in guinea pigs immunoreacted with an affinity-purified enzyme of 46–50 kDa on immunoblot. When total lung microsomes were used, a single broad polypeptide band of 45–50 kDa was observed. There was no cross-reactivity with rabbit CA II (data not shown). These results suggested that the antibody raised against rabbit lung CA IV in guinea pigs is a monospecific polyclonal antibody.

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Fig. 1. Carbonic anhydrase (CA) IV immunoblot of kidney cortex from 5 adult rabbits. A single band at 46–50 kDa was seen; signal was comparable for each rabbit except that it was somewhat more intense in kidney of 2.7-kg animal. Nos. on top, animal weight in kg; no. at right, molecular mass in kDa.
protein (up to ~60 kDa). Compared with the inner medulla and cortex, the outer medulla showed an intermediate amount and size of glycosylated protein (data not shown). Based on the molecular mass of the mature proteins, the oligosaccharide chains at the two glycosylation sites could add as much as 18 kDa to the cortical and 26 kDa to the inner medullary CA IV protein.

Maturation of CA IV expression in kidney cortex. The expression of CA IV in kidney cortical membranes of 1-wk-old animals was markedly less than that observed in adults (Fig. 3). The expression increased and was nearly at adult levels by 3–5 wk of life. Densitometric analysis of several gels revealed that during the first 2 postnatal wk, CA IV expression was one-third of the adult level, increasing during weeks 3 and 4 to two-thirds of the adult level, and reaching mature levels by 5 wk (Fig. 4).

When total kidney cortex protein was examined (Fig. 5), it was evident that cortical CA IV expression gradually increased with postnatal age. Densitometric analysis of several gels revealed that during the first 2 postnatal wk, CA IV expression was 20% of the adult level, doubling during weeks 3 and 4, and reaching two-thirds of the adult level by week 5 (Fig. 6).

Maturation of CA IV expression in kidney medulla. Because of the diffuse appearance of the CA IV band in tissue from the inner medulla, samples for densitometry were generally deglycosylated for 60 min with 100 mU of peptide-N-glycosidase F, then size fractionated on SDS-PAGE, transferred, and probed with the CA IV antibody. The densitometric analysis of the single deglycosylated band was facilitated by the collapse of the diffuse signal into a sharp band. Compared with the mature inner medulla, deglycosylated medullas from immature rabbits showed much less CA IV expression (Fig. 7). Densitometric analysis of several gels showed that during the first 2 postnatal wk, CA IV was one-fourth of the adult level, doubling during the third and fourth postnatal weeks, and reaching adult levels by 5 wk of age (Fig. 4). Comparable findings showing increasing glycosylation and intensity of signal during early maturation were obtained from total medullary proteins (data not shown).

In an additional experiment, we compared membranes from the whole medullas of maturing rabbit kidneys because the outer medulla was difficult to
distinguish from the inner medulla during the first 2 wk of life. Both the intensity and the apparent posttranslational processing of CA IV increased with maturation (Fig. 8A) and appeared to reach adult levels by 5 wk of age. Deglycosylated medullas showed a sharp increase during the third week of age (Fig. 8B). Densitometric analysis of three to four samples at each age group showed that medullary CA IV at 5 days was 6% and at 10 days was 22% of mature levels (Fig. 9). The levels at 3 and 5 wk were not statistically different from that of the adult kidneys.

Expression of SDS-resistant CA hydratase activity in maturing cortex. To verify that the maturational increase in CA IV protein was functional, we assayed SDS-resistant hydratase activity [presumably CA IV (8, 35)] in the membranes derived from homogenates of kidney cortex at postnatal weeks 1 and 3 and in adult rabbits. CA IV activity in 1-wk rabbits was 2.7 ± 1.3 EU/mg protein, 43% of the adult level of 6.3 EU/mg protein; in 3-wk animals, it was 75% of the adult activity (Fig. 10A).

To confirm the reliability of our method, we also measured SDS-sensitive hydratase activity in the cytosolic fractions of these cortical homogenates (Fig. 10B). The values closely compare with data published previously for 1-wk (17.4 ± 1.8 EU/mg protein) and adult (34.5 ± 3.5 EU/mg protein) rabbits (7).

Immunohistochemistry. Immature kidneys showed faint staining of the cortical CA IV in the labyrinths (Fig. 11A, arrow) but little staining in the medullary rays (*). The mature kidney showed heavy staining in the medullary rays (Fig. 11B, *) and in areas around the juxtamedullary nephrons but little staining in the cortical labyrinths (arrow). Despite identifying CA IV in the renal medulla by Western blot, our antibody did...
not detect medullary CA IV in mature kidneys by immunohistochemistry (data not shown).

Higher-power views of medullary rays of the mature kidney cortex showed heavy staining for CA IV on the apical brush border and basolateral plasma membranes of proximal tubules, primarily of S2 segments (Fig. 12B). Glomeruli and thick ascending limbs of Henle's loop were not stained. Proximal tubules near the glomerulus (S1; Fig. 13B) and in the medulla (S3) were less heavily labeled. Immature kidneys showed minimal staining of proximal tubules in medullary rays (Fig. 12A) and faint staining of proximal tubules in the juxtaglomerular region (Fig. 13A). Developing glomeruli in the nephrogenic zone did not stain for CA IV.

Mature cortical collecting ducts (CCDs) contained a small subpopulation of cells (10% of cells) that showed apical staining; these cells might be intercalated cells (Fig. 14B). Immature CCDs showed similar but less intense staining (Fig. 14A); however, in the medulla and deep cortex of the 5- and 10-day-old rabbits, several positive cells appeared to be insinuated in various regions of the walls of the medullary collecting ducts (Fig. 15) and deep CCDs. These cells appeared to be in various stages of extrusion and destruction because they were not visualized in the medullary collecting ducts of older rabbits (data not shown).

DISCUSSION

These studies show the maturational expression of CA IV in the developing rabbit kidney. CA IV levels in the neonatal kidney cortex are 20–30% of those in the adult, and mature levels are probably not reached until ~5 wk of age. This correlates well with previous histochemical analyses of the enzymes alkaline phosphatase, glucose-6-phosphatase, and 5'-nucleotidase, which show mature patterns of expression at ~28 days of age (47). Our findings also agree with previous observations in isolated tubules obtained from the juxtamedullary cortex of neonatal rabbits (38), which showed that the rate of HCO3\(^-\) absorption is approximately one-third of that observed in the adult. Bicarbonate reabsorption is mediated primarily by an apical Na\(^+\)/H\(^+\) exchanger (2, 4) in series with cytosolic and membrane-bound CA activities (10, 12, 23) and is driven by basolateral Na\(^+-\)K\(^+\)-ATPase (1). Proximal tubules comprise the major cell type expressing CA IV in the kidney cortex (5, 8, 10, 32). A maturational surge occurs between 4 and 6 wk of life such that mature levels of transport are reached by the end of this period (38). Functional evaluation of apical Na\(^+\)/H\(^+\) exchange activity in maturing rabbit proximal tubules (2) reveals that basal activity during the first 2 wk of life is one-third of mature levels. There is a doubling of activity in the third week; mature levels are reached by 6 wk. These observations suggest that the activity of the enzymes and transporters mediating HCO3\(^-\) absorption develop in parallel. In previous analyses by Schwartz (36) and Spitzer and Schwartz (41) of fluid (and NaCl) absorption in maturing proximal convoluted tubules, the postnatal surge in fluid absorption was accompanied by the maturational increase in proximal tubular basolateral surface area (14, 38) and Na\(^+-\)K\(^+\)-ATPase activity (11, 39).

The expression of cortical CA IV was found predominantly on the apical and basolateral membranes of S2 proximal tubules; there was much less expression by S1 and S3 segments, similar to what has been previously found in the rat (10). The expression in immature proximal tubular segments was quite reduced compared with mature segments as predicted from the Western analyses, and specific resolution over apical and basolateral membranes was not discernable until maturity. These findings suggest that the maturation of luminal and basolateral CA IV expression may parallel that of the functional maturation of NaCl and HCO3\(^-\) transport in the proximal tubule.

In contrast to the findings in rat kidney, we detected expression over the apical surface of a minority population of cells in the CCD, presumably α-intercalated cells, but staining was not consistently observed over
any population of cells in the outer medullary collecting duct. Interestingly, cells expressing CA IV were detectable in deep CCDs and medullary collecting ducts (Fig. 15). The appearance of these cells protruding in the walls of the collecting duct suggests, as originally proposed by Kim et al. (19), that α-intercalated cells are deleted from the maturing collecting duct by extrusion.

The abundance of CA IV in the medulla was unexpected. Previous functional studies (12, 17, 31) have shown species differences in the localization of the CAs among rodents and rabbits. For example, there is functional expression of luminal CA in the outer medullary collecting ducts of rabbits (42, 46) but not of rats (17). A previous immunofluorescence study of CA IV in the rat kidney (10) showed rather limited expression in the inner medulla. However, the present study shows abundant expression of CA IV protein in the rabbit inner medulla by Western blot. We have no explanation for why our antibody failed to detect CA IV in the medulla by immunohistochemistry. Perhaps the heavy glycosylation pattern prevented binding to the appropriate epitope. The findings in the medulla by Western blot agree with a recent Northern analysis of CA IV mRNA expression (50). We also demonstrated by in situ hybridization that CA IV mRNA was expressed by medullary collecting duct cells (50). We have subsequently used the reverse transcription-polymerase chain reaction to detect CA IV mRNA in isolated nephron segments (45). These studies clearly showed the presence of CA IV mRNA in the outer medullary collecting...

Fig. 11. Low-power views of 10-day (A) and adult (B) kidney cortices immunohistochemically stained for CA IV. Immature kidney stains lightly but primarily over cortical labyrinth (A, arrow) and not over medullary rays (*) or in nephrogenic zone (N). Mature kidney cortex shows heavy CA IV staining along medullary rays (B, *), probably in proximal straight tubules, and less in proximal tubules of cortical labyrinth (arrow). Glomeruli and thick limbs were not stained at either age.

Fig. 12. High-power views of 3-wk (A) and adult (B) kidney cortices stained for CA IV. Adult kidney shows heavy apical and basolateral staining of proximal straight tubules in medullary ray (B, solid arrows). A few intercalated cells (B, open arrow) are apically stained along adjacent cortical collecting duct (CCD). Immature kidney shows weak proximal tubule staining (A, solid arrows) in medullary ray; CCDs are also negative here. There is endothelial staining in a vessel (A, *) deep in the cortex. Bars, 10 µm.
duct and initial inner medullary collecting duct but, unlike in the rat, not in the medullary thick ascending limb.

Studies of HCO$_3^-$ transport in the outer medullary collecting duct show a small maturational increment in absorption (29), suggesting that H$^+$-secreting intercalated cells are relatively more mature than HCO$_3^-$-secreting intercalated cells in the neonatal period (36, 41). Indeed, using fluorescent dyes probing acidic cytoplasmic vesicles, mitochondrial potential, and cell pH, Satlin and Schwartz (34) suggested that the neonatal H$^+$-secreting cells were functionally mature. Immunocytochemical studies in the neonatal outer and initial inner medullary collecting ducts showed mostly apically polarized H$^+$ pumps, much as observed in the mature kidney, although the intensity increased greatly during postnatal life (26). The basolateral band 3-like anion exchanger (AE1) was more cytoplasmic and less polarized to the basolateral membrane in the neonatal medullary collecting duct (26), and there was a maturational increase in both the number and intensity of staining of H$^+$-secreting cells (26). These immunocytochemical studies are consistent with previous ultrastructural findings by Evan et al. (15) that showed intercalated cells of the outer medulla to have shorter apical perimeters, fewer vesicular profiles, and smaller mitochondrial volume percent than mature outer medullary intercalated cells. Overall, these studies would indicate that substantial maturation of transport processes also occurs in the renal medulla of early postnatal rabbits.

Further evidence showing significant postnatal kidney medulla maturation can be found in the histochemical analysis of succinic dehydrogenase and acid phosphatase, which have high activity in the neonate before decreasing to a mature pattern by ~28 days of life (47).

The neonatal loops of Henle have the configuration of short loops without thin ascending limbs (20). With maturation and zonal differentiation, there is a transformation of papillary portions of Henle's loops from relatively thick into thin limbs, and these enzymes are less active in the thin limbs compared with the thick limbs (47). This process of development involves apoptotic deletion of thick ascending limb cells and transformation into thin ascending limb cells, and this deletion gives rise to a well-defined boundary between inner and outer medullas by 21 days of age in the rat (20). Although there is no similar data in the rabbit, this maturational distinction in the rabbit probably occurs later than in the rat. Functionally, and with respect to urinary concentrating ability in the rabbit, this distinction is necessary before 21 days of age (18). Indeed, a clear-cut distinction between the inner and outer medullas was not seen in the 3-wk animals but rather in the 5-wk animals. For this reason, we repeated our examination of medullary CA IV expression using whole medullas, amputating only the papillary tip; this approach would help to eliminate any selection bias between the outer and inner medullas during maturation. Not surprisingly, we found that the maturational pattern for CA IV expression tends to
parallel that for the urinary concentrating system. Thus these data indicate that the maturational pattern observed in the inner medulla is rather similar to that for CA IV.

The observed size of rabbit CA IV was 46–50 kDa, substantially larger than the 34 kDa predicted from the nucleotide sequence (44, 50). This finding suggests that substantial posttranslational processing of the enzyme occurs. A previous study (48) showed that N-glycosylation accounts for some of this processing. The decrease in molecular mass from 46–50 kDa to 34–35 kDa suggests at least two oligosaccharide chains that must be rather large. Two oligosaccharide chains are predicted from the nucleotide sequence (44, 50). Two oligosaccharide chains on CA IV have been demonstrated by partial deglycosylation of rabbit CA IV expressed in COS 7 cells (S. Tamai and A. Waheed, unpublished observations). CA IV expressed from the immature rabbit kidney cortex showed the same size as that from mature kidney. With regard to CA IV maturation in the medulla, there was clearly an increase in abundance and some increase in posttranslational modification, which would tend to support the concept that cell differentiation and proliferation must occur in the maturing medulla. The diffuseness of the medullary CA IV band on the immunoblot suggests heterogeneity in the posttranslational modifications.

The high-molecular-mass peptides of ~68 and 69 kDa (seen in Figs. 2, 5, and 8) are thought to represent cross-linked CA IV that is insensitive to the reducing conditions of SDS-PAGE. The appearance of these polypeptides increases with aging of the tissues and the higher expression levels in the tissue extracts. We suspect that the 68- and 69-kDa polypeptides are due to covalent cross-linking of the CA IV by transglutaminase.

The abundance of medullary CA IV appeared to increase ~10-fold with maturation, more than what was observed in the cortex. Without data addressing the maturation of HCO₃⁻ transport in the inner medullary collecting duct, it is not possible to correlate changes in CA IV protein with those of transport. However, using titratable acid excretion as a marker of medullary collecting duct acidification, one observes that the mature rate (not corrected for body surface area) is five to six times that of the newborn human infant (13). Even allowing for the postnatal increases in collecting duct length, this indicates a large increase in absolute H⁺ secretion per millimeter of collecting duct. Thus the increase in H⁺ secretion is probably paralleled in part by the increase in CA IV on the apical membranes of the medullary collecting ducts. Further studies are needed to investigate the role of CA IV in the medulla during maturation.

We used two different methods to obtain protein from the kidney of maturing animals. In preliminary studies, we observed, as did Brosius et al. (9), that CA IV appeared to be susceptible to proteolysis during membrane fractionation. Despite the use of higher amounts of inhibitors, there were small differences in the patterns of maturation when CA IV expression was examined in the membrane fraction or in total cellular protein. It is possible that the recovery of intact CA IV might vary with the method of protein isolation as well as with postnatal age. Nevertheless, the major findings were comparable, showing low levels of CA IV during the first 2 wk of life followed by a large maturational increase. The maturational patterns in the cortex and medulla were also quite similar as well.

We confirmed our findings regarding the maturational increase in the abundance of CA IV protein in the kidney cortex by showing a nearly comparable postnatal increase in SDS-resistant (CA IV) hydratase activity of membranes fractionated from cortical homogenates. Indeed, the neonatal activity was 43% of the adult, whereas the protein abundance was 32% (see Fig. 4). These data, derived from two different methodologies, are in general agreement that the neonatal level of CA IV is substantially less than that of the adult.

In summary, using a polyclonal antibody directed toward rabbit CA IV, we have shown that the rabbit CA IV is heavily glycosylated in the kidney and is developmentally regulated. The enzyme was expressed at approximately one-fourth of the adult level during the
first 2 wk of life in the cortex and less so in the medulla before surging during postnatal weeks 3 and 4. This pattern appears to precede the maturation of renal acidification in the rabbit and is similar to what has been observed for other transport proteins in the maturing kidney. The maturational pattern also appears to involve programmed elimination of α-intercalated cells from the developing medullary collecting duct. It is probable that the maturation of renal CA IV expression contributes to the increase in HCO₃⁻ absorption and H⁺ secretion observed during infancy. The low level of CA IV in the newborn kidney could contribute to the difficulties neonates have in maintaining acid-base homeostasis.

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