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Mechanism of renal phosphate retention during growth

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Mechanism of renal phosphate retention during growth. We have previously demonstrated that the retention of phosphate required for growth is due to a a high V_{max} of the Na^+-Pi cotransport system located in the brush border membrane of the proximal tubule. Because of this and other similarities between adaptation of the kidney to a high Pi demand (growth) and that to low Pi supply, we measured the levels of NaPi-2 mRNA and cDNA present in kidney cortex of 3- and > 12-week-old rats. Like in Pi depletion, Western blots revealed that a 80 to 85 kDa protein recognized by a polyclonal antibody directed against the N-terminal region of the NaPi-2 protein was 2.3-fold more abundant in renal microvilli of the young than of adult animals. However, unlike in Pi depletion, Northern blot analysis failed to reveal a significant difference between mRNA levels at the two ages. Furthermore, suppression of NaPi-2 mRNA activity by annealing with antisense oligomers, or removal of the NaPi-2 transcripts by subtractive hybridization did not affect the rate of Na^+-Pi cotransport induced in oocytes by polyA RNA of rapidly growing animals, while abolishing the ability of the renal cortical polyA RNA of adult rats to encode for Na^+-Pi cotransport. RT-PCR of subtracted polyA RNA using primers specific for a region conserved in NaPi type II (Pi modulated) cotransporters yielded a product that was 98% homologous with that region, despite the absence of NaPi-2 cDNA. The results of these experiments demonstrate that the polyA RNA from kidneys of young animals contains unique mRNA transcripts able to encode for a NaPi protein homologous to, but distinct from NaPi-2.

The contribution of the kidney to the positive phosphate balance required for growth. Phosphate is of critical importance to body functions, particularly during periods of growth, owing to its role as a main constituent of the skeleton, of membrane phospholipids, nucleic acids and nucleotides, as well as to its participation in metabolic reactions and regulatory processes involving protein phosphorylation. The body content of phosphorus increases from 4 to 5 g/kg in the newborn to 10 to 12 g/kg in the adult, reflecting the increasing proportions of mineralized bone and cellular tissues per unit of body mass [1]. The infant retains ~40% (30 mg/kg per day) of the Pi absorbed from the gut, whereas the adult, by definition, retains none [2]. The kidneys contribute to the maintenance of the positive Pi balance required for growth by reabsorbing a high fraction of the filtered Pi (99% in newborns, 95% in infants fed human milk, and 80% in adults) [3]. This occurs in spite of the fact that what the kidney sees, namely the concentration of Pi in the extracellular fluid, is very high at birth (5.8 to 9.3 mg/dl), and decreases slowly thereafter, to reach adult values (3.0 to 4.5 mg/dl) as late as the second decade of life [4]. This intriguing observation prompted us to examine the mechanisms that account for the ability of the immature kidney to reabsorb Pi with such high efficiency.

Based on our previous work regarding the reabsorption of Na^+ by the immature kidney [5], we started with the assumption that the adaptive process allowing for a high fractional reabsorption of Pi is located in the distal segments of the nephron which are ontogenetically older, and therefore more mature, than the proximal segments. Micropuncture experiments revealed that ~85% of the age related difference in the fractional reabsorption of Pi was due to a higher rate of Pi reabsorption in the proximal segments of the nephrons [6]. Confirming our finding in guinea pigs [7], the rate of Na^+-dependent Pi uptake was found to be threefold higher in BBMV of younger than of older rats.

Similarities and differences between renal adaptive responses to Pi depletion and growth

Since then we learned that the renal adaptation to high Pi demand shares several characteristics with the adaptation to Pi deprivation: (1) high capacity (V_{max}) of the Na^+-Pi symport system(s) located in the brush-border of the proximal tubules [7]; (2) lack of an increase in the density of Pi-protectable, Na^+-dependent binding of phosphonoformate (PFA), presumably a measure of Na^+-Pi cotransporter abundance [8, 9]; (3) high membrane fluidity [10]; (4) a low intracellular concentration of Pi $[Pi]_i$ [11]; (5) an inversely proportional relationship between $[Pi]_i$ and V_{max} [11]; (6) a diminished phosphaturic response of the kidney to PTH [12, 13]; (7) abolishment of the adaptive response by inhibitors of protein synthesis [14, 15].

Recent work led to the identification of multiple NaPi transporters in renal tubules [16, 17]. The abundance of one of these transporters (NaPi-2) increases in Pi deprived rat [18, 19]. However, our studies and those of others make it unlikely that up-regulation of NaPi-2 is the sole, or even the most important cause of the high Pi reabsorption observed during the growth of the rat. These studies have shown that: (1) at comparable levels of $[Pi]_i$ the V_{max} of the Na^+-Pi cotransport system is substantially higher in the newborn than in the adult [11]; (2) increments in Pi intake have a substantially lower effect on V_{max} in the newborn than in the adult [7, 20]; (3) growth hormone (GH) or insulin-like growth factor 1 (IGF1) have a larger effect on Pi reabsorption in growing than in fully-grown animals [21]; and (4) the effect of GH (IGF1) on Pi transport is complementary to that of Pi deprivation and is not mediated by changes in $[Pi]_i$ [22]. Thus, the high rates of Pi reabsorption observed in Pi deprivation appear to be due to a low $[Pi]_i$, whereas those required for growth appear to be consequent to the action of two independent factors: high circulating levels of GH (IGF1) and low $[Pi]_i$. These factors, acting apparently via different pathways, increase V_{max}.

Abundance of NaPi-2 mRNA in renal cortex of newborn and adult rats

Because of the similarities between adaptation of the kidney to a high Pi demand (growth), and that to low Pi supply, we started the inquiry by measuring the abundance of NaPi-2 mRNA transcripts present in kidney cortex of 3-week-old and > 12-week-old rats. The mRNA was detected by Northern analysis using a full length cDNA NaPi-2 probe. RNA obtained from renal cortex of adult rats showed a strong hybridization with the NaPi-2 probe, at 2.7 kb. The hybridization signals detected with renal cortical RNA derived from 3-week-old rats were of similar or lower intensity than those observed with RNA from adult rats, even when normalized for the amount of RNA loaded, as assessed by reprobing the blots with β-actin cDNA.

Effect of age on mRNA expression and ^{32}Pi uptake in oocytes

To evaluate the possibility that transcriptional (mRNA) events contribute to the high up-regulation of Na^+-Pi transport in kidneys of rapidly growing animals, we measured the effects of renal cortical polyA RNA derived from young and adult rats on Na^+-Pi cotransport in oocytes. Oocytes injected with mRNA exhibited threefold (3-week-old) and fourfold (> 12-week-old) higher Na^+-dependent ^{32}Pi uptake than oocytes injected with water. Furthermore, Pi uptake (corrected for the endogenous level) was substantially higher in oocytes injected with mRNA derived from 3- than from > 12-week-old rats. Thus, transcriptional events appear to be, at least in part, responsible for the high renal Na^+-Pi cotransport observed in this age group.

Abundance of NaPi-2 protein in renal cortex of young and adult rats

The apparent discrepancy between the NaPi-2 mRNA levels and the high rates of Na^+-Pi cotransport in renal BBMV observed in the young, prompted us to determine the expression of NaPi-2 protein in BBMV and renal cortex of 3-week-old animals, and compare the results with those observed in the > 12-week-old rats. A single protein, between 80 to 90 kDa, that reacted strongly with NaPi-2 antiserum, was more abundant in 3- than in > 12-week-old animals. The 75 to 80 kDa protein recognized by a γ-GT specific antibody in the same BBM preparations did not change with age. Immunohistochemical studies confirmed these findings: the immunofluorescence was more intense in the subcortical and midcortical regions of kidneys from young than from adult rats.

Effect of NaPi-2-depleted polyA RNA transcripts on the induction of Na^+-Pi cotransport in oocytes

To assess the contribution of NaPi-2 transcripts to the age-related difference in Na^+-Pi cotransport observed in oocytes injected with polyA RNA, we hybridized an oligodeoxynucleotide capable of annealing (antisense) to the open reading frame of the NaPi-2 mRNA transcript, to polyA RNA derived from 3- and > 12-week-old rats, or to NaPi-2 cRNA, and assessed the effect of the hybrids on induction of Na^+-Pi cotransport in oocytes. In the controls, the sense 16 mer was substituted for the antisense 16 mer in the annealing reaction. The hybridized polyA RNA samples were also digested with RNase H and used for RT-PCR using a primer pair to amplify a 75 bp NaPi-2 specific product. PolyA RNA of 3- as well as of > 12-week-old rats exposed to the sense primer generated the expected 75 bp specific PCR product. Annealing the polyA RNAs to the antisense oligomer followed by RNase H digestion of the hybrids, annihilated their ability to serve as templates for the generation of the NaPi-2 specific product. Yet, annealing with the antisense abolished only the ability of polyA RNA of adult (and that of the cRNA), but not that of the young rats, to induce Na^+-Pi cotransport in oocytes.

Subtractive hybridization of renal cortical polyA RNA

The annealing experiments, which result in suppression of NaPi-2 activity, were supplemented by experiments in which the NaPi-2 transcripts were removed from the cortical polyA of the young. To this end, polyA RNA derived from 3-week-old rats was hybridized with cDNA generated from renal cortex polyA RNA of adult rats. To test for the effectiveness of the subtractive hybridization, renal cortex polyA RNA from > 12-week-old rats was hybridized with cDNA generated from rats of the same age. One subtraction cycle was sufficient to remove the NaPi-2 transcripts and abolish the ability of this polyA RNA to induce Pi uptake in oocytes (Fig. 1). To determine whether the transcript responsible for the high rate of Pi uptake was present at both ages, but was more abundant in the young, we hybridized polyA RNA of growing rats to cDNA derived from a fivefold excess of renal cortical polyA RNA from > 12-week-old rats. Exposure to excess adult cDNA did not reduce the ability of subtracted polyA RNA from growing animals to induce Pi uptake (Fig. 2). To assess further the effectiveness of the subtraction, β-actin and NaPi-2 specific primers were used to check for amplification of any residual β-actin and NaPi-2 mRNA. The β-actin mRNA, a very abundant transcript, was still detectable, albeit in small quantities, after three hybridization passages. NaPi-2 mRNA, an uncommon transcript, was completely depleted from polyA RNA after three passages (Fig. 3). Because NaPi-2 specific RT-PCR product may have escaped detection due to lack of sensitivity of the ethidium bromide staining, we carried out Southern hybridization using a ^{32}P-CTP labeled full length cDNA NaPi-2 probe. The results confirmed that polyA RNA subjected to three subtraction cycles is completely depleted of NaPi-2 transcripts. Yet, Na^+-dependent Pi uptake was similar in oocytes injected with polyA RNA harvested after the third subtractive passage and in those injected with polyA prepared prior to subtraction. Both these uptake rates were greater than that observed in water injected oocytes. Thus, polyA RNA from young rats in which NaPi-2 mRNA was either inactivated by annealing with an antisense probe, or removed by subtractive hybridization, preserved its ability to encode for Na^+-Pi transport in oocytes. These results demonstrate that the induction of Na^+-Pi cotransport by renal cortex polyA$^+$ RNA of young rats is largely independent of the presence of NaPi-2 mRNA.

Identification of a conserved region of a NaPi type II transcript in polyA RNA depleted of NaPi-2

We next embarked on studies leading to the identification of the growth-specific Na^+-Pi cotransporter. We reasoned that any Na^+-dependent Pi transporter specific to the kidney of growing rats should share sequences with other NaPi type II transporters. To test this hypothesis, the mRNA transcripts that remained unbound after three subtraction passages, were used for: (a) RT-PCR with primers for a region of the NaPi-2 sequence highly

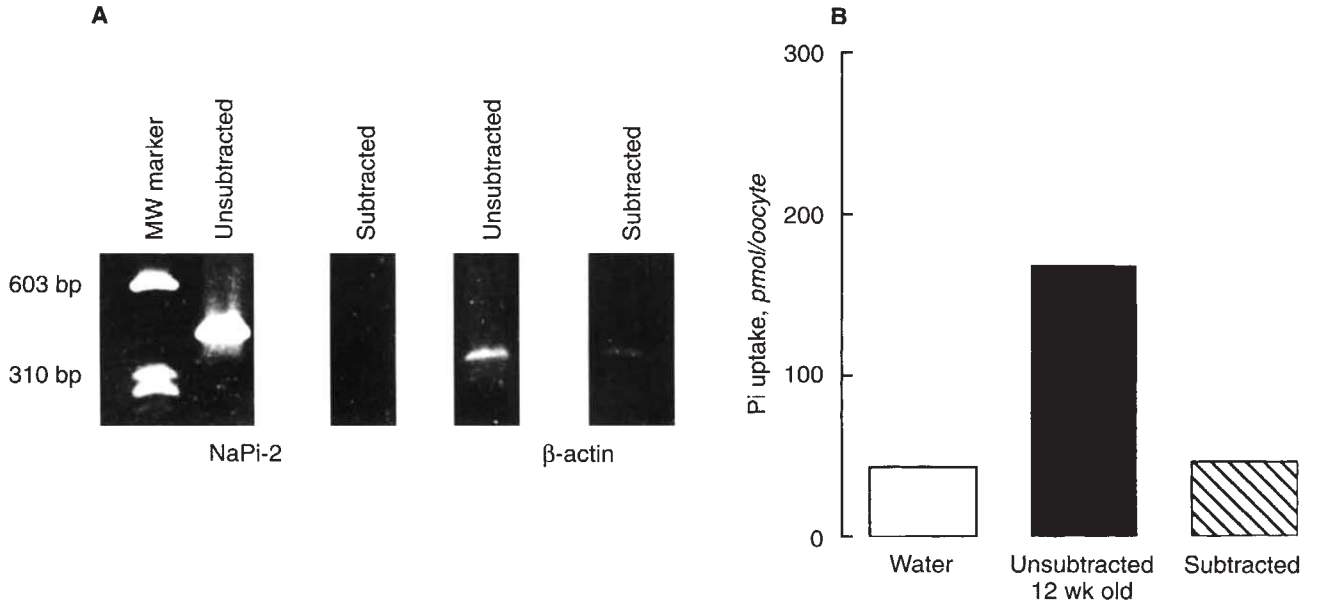

Fig. 1. *Effect of one cycle of subtractive hybridization of mRNA versus cDNA prepared from the renal cortex of adult animals.* (**A**) RT-PCR amplification products using primers specific for NaPi-2 and β-actin; (**B**) Pi uptake in oocytes injected with water, unsubtracted or subtracted polyA RNA.

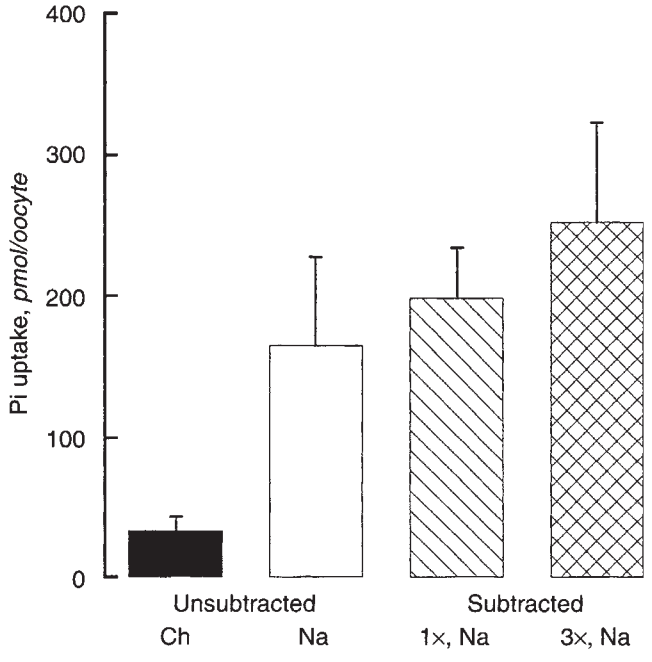

Fig. 2. *Pi uptake in oocytes injected with polyA RNA unsubtracted or subtracted using excess (5-fold) adult cDNA, after one (1 ×) or three (3 ×) cycles of hybridization, in the presence of choline (Ch) or sodium (Na).*

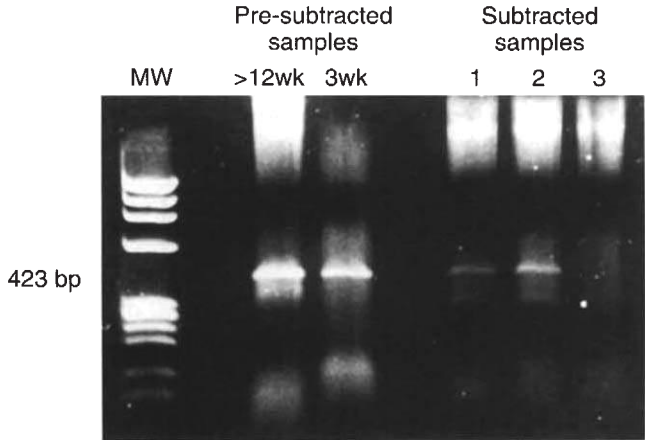

Fig. 3. *NaPi-2 RT-PCR amplification products generated from cortical polyA RNA derived from 3-week-old rats, after one, two, or three cycles of hybridization with cDNA from > 12-week-old rats.*

conserved in several renal Na^+-Pi type II cotransporters; or (b) RT-PCR with NaPi-2 specific primers. RT-PCR using primers to amplify the highly conserved region resulted in an abundant signal of the expected size, while RT-PCR using primers specific for NaPi-2 cDNA (423 bp) did not generate the expected product. Sequence analysis of a segment of this product revealed a 98% homology with the corresponding NaPi-2 region (Fig. 4). These results demonstrate that renal cortical polyA RNA specific to growing rats contains a transcript with a nucleotide sequence nearly identical to that present in all known type II Na^+-Pi cotransporters. This must represent part of the message for a NaPi transporter isoform unique to the kidney of growing rats.

Thus, in spite of the apparent direct proportionality between the quantity of NaPi-2 like proteins and Na^+-Pi cotransport rates in BBM of newborn animals, there are discrepancies that point towards a more complex mechanism that regulates the adaptation of this transport system to the needs of the growing organism. Demonstrative of transcriptional regulation is the induction of Na^+-Pi cotransport in oocytes injected with NaPi-2 depleted polyA RNA prepared from the renal cortex of the young rat; suggestive of post-transcriptional regulation is the discrepancy observed in the newborn between the levels of NaPi-2 mRNA and

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gb|L13257|RATNAPI2A Rattus norvegicus, renal Na/Pi-cotransport mRNA, complete
                    cds.              Length = 2440

Plus Strand HSPs:   Score = 930  (257.0 bits), Expect = 8.9e-70, P = 8.9e-70
Identities = 186/190 (98%), Positives = 186/190 (98%), Strand = Plus / Plus
Query:    1 GCCCCTCTGCCACTTCTTCTTCAACATCTCGGGCATCCTCCTGTGGTACCCGCTGCCCTGCA 62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1466 TGCCCTCTGCCACTTCTTCTTCAACATCTCGGGCATCCTCCTGTGGTACCCGCTGCCCTGCA 1527

Query:   63 CACGTCTGCCCATCCGCATGGCCAAGGCACTGGGCAAACGCACTGCCAAGTACCGCTGGT 122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1528 CACGTCTGCCCATCCGCATGGCCAAGGCACTGGGCAAACGCACTGCCAAGTACCGCTGGT 1587

Query:  123 TTGCCGTCCTCTACCTCCTCGTGTGCTTCCTGCTCCTGCCCTCACTGGTGTTTGGCATTT 182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1588 TTGCCGTCCTCTACCTCCTCGTGTGCTTCCTGCTCCTGCCCTCACTGGTGTTTGGCATTT 1647

Query:  183 CTATGGAG 190
            ||||||||
Sbjct: 1648 CTATGGCA 1655
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Fig. 4. *Homology between an RT-PCR amplification product of NaPi-2 depleted polyA RNA (Query), using primers specific to a highly conserved region of NaPi type II transporters, and the corresponding segment of the NaPi-2 mRNA (Sbjct).*

those of NaPi-2 like protein. As in Pi depletion, transcriptional as well as post-transcriptional events may mediate the up-regulation of Na^+-Pi cotransport during growth.

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