The kidney contributes to a positive phosphate balance for organisms during growth. Phosphorus plays an important role as a constituent of bones, membrane phospholipids, nucleic acids and is involved in phosphorylation processes. The bulk of phosphate is reabsorbed in the proximal tubule (7). The abundance of phosphate reabsorption, which is possibly correlated with alterations during aging. It shows an age-related decrease in renal phosphate reabsorption via the type II sodium/phosphate cotransporter (NaPi-2) in the brush border membrane (BBM) of proximal tubules underlies alterations during aging. The ontogeny of NaPi-2 in kidneys from newborn to 6-wk-old rats was investigated. NaPi-2 protein distribution in the kidneys of neonatal, 13-d-old, 22-d-old, and 6-wk-old rats was immunohistochemically analyzed, and NaPi-2 mRNA distribution in neonatal and 6-wk-old rats was analyzed by in situ hybridization. In kidneys of newborn rats, the appearance of NaPi-2 protein and mRNA coincided with the development of the brush border (assessed by actin staining) on proximal tubular cells. NaPi-2 was not detectable in the nephrogenic zone or in the outgrowing straight sections of proximal tubules, which lack a brush border. In 13-d-old suckling rats, strong NaPi-2 staining was seen in the BBM of convoluted proximal tubules of all nephron generations. In contrast, in 22-d-old weaned rats, NaPi-2 staining in the BBM of superficial nephrons was weaker than that in the BBM of juxtamedullary nephrons. Western blotting demonstrated that the overall abundance of NaPi-2 protein in the BBM of 22-d-old rats was decreased to approximately 70% of that in 13-d-old rats. In kidneys of 6-wk-old rats, the internephron gradient for NaPi-2 abundance in the BBM corresponded to that in adult rats. The data suggest that the NaPi-2 system in the kidney is fully functional and possesses the capacity for regulation as soon as nephrogenesis is completed. The manifestation of NaPi-2 inter nephron heterogeneity immediately after weaning might be related to the change in dietary inorganic phosphate content.
those observed in 6-wk-old rats, i.e., NaPi-2 abundance is downregulated in superficial nephrons.

Materials and Methods

Experimental Animals

The studies were performed with Wistar rats (BRL, Basel, Switzerland) of four different ages (four animals in each group), as follows: (1) newborn rats (5.5 g body wt); (2) 13-d-old rats (34 g body wt), which were still suckling; (3) 22-d-old rats (55 g body wt), which had been removed from their mothers on day 20 and were fed a standard laboratory diet; (4) 6-wk-old rats (150 g body wt), which were fed a standard laboratory diet (approximately 0.6% P). The gender of the newborn rats was unknown, and the older rats were all male.

Fixation

The fixation procedures for the kidneys differed according to the age of the rats. After administration of anesthesia (thiopental at 100 mg/kg body wt, intraperitoneally), the newborn and 13-d-old rats were perfused with a hypodermic syringe via the left cardiac ventricle. The older rats were fixed by retrograde perfusion of fixative, via the abdominal aorta, at 1.38 times hydrostatic pressure, as described previously (12). The fixative consisted of 3% paraformaldehyde and 0.05% picric acid in a 6:4 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol with sucrose) plus 0.066% MgCl₂, 1 H₂O, and 10% hydroxyethyl starch (HAES steril; Fresenius, Stans, Switzerland). After 5 min, the fixative was washed out at hydrostatic pressure (70 cm) by perfusion with cacodylate buffer for 5 min. The kidneys were then removed and cut into slices, which were mounted onto thin cork plates and immediately frozen in liquid propane, cooled with liquid nitrogen.

Immunohistochemistry

Sections (4 μm thick) were cut at −22°C with a cryomicrotome. The sections were mounted on chromalum/gelatin-coated glass slides, thawed, and stored in 4°C cold phosphate-buffered saline (PBS) until use. For immunofluorescence staining, sections were pretreated for 10 min with 10% normal goat serum in PBS. They were then incubated overnight at 4°C with an anti-rat NaPi-2 rabbit antisem (12) diluted 1:500 in 3% powdered milk in PBS containing 0.3% Triton X-100. Sections were then rinsed three times with PBS and covered for 45 min at 4°C with the secondary antibody, which was a FITC-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) diluted 1:50 in PBS/powdered milk. Double staining of actin filaments and NaPi-2 was performed by adding rhodamine-phalloidin (Molecular Probes, Eugene, OR), at a dilution of 1:50, to the secondary antibody. Double staining for NaPi-2 and the Golgi apparatus was performed by adding mouse monoclonal antibodies against the Golgi M₅ 58,000 protein (Sigma, Buchs, Switzerland) to the working dilution of the NaPi-2 antisem. The final dilution of the anti-Golgi M₅ 58,000 protein antibody was 1:75. Finally, the sections were rinsed three times with distilled water, coverslipped using DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma Chemical Co., St. Louis, MO) as a fading retardant, and studied with an epifluorescence microscope (Polyvar, Reichert-Jung, Austria).

The unpurified rabbit antisem used was directed against 13 amino acids (Met-Met-Ser-Tyr-Ser-Glu-Arg-Leu-Gly-Gly-Pro-Ala-Val) of the amino terminus of the rat Napi-2 molecule. The specificity of this antisem was proven previously by Western blotting, by complete blocking of the antigen by addition of the peptide to the antiserum, and by incubation with preimmune serum instead of antiserum (7). Nonspecific binding of the secondary antibodies to the tissue was assessed by omitting the primary antibody. All control incubation results were clearly negative.

In Situ Hybridization

Digoxigenin-11-UTP-labeled riboprobes were synthesized by in vitro transcription (DIG RNA labeling kit [Sp6/T7]; Boehringer) from the full-length rat NaPi-2 cDNA (3). After linearization with BamHI and SalI, sense and antisense riboprobes were generated with T7 and SP6 RNA polymerases, respectively. RNA probes were degraded by alkaline hydrolysis, at 60°C for 45 min, to fragments of approximately 200 to 500 bp.

The cryostat sections were post-fixed for 15 to 20 min at room temperature with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. The slides were then rinsed with diethylpyrocarbonate-treated water, pretreated for 10 min with proteinase K (10 μg/ml; Sigma) in buffer containing 20 mM Tris-HCl and 2 mM CaCl₂ (pH 7.4), acetylated for 10 min with 0.1 M triethanolamine and 0.25% acetic anhydride, and rinsed twice with water. The sections were then prehybridized at 68°C for 1 h in PBS containing 0.025 M ethylenediaminetetraacetic acid, 2.5× Denhardt’s solution, 250 μg/ml salmon sperm DNA, 250 μg/ml tRNA, and 50% formamide.

Hybridization was performed at 68°C for 18 h, in a solution containing 10 ng/μl of the probe, 0.5× PBS, 0.3 M NaCl, 1× Denhardt’s solution, 100 μg/ml salmon sperm DNA, 250 μg/ml tRNA, 10% dextran sulfate, and 50% formamide. The sections were then rinsed once with 2× SSC for 15 min at room temperature and once with 2× SSC for 30 min at 68°C. Nonhybridized single-stranded mRNA was digested with 40 μg/ml RNase A in 1× SSC for 2 min at room temperature. This treatment was followed by rinses with 1× SSC, 0.5× SSC, and 0.2× SSC, each for 30 min at 68°C.

Digoxigenin-labeled probes were detected by using the DIG RNA labeling and detection kit (Boehringer), according to the instructions provided by the manufacturer. The alkaline phosphatase-linked sheep anti-digoxigenin antibody was diluted 1:4000. Nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate served as chromogenic substrates for the alkaline phosphatase-catalyzed color reaction. Endogenous alkaline phosphatase activity in the tissue was blocked by the addition of 5 mM levamisole to the substrate solution. The sense probe showed no reaction product at all (Figure 1).

Western Blotting

Proximal tubular BBM from three 13-d-old and three 22-d-old rats were isolated as described previously (13). Western blotting was performed as described previously (7), using the anti-NaPi-2 polyclonal antisem. Equal loading was assessed by staining the blots with ponceau rouge (Sigma). The immunoreactive NaPi-2 band was quantified relative to the β-actin band, which was observed using ponceau rouge.

Results

Newborn Rats

In newborn rat kidneys (diameter, approximately 3 mm), the most peripheral tissue layer is the nephrogenic zone (NZ), containing induced mesenchyme, renal vesicles, and “S-shaped bodies,” which develop into functional nephrons within the first postnatal week. The NZ covers the inner cortex, which contains intermediate and juxtamedullary nephrons. The juxtamedullary nephrons are the oldest generation of nephrons and
are fully differentiated and functional at birth. Their descending and ascending limbs run along the periphery of the wide medullary rays and penetrate for a short distance into the medulla. The medullary rays in the cortex contain the outgrowing loop limbs of younger nephron generations and the collecting ducts, surrounded by a loose meshwork of mesenchymal tissue (14). Only the descending limbs of the juxtamedullary nephrons display a brush border at this stage, whereas the outgrowing descending limbs of the younger intermediate nephrons are even less differentiated and still lack brush border microvilli. Zonation of the medulla is still nonexistent.

NaPi-2 protein and mRNA were seen in proximal tubular profiles located in the labyrinth of the inner cortex (Figure 2, A and B). Double staining for actin filaments (with rhodamine-phalloidin) (Figure 3, A and D) and for NaPi-2 demonstrated that NaPi-2 protein (Figure 3, B and E) was present exclusively in the proximal tubule cells that displayed brush borders at their apical poles. NaPi-2 protein staining of the BBM began abruptly at the urinary pole (Figure 2C). In addition, there was rather strong background staining for NaPi-2 in the cytoplasm. Higher magnifications revealed that cytoplasmic NaPi-2 was present in the Golgi apparatus and in abundant small intracellular vesicles (Figure 4, A and E). Within the medullary rays, NaPi-2 staining was absent. NaPi-2 protein abundance seemed higher in juxtamedullary nephrons than in intermediate nephrons (Figure 2A).

The distribution patterns for NaPi-2 protein and mRNA were fully congruent (Figure 2B). The ISH reaction product for NaPi-2 mRNA was located exclusively in the cytoplasm of proximal tubule cells. Staining was intense in the proximal convoluted portions of juxtamedullary and intermediate nephrons (Figure 2D) and disappeared along the straight portions of the proximal tubules (Figure 3F). Neither NaPi-2 mRNA nor protein was detected in glomerular cells, any distal cells, or any structures in the NZ (Figure 3, B and C).

13-Day-Old Rats

The 13-d-old rats were still being fed exclusively with the milk of their mothers. In the kidneys of 13-d-old rats (kidney diameter, approximately 7 mm), the NZ has been completely transformed to nephrons, which at this age are all functional. The cortical labyrinth has broadened, because of the outgrowth of the convoluted tubular portions, and the medullary rays appear relatively narrower than in kidneys of younger rats (Figure 5A).

NaPi-2 abundance seemed to be similarly high in the convoluted portions of all nephron generations (juxtamedullary, intermediate, and superficial). No gradient of NaPi-2 distribution was apparent in the labyrinth, including the narrow cortex corticis. The proximal tubular portions in the medullary rays were comparably weakly stained. Higher magnification showed that, in addition to the brush border, NaPi-2 was present in the Golgi apparatus and in intracellular vesicles (Figure 4, A and E).

22-Day-Old Rats

The 22-d-old rats had been removed from their mothers on day 20; they were fed from that day forward with standard laboratory chow with a Pi content of approximately 0.6%. The overall organization of kidneys in 22-d-old rats (kidney diameter, approximately 8 mm) corresponded to that of young adult rats. The thickness of the cortex was further increased, the zonation of the medulla into outer and inner stripes was apparent, and the inner medulla was recognizable (Figure 5B).

NaPi-2 staining was seen in the brush border all along the proximal tubule. In contrast to the 13-d-old rats, NaPi-2 staining was heterogeneous among nephron generations. Brush border staining was slightly weaker in superficial nephrons than in juxtamedullary nephrons. Within the nephron, the brush border in straight portions in the medullary rays was less immunoreactive than that in the convoluted portions. Intracel-
lular staining was apparent in the Golgi apparatus and in a few intracellular vesicles (Figure 4, C and G).

6-Week-Old Rats

The 6-wk-old rats (kidney diameter, approximately 10 mm) were fed standard laboratory chow. The kidney organization and NaPi-2 expression at the protein and mRNA levels resembled in all respects those of adult rats. NaPi-2 abundance in the brush border of convoluted proximal tubules of superficial nephrons was markedly less than that in juxtamedullary nephrons. This internephron gradient with respect to NaPi-2 BBM staining was slightly more pronounced in 6-wk-old rats (Figure 5C) than in 22-d-old rats (Figure 5B). Intracellular staining in 6-wk-old rats was less prominent than in younger rats (Figure 4, D and H) and was essentially restricted to the Golgi apparatus. The NaPi-2 protein and mRNA distribution

Figure 2. NaPi-2 protein, detected by immunofluorescent staining using a rabbit anti-rat NaPi-2 antiserum (A and C), and NaPi-2 mRNA, detected by in situ hybridization (ISH) using a digoxigenin-labeled riboprobe from rat NaPi-2 cDNA (B and D), in cryostat sections of newborn rat kidneys. (A and B) The protein and the mRNA are restricted to tubular profiles in the cortical labyrinth. The medullary rays, the medulla (M), and the nephrogenic zone (NZ) lack specific signals. (C and D) NaPi-2 protein and mRNA staining begins abruptly at the beginning of the proximal tubule at the urinary pole (arrows). The protein is seen in the brush border and at intracellular sites in the region of the Golgi apparatus (arrows); NaPi mRNA is present in the cytoplasm of proximal tubule cells. Magnification, ×90 (A and B) or ×270 (C and D).
patterns in the kidney were congruent, *i.e.*, NaPi-2 mRNA appeared more abundant in convoluted proximal tubules of juxtamedullary nephrons than of superficial nephrons (Figure 5D).

**Western Blot Analysis of NaPi-2**

In Western blots of rat BBM, the NaPi-2 antiserum recognized proteins with an apparent molecular mass of 80 to 90 kD (Figure 6). Only this region is shown in Figure 6, because this is the location of the major signal detected after protein fractionation on 10% gels under nonreducing conditions, as used in this study. The staining pattern was specific, because these protein bands were not detected in the presence of preimmune serum and were completely blocked by antigenic peptide (7). Densitometric analysis of the blots showed no difference in the

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**Figure 3.** NZ (A through C) and corticomedullary border (D to F) in cryostat sections of newborn rat kidney, with detection by double labeling with rhodamine-phalloidin (for actin measurement) (A and D) and anti-NaPi-2 antiserum (B and E) or ISH analysis for NaPi-2 mRNA (C and F). (A through C) Actin staining (A) at the apical pole of proximal tubule cells indicates the formation of the brush border; S-shaped bodies (outlined) do not display NaPi-2 protein (B) or mRNA (C), and NaPi-2 immunostaining at the apical pole of proximal tubule cells is colocalized with the brush border. (D through F) In the most distal portions (arrows) of the nascent straight proximal tubules, the brush border is not developed, as evident by the lack of apical actin and NaPi-2 staining; NaPi-2 mRNA disappears at these sites. Magnification, ×360.
amounts of β-actin detected. In 22-d-old rats, the abundance of NaPi-2, when normalized to β-actin levels, was approximately 70% of that in 13-d-old rats.

Discussion

The P_i demand of growing organisms is particularly high (1). However, no data are currently available regarding at what stage of nephrogenesis the sodium/phosphate cotransporter, NaPi-2, which contributes to the major part of renal phosphate reabsorption, is first apparent and at what age the distribution pattern of NaPi-2 protein in the renal cortex corresponds to that in adult animals. Furthermore, we were interested in whether the naturally occurring change in the dietary Pi content with weaning affects the abundance of NaPi-2 in the BBM. In this study, we investigated these questions in kidneys of newborn, preweaning, postweaning, and young adult rats, using IHC and ISH analyses.

In the kidneys of newborn rats, all developmental stages of nephrons are displayed; the juxtamedullary nephrons (the first generation of nephrons) are functional, in intermediate nephrons glomeruli have been formed but the epithelium of the tubular system is not yet fully differentiated, and in the most peripheral tissue layer, the NZ, nephrogenesis is proceeding. Therefore, in the latter zone, induced mesenchyme, vesicular bodies, and S-shaped bodies are still present. At this age, NaPi-2 is detectable by IHC and ISH analyses exclusively in proximal tubules of juxtamedullary nephrons and intermediate nephrons and is completely absent in all structures of the NZ. Although in the fully developed juxtamedullary nephrons of newborn rats NaPi-2 is seen in the BBM all along the convoluted and straight portions of proximal tubules, in intermediate nephrons both NaPi-2 protein and mRNA levels progressively decrease along the nascent straight portions of the proximal tubules, in parallel with the decreasing differentiation of microvilli on the apical cell pole. NaPi-2 is absent at all of the sites where microvilli are not yet formed. Therefore, also in the morphologically well polarized prospective proximal tubule cells of the S-shaped bodies, which lack microvilli, NaPi-2 mRNA and protein are not detectable. This contrasts with other BBM transport proteins, e.g., Na^+/H^+ exchanger-3. Biemesderfer et al. (15) described the occurrence of Na^+/H^+ exchanger-3 in the luminal membranes of those cells in the S-shaped bodies that will differentiate to proximal tubule cells. Interestingly, in other NaPi-2-expressing cells (OK cells), the cotransporter is found only at sites where bundles of microvilli are present and not in the membranes of the smooth areas of the cells (16). These observations suggest that NaPi-2 expression is closely correlated with the formation of brush border microvilli and that it might depend on the coexpression of specific, as yet undetermined, cytoskeletal elements.

Intracellular staining for NaPi-2 in adult rats is seen in compartments that are involved in the processing, storage, and degradation of NaPi-2, i.e., in cisterns of the endoplasmic reticulum (M. Traebert, unpublished observations) and of the Golgi apparatus (17) and in lysosomes (18). Intracellular staining for NaPi-2 is particularly extensive in developing

Figure 4. Double immunofluorescence staining for NaPi-2 (A through D) and for the Golgi Mr 58,000 protein (E through H) in S1 segments from proximal tubules of intermediate nephrons of newborn (A and E), 13-d-old (B and F), 22-d-old (C and G), and 6-wk-old (D and H) rats. NaPi-2 staining in the Golgi apparatus remains high in all stages, whereas intracellular staining becomes less abundant during aging. Note the increase in the cross-sectional dimensions of S1 segments from newborn to 6-wk-old rats. Arrows, Golgi areas. Magnification, ×900. Bar = 20 μm.
nephrons, and it is more pronounced in younger nephrons. The strong intracellular NaPi-2 staining in developing nephrons might indicate synthesis and intracellular storage, from which NaPi-2 could be translocated to the BBM, rather than degraded.

In adult and 6-wk-old rats fed a standard laboratory diet (0.6% P_i), NaPi-2 immunostaining in the brush border and NaPi-2 mRNA in the cytoplasm exhibit a marked internephron gradient, decreasing from the highest staining intensity in juxtamedullary nephrons toward very low staining intensity in juxtamedullary convoluted proximal tubules than in superficial ones. MR, medullary ray. Magnification, ×90. Bar = 200 μm.
the BBM of superficial nephrons. A similar but somewhat less pronounced internephron gradient is apparent in the kidney cortex of 22-d-old rats. In marked contrast, in the renal cortex of 13-d-old rats all nephron generations, including the superficial nephrons formed last, exhibit equally strong NaPi-2 immunostaining in the BBM. Western blot analysis of NaPi-2 in BBM vesicles from 13-d-old and 22-d-old rats extend the IHC data, demonstrating that the immunoreactivity in the BBM of older rats is decreased to approximately 70% of that in younger animals. Therefore, the internephron gradient for BBM NaPi-2 in 22-d-old rats does not reflect the different developmental ages of the nephrons but seems to reveal down-regulation of NaPi-2 in the BBM of superficial nephrons. Our observations agree well with the data of Pegorier and Merlet-Benichou (19), who reported that BBM vesicles from 14-d-old rats exhibited greater P$_i$ uptake than did those from 21-d-old rats, and with the previously described decrease in NaPi-2 activity during maturation (20,21).

The high NaPi-2 abundance in the cortex of 13-d-old rats and the downregulation of NaPi-2 observed in 22-d-old rats are similar to findings for adult rats chronically fed a P$_i$-poor diet and then switched to a P$_i$-rich diet. In rats receiving a P$_i$-poor (0.1%) diet, NaPi-2 abundance is high in all nephron generations; in rats receiving a P$_i$-rich (1.2%) diet, NaPi-2 is massively downregulated in superficial and intermediate nephrons (8,10). Acute changes in the dietary P$_i$ content reverse the distribution patterns of NaPi-2 abundance in the brush border in <2 h, whereas the corresponding adaptation of mRNA levels is more sluggish (10). It is possible that the absence of an internephron gradient in the 13-d-old rats and the downregulation of NaPi-2 in superficial nephrons in the 22-d-old rats not only may be related to the developmental stage, but also may reflect the different P$_i$ contents of the available food. According to Luckey et al. (22), rat milk has a P$_i$ content of approximately 0.25% of dry weight. The 13-d-old rats were fed exclusively with rat milk; from day 20 on, the rats were fed the standard laboratory diet, with a P$_i$ content of 0.6%.

In addition, hormonal changes occurring around the time of weaning might contribute to the observed changes in NaPi-2 abundance in the BBM. In addition to parathyroid hormone (9,17), glucocorticoids downregulate NaPi-2 in the brush border (23,24). Henning (25) reported that glucocorticoid levels are low in newborn rats, start to increase by day 14, and reach a peak at day 24.

In summary, we show that the first expression of NaPi-2 during nephrogenesis is correlated with the morphologic differentiation of the brush border in the proximal tubular epithelium. In 13-d-old preweaning rats, NaPi-2 protein levels in the BBM of all nephron generations seem to be equally high, indicating a high capacity for P$_i$ uptake via NaPi-2. In 22-d-old weaned rats, NaPi-2 is downregulated in superficial nephrons. The high NaPi-2 abundance in the BBM of the younger rats might be interpreted as a way to meet the high P$_i$ demand of the growing animals, as well as an adaptation to the low P$_i$ content of rat milk. The downregulation of NaPi-2 observed after weaning might be seen as a response to the higher P$_i$ content of the diet. The data suggest that the NaPi-2 system in the kidney is fully functional and possesses the capacity for regulation as soon as nephrogenesis is completed.

**References**


