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MOLECULAR CHARACTERIZATIONS OF TYPE IIb SODIUM DEPENDENT PHOSPHATE COTRANSPORTER IN MOUSE INTESTINE

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by KAYO ARIMA

entitled MOLECULAR CHARACTERIZATIONS OF TYPE IIb SODIUM DEPENDENT PHOSPHATE COTRANS PORTER IN MOUSE INTESTINE

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LIST OF ABBREVIATIONS

ADHR	autosomal dominant hypophosphatemic rickets
AQP	water channels of the aquaporin family
ATCH	adrenocorticotrophic hormone
BBM	brush-border membrane
BBMV	brush-border membrane vesicles
BLM	basolateral membrane
bp	base pair
Caco-2 cells	colonic epithelium cells
CNX	membrane-bound calnexin
cRNA	complimentary RNA
CRT	lumenal calreticulin
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethylether) tetraacetic acid
EMSA	electrophoretic mobility shift assay
Endo H	endoglycosidase H
ER	Endoplasmic reticulum
FGF-23	fibroblast growth factor 23
GC	glucocoricoid
GLUT	fructose transporter

GRE	glucocorticoid response element
HBD	hypophosphatemic bone disease
HEPES	N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid
HHRH	hereditary hypophosphatemic rickets with hypercalcuria
HNF-1	hepatic nuclear factor-1
Hyp	hypophosphatemic
kb	kilobase
kDa	kilo-Dalton
K_m	Michaelis constant
LPH	lactose-phlorizin hydrolase
MDCK cells	Madin-Darby canine kidney cells
MP	methylprednisolone
mRNA	messenger RNA
Na/P_i	sodium-dependent phosphate
NF	nuclear factor
NHE	Na^+-H^- exchanger
NPT1	sodium-dependent P_i symporters, type I cotransporters
OK cells	opossum kidney cells
PepT1	oligopeptide transporter 1
PHEX	phosphate regulating gene with homologies to endopeptidases on the X chromosome
P_i	inorganic phosphate
PMSF	phenymethylsulfonylfluoride

PNGase F	peptide N-glycosidase F
PTH	parathyroid hormone
RDA	Recommended dietary allowances
RIE-1 cells	rat intestinal epithelial cells.
RNA	ribonucleoic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulphate
SGLT	sodium-glucose cotransporter
SSC	salt and sodium citrate
TGN	trans-Golgi network
UV	ultraviolet
XLH	X-linked hypophosphatemic rickets

ABSTRACT

Inorganic phosphate (P_i) homeostasis is mainly regulated by absorption of dietary P_i in the small intestine and reabsorption of filtered P_i in the kidney. To date, the renal sodium-phosphate (Na/P_i) cotransporters have been intensively studied at the molecular level. However, most studies of intestinal apical Na/P_i cotransporter were biochemical and/or functional assays that did not describe molecular mechanisms of phosphate transport. To address this issue, I have characterized the 5'-flanking region (1018bp) and overall gene structure of the murine type IIb sodium-phosphate cotransporter (Na/P_i IIb), a major apical phosphate transporter. The Na/P_i-IIb cotransporter gene spans more than 18 kb and consists of 12 introns and 13 exons. Three promoter/reporter gene constructs, −159/+73, −429/+73 and −954/+73, showed significant luciferase activity (22-82 fold over background) when transfected into in rat intestinal epithelial (RIE-1) cells.

P_i requirement during development is much higher than in adult life. In the next set of experiments, I sought to characterize expression of the intestinal Na/P_i-IIb cotransporter during mouse ontogeny and to assess the effects of methylprednisolone (MP) treatment, a glucocorticoid frequently used for high-dose therapy. In control mice, sodium-dependent P_i (Na/P_i) uptake by intestinal brush-border membrane vesicles was highest at 14-days-of-age, lower at 21 days and further reduced at 8 weeks and 8-9 months of age. Na/P_i-IIb mRNA and immunoreactive protein levels in 14-d animals were markedly higher than in older groups. MP treatment significantly decreased Na/P_i uptake, and Na/P_i-IIb mRNA and protein expression in 14-d mice. Additionally, the size of the protein was smaller in 14-d mice. Deglycosylation of protein from 14-d and 8-wk old

animals with PNGase F reduced the molecular weight to the predicted size. I conclude that intestinal Na/P_i uptake and Na/P_i-IIb expression are highest at 14-d and decrease with age. Furthermore, MP treatment reduced intestinal Na/P_i uptake ~3-fold in 14-d mice and this reduction correlates with reduced Na/P_i-IIb mRNA and protein expression. I also demonstrate that Na/P_i-IIb is an *N*-linked glycoprotein and that glycosylation is age-dependent.

In conclusion, the mouse intestinal Na/P_i-IIb cotransporter is developmentally regulated at mRNA and protein levels. MP-treatment also reduces mRNA and protein expression during development. The Na/P_i-IIb gene promoter constructs identified in the first study will be a useful tool to investigate the possible transcriptional regulations.

Furthermore, studying post-translational regulation including glycosylation will reveal developmental effects on Na/P_i-IIb cotransporter protein. These studies will help to decipher molecular mechanisms of P_i absorption in mammalian small intestine.

CHAPTER I

INTRODUCTION

Phosphorus is required by all organisms because of its structural and functional roles. In mammals, plasma concentrations of inorganic phosphate (P_i) are tightly regulated by the intestinal and renal P_i absorption by means of sodium-dependent phosphate (Na/P_i) cotransporters. Numerous factors that perturb P_i homeostasis have been studied. In particular, very low birth weight infants are most susceptible to P_i deficiency that can cause hypophsophatemic rickets and osteopenia because of their rapid growth, limited dietary P_i intake, immature gastrointestinal (GI) tract and occasional glucocorticoid (GC) treatment. In addition, in order to transfer the anionic P_i through the plasma membrane against its electrochemical gradients, secondary active transport systems are required. The intestinal and renal Na/P_i cotransporters have been identified at the molecular level and their connection with inherited hypophosphatemic rickets has been gradually revealed.

In this chapter, I will discuss P_i homeostasis, abnormal P_i metabolism in prematurities and the effects of glucocorticoids. I will also review developmental effects on nutrient transporters and enzymes in intestine to obtain underlying knowledge of developmental defects in P_i homeostasis. Subsequently, I will discuss the Na/P_i cotransporters and genetic abnormalities causing hypophosphatemic rickets. Finally, I will review glycosylation, which is the most common posttranslational regulation in the intestine and is frequently regulated during development.

Phosphate Homeostasis

Inorganic phosphate (P_i) is an essential nutrient in mammals. The RDA (Recommended Dietary Allowances) for phosphate intake including the organic and inorganic forms is 800 mg for children 1 to 10 years, 1,200 mg for age 11 to 24 years, and 800 mg for ages beyond 24 years (232). The organic forms are degraded in the intestinal lumen to yield inorganic phosphate species, $H_2PO_4^-$ and HPO_4^{2-}, depending on pH. $H_2PO_4^-$ is predominant in the proximal duodenum (~ pH 4), but is converted to HPO_4^{2-} in the jejunum and ileum (~ pH 7.5).

P_i homeostasis is tightly regulated by intestinal and renal P_i absorption. Dietary P_i is absorbed primarily in the proximal small intestine, and the excess P_i is excreted in the urine. Approximately 85% of P_i in adult body is found in the skeleton, where P_i is an important component of bone mineral in the mass ratio of 1 P_i to 2 calcium (199). The skeleton provides structural and mechanical support, and serves as a reservoir for mineral homeostasis. In P_i deficiency, bone demineralization in addition to the renal P_i reabsorption and intestinal absorption is increased. Plasma P_i concentrations may be maintained at the expense of skeletal mineral content. However, prolonged phosphate deficiency results in skeletal abnormalities such as short stature, femoral and tibial bowing, osteopenia, osteomalacia and even rickets (13, 61, 241, 244).

Intestine

In the intestine, dietary P_i is absorbed predominantly in the jejunal brush-border membrane (BBM) by a sodium-dependent phosphate (Na/P_i) transport mechanism (173,

178). The apical Na/P_i cotransport has been studied in brush-border membrane vesicles (BBMVs) isolated from the small intestine of various species. The Na/P_i cotransporter exhibits an apparent affinity [Michaelis constant (K_m)] for P_i of 0.1-0.2 mM and a coupling stoichiometry of 2 Na ions to 1 phosphate molecule (38, 200, 216), the P_i transport activity is higher at pH 6.1-6.6 than pH 7.0-7.4 (23, 137), and the intestinal Na/P_i cotransporter is electroneutral at pH 7.4 and electrogenic at pH 6.1 (23). Biochemical and functional studies have revealed that the intestinal P_i transport system is enhanced by vitamin D_3 and dietary P_i (30, 161, 176, 177, 200, 259) but is not responsive to parathyroid hormone (PTH) (137).

Diffusion possibly occurs only when the concentration of dietary P_i in mucosa is high enough to achieve its chemical gradient to overcome the inside-negative transmembrane potential difference. However, it is trival pathway for P_i entry in the jejunum, where P_i is mainly absorbed (53). Intracellular concentration of P_i is calculated to be ~ 1 mM and plasma P_i is ~ 0.5 mM (193). Therefore, basolateral exit is likely via P_i channel and/or facilitated P_i diffusion along with its electrochemical gradient. The scheme for P_i absorption is described in Figure 1.

To date, two Na-dependent P_i cotransporters, type IIb (Na/P_i-IIb; GenBank accession number AAC80007) and type III Na/P_i cotransporter (also called PiT-2; GenBank accession number AF196774), have been identified in mouse small intestine (10, 100). Na/P_i-IIb is expressed in enterocytes and is located in BBMs (100). Apical location, kinetic characteristics, and pH dependency suggest that type IIb protein is likely a major Na/Pi cotransporter in the mammalian intestine (100).

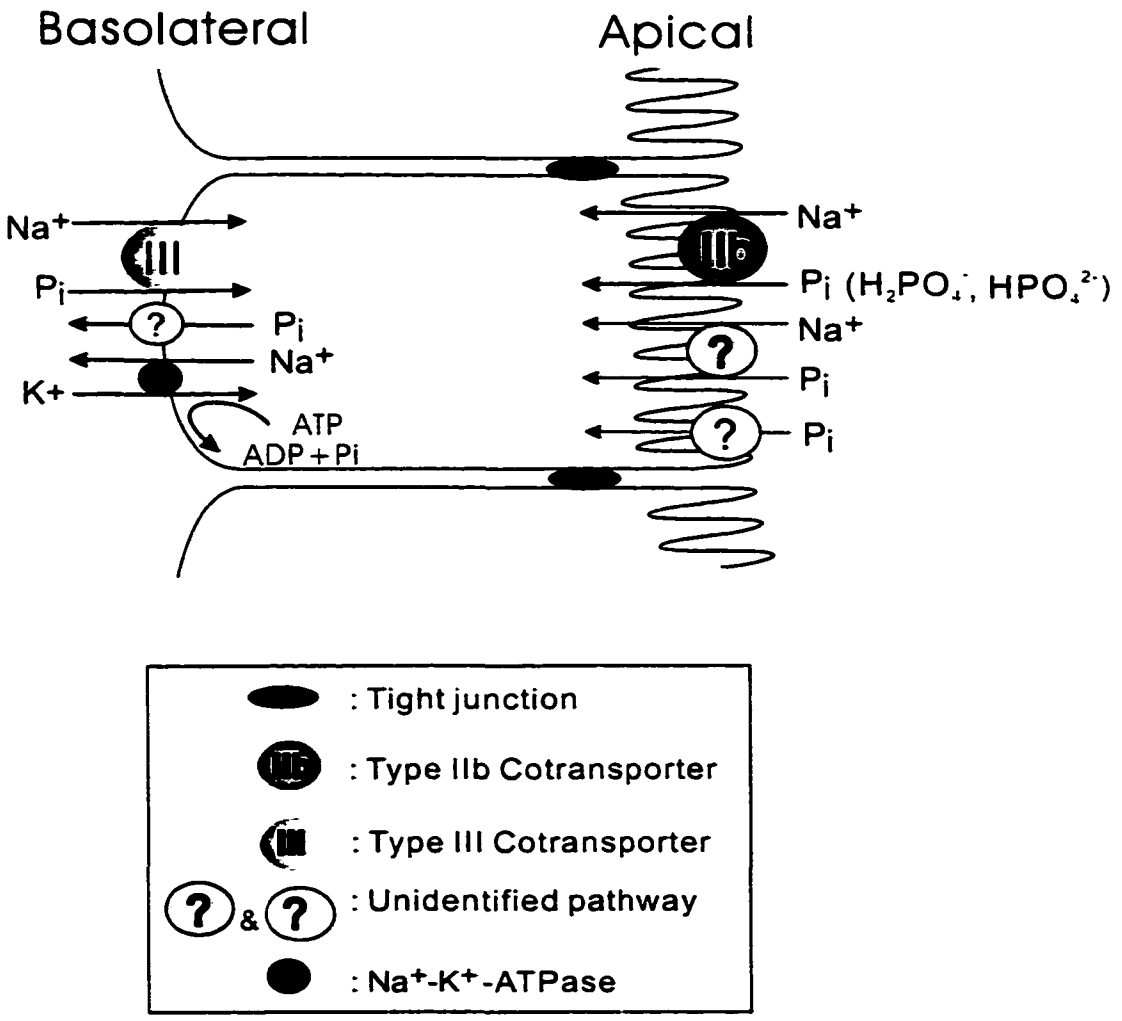

Figure 1. Scheme for inorganic phosphate absorption in the intestinal epithelial cells. This diagram shows known two Na/P_i cotransporters and possible P_i pathways in the enterocyte. Type IIb (Na/P_i-IIb) cotransporter is located in the BBM (100) and type III cotransporter is though to be located in the BLM (10). The direction of movement of transported molecules is indicated by arrows. Also shown Na^+-K^+-ATPase transfers K^+ ions into the cytosol from the extracellular fluid and Na^+ ions out. As a result, the concentration of K^+ is 20-40 times higher, and the concentration of Na^+ is 8-12 times lower in the cells than in the blood. Because of the inside low Na^+ gradient, inward movement of Na^+ ions is thermodynamically favored and is used as force to move P_i ions against their gradients. Additionally, the inside-negative membrane potential is mainly maintained by constant release of cytosol K^+ ions, which are provided by Na^+-K^+-ATPase (145).

Kidney

Three Na/P_i P_i symporters, type I (NPT1), type II (Na/Pi-IIa), and type III Na/P_i cotransporters (also called Glvr-1 and Ram-1), have been identified in the renal proximal tubules (170, 171, 174, 175). P_i is taken up from the tubular fluid mainly by type IIa Na/P_i cotransporter (Na/P_i-IIa) and exits the cell via an undetermined basolateral transport pathways. The brush-border entry step via Na/P_i-IIa is the rate-limiting step and is regulated by several physiological and dietary factors including glucocorticoid (83), epidermal growth factor (EGF) (3, 4), parathyroid hormone (PTH) (108, 174, 194), thyroid hormone (2, 225), vitamin D_3 (236), and dietary phosphate (41, 49, 101, 118, 174, 239, 265). Besides Na-dependent P_i cotransporters, a small amount of P_i is taken up by a Na-independent pathway(s) in the BBM of proximal tubules. Absorbed P_i is exported from the basolateral membrane (BLM) into the circulation. Possible basolateral P_i export includes anion exchange, P_i channel and facilitated P_i diffusion along its electrochemical gradient. BLM also imports P_i via type III Na/P_i cotransporter when BBM P_i entry is insufficient to satisfy cellular requirements (126). Thus, the type III cotransporter is likely involved in intracellular P_i homeostasis.

Ontogeny of Phosphate Absorption

Plasma P_i levels decrease with age and this age-related reduction may result from decreased renal and intestinal P_i absorption (7). Several studies have suggested that the age-related decline in renal P_i absorption is due to lower Na/P_i-IIa expression (2, 83,

238). On the other hand, a correlation between ontogeny of intestinal Na/P_i cotransport and expression of the Na/P_i-IIb cotransporter has not yet been documented.

Abnormal P_i Homeostasis

Although intestinal and renal Na/P_i uptake is well regulated by physiological factors, P_i homeostasis can be altered by genetic and environmental factors. Genetic abnormalities include X-linked hypophosphatemic rickets (XLH; OMIM #: 307800), hypophosphatemic bone disease (HBD; OMIM #: 146350), hereditary hypophosphatemic rickets with hypercalcuria (HHRH; 241530), and autosomal dominant hypophosphatemic rickets (ADHR; OMIM #: 193100). The inherited hypophsophatemic reckets are intensively described below.

Environmental factors that cause hypophsophatemic reckets include tumor-induced osteomalacia and other physiological conditions, such as gastrointestinal disorders, and severe decrease in dietary availability of vitamin D, calcium and P_i. Limited dietary supply of P_i and immature digestive systems may partially explain the prevalence of hypophsophatemic rickets and osteopenia in preterm infants.

Phosphate Deficiency and Rickets in Premature Infants

P_i Homeostasis in Infants

The skeletal development begins in early embryonic life and continues throughout fetal life, infancy and childhood (228). Especially high growth velocity is observed during the late stage of gestation and the first year. Thus, plasma P_i concentrations are

maintained at a higher level to fulfill P_i requirements during development than in adults (248). Adequate P_i absorption is critical to provide for the rapid cellular and skeletal growth in both preterm and full-term infants.

Plasma P_i levels are highest during infancy and then gradually decline until adulthood (248). The decline in plasma P_i concentration may result from a decrease in intestinal and renal P_i (re)absorption (7). After weaning of laboratory animals, both intestinal and renal Na/P_i uptake activities decrease with age (22, 25, 78, 238). Intestinal Na/P_i absorption across the apical and endoplasmic reticulum membranes of rat enterocytes is highest during the suckling period and decreases with age (22, 25, 78). Conversely, renal Na/P_i uptake in rats is lowest in suckling animals, highest at weaning and declines with age (238). These observations suggest that high P_i levels in plasma are maintained by upregulated intestinal Na/P_i uptake in infants.

Preterm infants are more susceptible for P_i deficiency because human milk does not fully support P_i requirement to support their rapid growth (234). In addition, hypomineralization and growth retardation are frequently seen in premature infants with chronic lung disease and gastrointestinal problems (9). Furthermore, damage and immaturity of BBM and/or Na/P_i cotransporter proteins can cause a massive reduction of P_i absorption from the intestine.

Abnormal P_i Physiology in Premature Infants

Osteopenia and rickets are very common nutritional problems in prematurity (40, 67). P_i requirements of prematurity are higher than requirements of full-term infants

perhaps because of an immature gastrointestinal tract and decreased active nutrient absorption after abrupt interruption of maternal nutrient supplies through the placenta at birth (228). There is a high correlation between bone mineral content, gestational age, birth weight, and bone width (162, 229). The frequency of osteopenia and rickets is inversely correlated with gestational age and directly correlated with postnatal morbidity of bronchopulmonary dysplasia, necrotizing enterocolitis, prolonged malnutrition, delay in full enteral feeding, and prolonged parenteral nutrition (31). Rickets has been found in 55% of preterm infants (a birth weight less than 1000 g) and in 23% of infants with a birth weight less than 1500 g (123, 158). Infants who do not tolerate enteral feeding represent a special challenge to provide sufficient calcium and Pi and are at the highest risk for osteopenia and rickets.

Chronic lung disease and gastrointestinal problems are frequently seen in premature infants (9). Hypomineralization and growth retardation have been reported in prematurity under GC treatment, which is commonly utilized for preterm infants with chronic lung disease to improve pulmonary compliance (218, 264). Damage and immaturity of BBM and/or Na/P_i cotransporter proteins can cause a massive reduction of P_i absorption in the intestine. Furthermore, rickets caused by phosphate deficiency has been found in prematurity with aluminum-containing antacid therapy (196), and in prematurity with prenatal P_i deficient due to abnormal placenta (102). In addition, hypophosphatemia is frequently associated with gastrointestinal disorders such as inflammatory bowel disease, celiac disease, and cholestatic liver disease (226).

Because the defects in P_i metabolism are developmental, better understanding of developmental changes in nutrient transports is necessary.

Developmental Effects on Intestinal Nutrient Transporters

Because of need for efficient utilization of nutrients for massive growth, most nutrient transport activities are highest in neonates and decrease during development (146). Developmental changes also affect the diversity of intestinal gene expression along the crypt-villus and duodenal-colonic axis. In the neonatal intestine, nutrient transport occurs along the entire crypt-villus axis, whereas in the adult intestine the absorption of nutrients is shifted to the upper part of villi (222). Na^+-dependent glucose and alanine transporters were observed in neonatal colon during the first postnatal days and rapidly disappear by 6-8-day (197, 198). Although the physiological significance of this phenomenon remains under speculation, the presence of nutrient transporters in colon can compensate for the low capacity of the small intestine to absorb nutrients. The colon also appears to have a significant role in absorption of water and ions and in maintenance of normal fluid and electrolyte homeostasis in neonatal mammals. Colonic nutrient absorption may be extremely important during the rapid postnatal growth when the small intestine absorption and renal reabsorption functions are not fully developed (94).

Sugar Transport

In neonates, sugars and amino acids seem to be absorbed by the same mechanisms as in adults although the relative contribution of individual transporters may change

substantially during maturation. SGLT1, which actively transports glucose and galactose across the apical membrane in adults (274), is present long before birth. SGLT1 expression level increases during fetal development and in preparation for absorption of glucose and galactose released from hydrolyzed lactose during the suckling period (261). SGLT1 cotransporter mRNA levels are developmentally regulated after birth (73, 139, 165). SGLT1 transport activity decreases during the weaning period. The decrease is associated with disappearance of an activity distal segment (182, 250). In contrast, fructose transporter, GLUT5, is expressed at low levels during the suckling period and increases its activity during the weaning period (32). The enhancement of GLUT5 activity correlates with the onset of fructose appearance in the intestinal lumen as a result of an increase in sucrase activity during the weaning period. GLUT5 expression can be precociously induced by dietary fructose administration (219). Although absorption of glucose, galactose, and fructose across the apical membrane is mediated by two different proteins, their transport across the basolateral membrane (BLM) may be mediated by a single protein, the facilitated glucose transporter GLUT2, that decreases during the late suckling period (165).

Protein and Amino Acid Transport

The presence of Na^+-dependent and independent pathways for amino acid transport was observed in human small intestine before birth (152). The prenatal presence of nutrient transporters is critical for absorption of the dilute nutrients existing in swallowed amniotic fluid. The nutrient stimulation is critical for normal growth and

maturation of the intestine in fetus although major pathways of nutrient absorption for fetus mediate the placenta and umbilical vein (195). Suckling animals show quite distinct protein absorption from mature animals. During the suckling period, the majority of proteins from colostrum and milk are transferred across the intestinal wall as intact molecules. The specific transport is achieved in many cases by binding of luminal factors to specific receptors that shuttle them across the intestinal mucosa without intracellular hydrolysis. Intact food proteins cross the epithelium predominantly through specialized M cells. The effective pynocytosis of macromolecules is facilitated by decreased proteolytic degradation of proteins due to the presence of colostral protease inhibitor, and low secretion of gastric and pancreatic protease. These unique transport pathways are important to gain passive immunity for the newborns and thus, the pathways disappear after the suckling period.

Developmental effects on intestinal apical amino acid absorption were intensively characterized in pigs (33). Absorptive rates of amino acids generally declined during the first 24-h after birth. Absorption of neutral (leucine and methionine), and imuno (proline) amino acids continuously declined although acidic (aspartate) and basic (lysine) amino acids did not decline during the suckling period. Besides single amino acid transport systems, there is an identified intestinal oligopeptide transporter (PepT1), which is highest in 4-day-old rats and decreases to adult levels by day 28 after birth (166).

Water and Salt Absorption

Water absorption during the suckling period is higher than in adult life. At all ages, water is passively absorbed via water channels of the aquaporin family (AQP) in response to osmotic gradients created by transcellular absorption of Na^+ and other solutes. Previous studies discovered AQP1 throughout the GI tract, AQP2 in apical membranes of rat distal colon epithelium, AQP4 in BLM in the small intestine (76, 128). In addition, recently cloned AQP10 is exclusively expressed in the duodenum and the jejunum and suggested to be important for intestinal water absorption (89). However, the developmental pattern of APQ expression in the intestine has not been addressed.

The high water absorption during early postnatal life may reflect leaky paracellular pathways (188), and colonic water absorption is predominant to small intestinal absorption during the suckling period, and then decreases (68, 160). In addition, other study has suggested that water transport is directly coupled to SGLT1, which is highly expressed in newborns and transfer 260 water molecules per each sugar molecule transport (147). Therefore, SGLT1 may be the major player in intestinal water absorption.

Na^+ absorption is also high during early suckling and decreases with age (188). The major Na^+ uptake pathway in the colon is electrogenic Na^+ transport via amiloride-sensitive Na^+ channels, which increase in abundance from fetal life to the end of the weaning period (185). The capacity of electrogenic Na^+ transport increases during the suckling period, decreases during the weaning period, and then is replaced by Na^+-H^+ exchanger (NHE) family. NHE-3, which is expressed in the apical membrane of

enterocytes in the small intestine and epithelium of the colon, contributes 59% of total Na^+-H^+ exchange in suckling and 92% in 6-wk-old rats (119). Intestinal BLM expresses two NHE isoforms, NHE-1 and NHE-4, whose expression is lowest in suckling and highest in adult rats (48). Na^+ is extruded across BLM by Na^+ pump, Na^+-K^+-ATPase, which increases during the suckling and weaning period (189). The expression of Na^+-K^+-ATPase is regulated at mRNA levels and reaches adult levels at weaning (75, 282).

In contrast to NHE exchangers, the developmental pattern of Cl^-/HCO_3^- exchanger is decreased with age although Cl^-/HCO_3^- exchanger is considered to have functional coupling with Na^+-H^+ exchangers (205). In addition to Cl^-/HCO_3^- exchanger, there are other Cl^- transport systems to compensate for the developmental decrease in Cl^-/HCO_3^- exchanger during early postnatal life (57).

Calcium Transport

Adequate calcium absorption is also important to ensure normal bone development in earl infancy. The major calcium uptake system is a mediated vitamin D-independent, nonsaturable pathway in newborn and suckling rats, and is replaced by a combination of a saturable vitamin D-dependent and –independent absorption at weaning (213).

Enzymes involved in intestinal nutrient absorption also play important roles in developmental changes in the GI tract. During the suckling/weanling transition period, the small intestine prepares for solid food entry by enhancing mucosal proliferation and altering activities of digestive enzymes, such as lactase and sucrase-isomaltase.

Glycosylation of these enzymes is also regulated during this period although the role of glycosylation is not well understood. The developmental changes in the enzyme activities are described below.

Developmental Effects on Intestinal Enzymes

Lactose-Phlorizin Hydrolase (Lactase)

Lactose-phlorizin hydrolase (lactase, LPH) is necessary to digest lactose, the main carbohydrate in milk. Thus, lactase plays a critical role in carbohydrate intake of mammalian neonates, and then its activity declines after the weaning period when lactose is no longer the main part of the diet. Although congenital lactose deficiency is extremely rare, lactose intolerance is very common in adult population.

The mechanisms involved in age-dependent down-regulation of lactase activity have not been fully understood. At least, down-regulation is not regulated by dietary lactose (69, 209) but seems to be genetically programmed (63, 227, 253, 262). Lactase mRNA levels are markedly higher in suckling rats than in adult rats and the decrease in lactase mRNA is due to decreased transcription rate (34, 129). Wang et al. (1998) demonstrated that genetically programmed lactose intolerance was detectable by patterns of genetic polymorphism in the LPH gene, which determined lactase mRNA levels and subsequent enzyme activity (262). Lactase gene promoter region include *cis*-regulatory elements that interact with intestinal nuclear factor NF-LPH1 in pigs (253), the intestine-specific homeodomain protein Cdx2 in rats (63), and hepatocyte nuclear factor HNF-1 in Caco-2 cells (227). Among them, mRNA level of NF-LPH1 coincided to post-weaning

decline of lactase (253). Meanwhile, other studies suggest posttranscriptional down-regulation of lactose activity at weaning (201, 254). Additionally, terminal sugars of glycosylation shift from predominantly sialic acid to fucose during the suckling/weaning transition, although this shift is probably not responsible for the enzyme down-regulation (35).

Sucrase-Isomaltase

Sucrase-isomaltase, which is required for digestion of sucrose and starch in the weaning diet, is very low or undetectable during the first two postnatal weeks and increases expression during weaning (97). A previous study using transgenic mice suggested that increased expression of the sucrase-isomaltase gene at the suckling-weaning transition is due predominantly to induction of transcription (255). Further studies of the human sucrase-isomaltase promoter in cell lines have identified three major positive regulatory elements, sucrase-isomaltase footprint 1 (SIF1), SIF2, and SIF3 (252). Cdx1 and Cdx2 interact with the SIF1 element and induce gene transcription *in vitro* (233, 240). The SIF2 and SIF3 elements interact with HNF-1 proteins to regulate transcription (275). However, it is currently unclear whether these proteins interact with the sucrase-isomaltase gene promoter *in vivo* to enhance gene transcription during intestinal development.

Glycosydases

Many intestinal proteins located in the BBM are glycoproteins and their glycosylation is also developmentally regulated (81). A shift from high sialylation during suckling to high fucosylation after weaning has been reported in rat intestinal glycoproteins (35, 127, 251). This shift is associated with a parallel decline in the activity and the mRNA level of α-2,6-sialyltransferase from birth to weaning (18, 40, 258), and by a large increase in the activity of α-1,2-fucosyltransferase just after weaning (16, 19). Therefore, a developmental increase in α-1,2-fucosyltransferase activity during suckling/weaning transition is necessary for intestinal glycoproteins to be present in their adult forms.

The intestinal transferase activities may be modulated by hormonal factors and dietary factors. Polyamines in human and animal milk are essential for the intestinal maturation (148). Exogenous spermine, a dietary polyamine, induced precocious intestinal maturation in suckling animals (267, 270). Oral administration of spermine enhanced α-1,2-fucosyltransferase activity and fucosylation rate of apical glycoproteins in suckling rats although it did not decrease sialytransferase activity (81). Kaoiass et al (1994) proposed that polyamine-induced intestinal maturation in suckling animals was secondary to enhanced adrenocorticotrophic hormone (ATCH) (111). Their hypothesis is supported by a previous study in which cortisone injection precociously induced a decreased sialyltransferase activity and an increased fucosyltransferase activity in suckling rats (40). Biol et al. (1991) demonstrated that hydrocortisone administration to suckling rats induced a precocious activation of fucosyltransferase to adult levels.

However, antiglucocorticoid drug, RU38486, failed to prevent the developmental rise of fucosyltransferase activity in weanling rats (19). Therefore, exogenous GCs or polyamines can accelerate velocity of development in suckling animals although they may not be sufficient to block developmental changes in weanling animals.

Use of GC as a tool to study intestinal maturation allows increased understanding of these complex processes. Possible involvement of endogenous GCs in intestinal maturation, exogenous GC-induced maturation and adverse effects of GCs are summarized below.

Effects of Glucocorticoids on Intestinal Maturation

GCs are considered as the most potent regulators among physiological factors involved in intestinal maturation (94). Glycosylation (fucosylation) in rat small intestine is increased by GC administration during the suckling period through enhanced fucosyl-transferase activity (17). Jejunal sucrase activity is precociously increased by dexamethasone between the postnatal ages of 4 and 15 days (96), and the precocious induction is likely due to an increase in sucrase-isomaltase mRNA levels (138). Pharmacological doses of cortisone also induced coordinated increases in jejunal sucrase and lactase activities and in corresponding mRNA levels in 6-days-old rats (279). In addition, fetal lactase activity was also enhanced by cortisol-infusion (210). Meanwhile, GC antagonist treatment significantly inhibited GC-induced elevation of sucrase, trehalase, and glucoamylase (179). Moreover, adrenalectomy and GC antagonists delay intestinal maturation (94).

Endogenous plasma GC levels markedly change during development. Rat plasma corticosterone, the principal GC in rats and mice, was very low during the first two postnatal weeks, rose during the third postnatal week, and then gradually decreases into adulthood (95). In the first two postnatal weeks, exogenous GC administration induces precocious intestinal maturation by modulating gene expression, membrane fluidity and patterns of protein glycosylation (17, 40, 98, 176, 207) but the GC responsiveness is lost after day 17-18 in rats (138).

Effects of Glucocorticoids on P_i Homeostasis and Bone Mineralization

Chronic GC treatments are the most common risk factors in the development of osteoporosis. Marked bone loss has been described in adult, children and premature infants who receive exogenous GCs for its anti-inflammatory action (51, 60, 62, 133, 144, 163, 264, 272). However, dexamethasone is commonly used for preterms with chronic lung disease to improve pulmonary compliance and facilitate weaning from the ventilator (110, 181). Prolonged GC-treatment causes growth impairment, a delay in bone formation and skeletal maturation (12, 39, 168, 263). GCs are reported to induce disturbances in mineral and vitamin D metabolism (39, 121, 164), bone mineralization and collagen synthesis (52). Although precise mechanisms remain unknown, GC-induced bone loss was protected by an estrogen analog (tamoxifen) in adult patients as well as piglets (65, 74). These results imply that specific genomic actions are involved in GC-induced abnormalities.

Clinical observations showed that chronic GC administration enhances urinary P_i excretion and reduces plasma P_i levels in adult patients (122, 130) and premature infants (218). Interestingly, *in vivo* studies have reported that the renal P_i reabsorption mediating Na/P_i-IIa cotransporter is reduced by GC treatment in adult rats but not in suckling/weanling rats (83, 140). Meanwhile, another study reported that pharmacological doses of GCs markedly reduced intestinal Na/P_i transport in suckling rabbits (24). Therefore, GCs appear to play a crucial role in regulating P_i homeostasis by control of intestinal P_i absorption in young animals. However, the specific effects of GCs on Na/P_i-IIb expression have not been addressed.

Besides hormonal controls, studying genetic defects that lead to hypophosphatemic rickets also help to understand P_i metabolism. Known inherited disorders and a possible mechanism inducing hypophosphatemic condition are described below.

Genetic Abnormalities Causing Hypophosphatemic Rickets

X-linked hypophosphatemic rickets (XLH), autosomal dominant hypophosphatemic rickets (ADHR), and oncogenic osteomalacia are phenotypically similar disorders characterized by hypophosphatemia, renal phosphate wasting, normal or low serum calcitriol concentrations, normal serum concentrations of calcium and parathyroid hormone (PTH), and defective skeletal mineralization (203).

XLH results from mutations in PHEX (Phosphate regulating gene with Homologies to Endopeptidases on the X chromosome; formerly PEX) gene, encoding a

membrane-bound endopeptidase, whereas ADHR is caused by mutations of the gene encoding a member of the fibroblast growth factor family, FGF-23 (1). Tumor-induced osteomalacia is invariably curable if the tumor can be found and resected, indicating that phosphate wasting is induced by humoral factors secreted from the tumor. The humoral factor that is responsible to cause tumor-induced osteomalacia has been recently cloned and identified as FGF-23, the gene mutated in ADHR (215). FGF-23 has been proposed as the phosphaturic factor, previously termed "phosphatonin" (230).

Both the wildtype FGF-23 and the ADHR mutant FGF-23 (R179Q) inhibit phosphate uptake in renal epithelial cells (26). In the same study, PHEX cleaved native FGF-23 but not the mutant R179Q, which has a missense mutation at cleavage site. Therefore, overproduction of FGF-23 by tumors, mutations that prevent cleavage of FGF-23, and PHEX mutations that fail to cleave FGF-23 would all increase the level of uncleaved FGF-23. In the proposed hypothesis, FGF-23-induced renal abnormalities lead to phosphaturia, hypophosphatemia, and rickets/osteomalacia. Possible mechanism underlying the hypophosphatemic condition of XLH, ADHR and tumor-induced osteomalacia is summarized in Figure 2.

X-linked Hypophosphatemic Rickets (XLH)

X-linked hypophosphatemic rickets (XLH) is the most prevalent (1 in 20,000 live births) inherited hypophosphatemia (214). Phenotypic characteristics include rickets, osteomalacia, growth retardation, and lower body skeletal abnormalities (244).

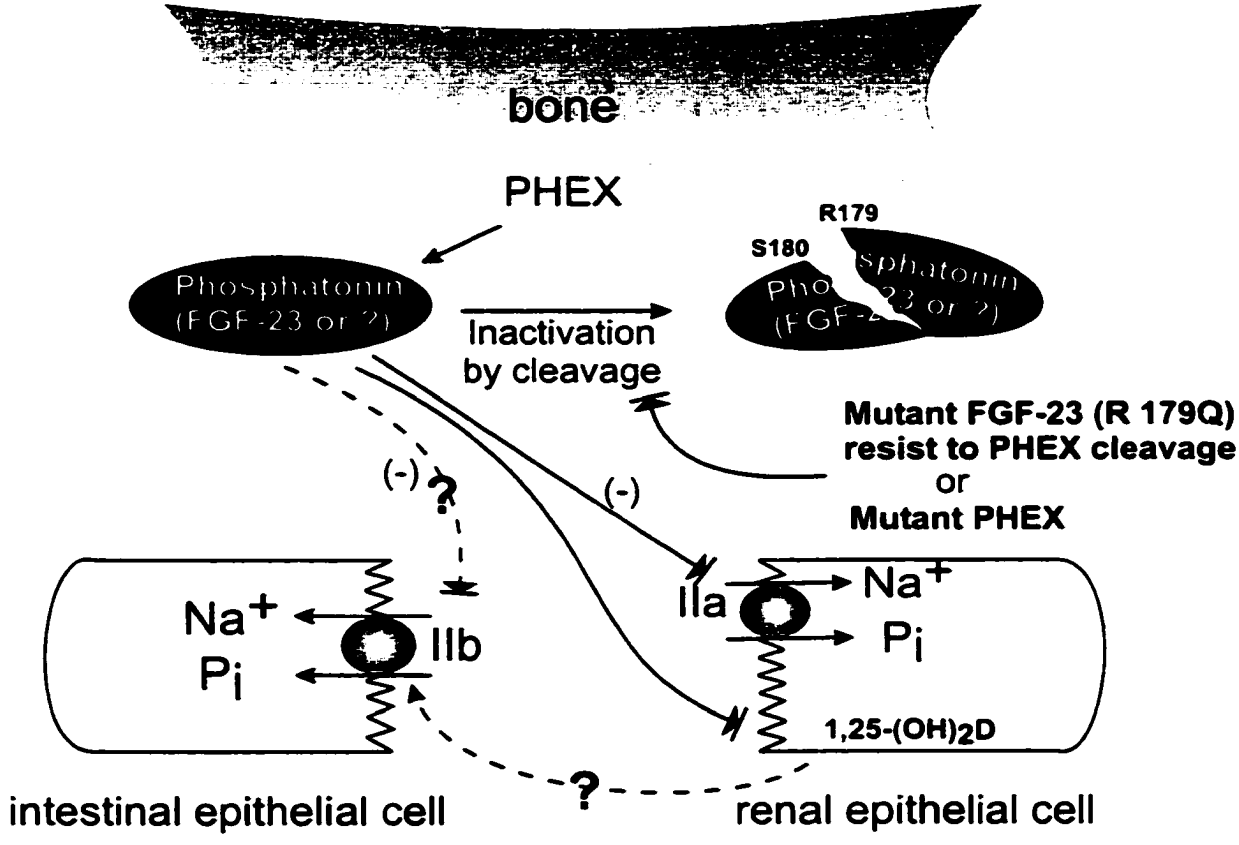

Figure 2. Hypothetical role of PHEX, phosphatonin and FGF-23 in phosphate homeostasis. Both FGF-23 mutant (R179Q) that resists to PHEX cleavage in ADHR and overproduction of FGF-23 in tumor-induced osteomalacia increase circulated FGF-23 level, resulting in increased phosphatonin-receptor interaction. In XHL, the defective PHEX gene products fail to inactivate a bone-derived peptide factor, phosphatonin (FGF-23), and result in increased phosphatonin-receptor interaction, which decreases Na/P_i-IIa gene transcription (41), possibly decreases intracellular translocation of the gene products and impairs $1,25(OH)_2D$ metabolism in the renal epithelial cells (244). In young XLH patients, phosphatonin-receptor interaction or reduction of plasma $1,25(OH)_2D$ level may lead to decrease Na/P_i-IIb expression resulting in defective Pi absorption (217) in the intestinal epithelial cells (30).

The most common features of XLH are short stature and femoral and/or tibial bowing presenting early in life (1 to 2 years) without muscle weakness, tetany, or convulsions. These symptoms appear to result from combined defects in renal tubular Pi transport and abnormal regulation of renal 25-hydoroxyvitamin D [25(OH)D] metabolism. In XLH patients, plasma 1,25-dihydroxyvitamin D does not increase in response to increased PTH as well as P_i deprivation. Thus, biochemical manifestations include high serum alkaline phosphatase levels, abnormal vitamin D metabolism (203) and hypophosphatemia, mainly resulting from decreased phosphate reabsorption in the renal proximal tubules (176, 178, 243) by Na/P_i-IIa cotransporter (13, 42). Interestingly, intestinal P_i absorption is not affected in adult XHL patients. Defective P_i transport of the small intestine is only reported in juvenile XLH patients (50, 217), and young mouse homologue of this genetic defect (161).

Insight into the pathogenesis of this disorder has been facilitated by studies on the *Hyp* mouse, which displays many of the phenotypic and biochemical manifestations of XLH along with an X-linked mode of inheritance. *Hyp* mice studies have indicated that the defect responsible for hypophosphatemia is localized mainly the BBM of the proximal tubule (176). However, *Hyp* mice express normal Na/P_i-IIa cotransporter mRNA and protein (43). Collins et al. (1995) reported that the defective renal P_i absorption seen in *Hyp* mice is not because of abnormal expression of Na/P_i-IIa protein but because of reduced transcription level and resulting decrease in Na/P_i-IIa mRNA levels. Furthermore, Na/P_i-IIa cotransporter gene localizes chromosome 5q35 in

human (126), and chromosome 13B in mice (284). These observations define that Na/P_i-IIa gene is not the cause of XLH but is inhibited by *Hyp* mutant gene.

Positional cloning of the gene responsible for XLH identified PHEX in chromosomal region Xp22.1 (104, 257). The human and murine PHEX cDNAs have been cloned (13, 59, 82, 84, 142, 231) and they are believed to encode membrane bound proteins with homology to members of the neutral endopeptidase (NEP) family (104, 257). NEP family is a widely expressed proteolytic inactivator for various small peptides (208).

Abnormal Metabolism of Vitamin D in XHL

Vitamin D_3 (cholecalciferol) is produced in the skin from pre-vitamin D. The initial step of bioactivation of vitamin D_3 is the 25-hydroxylation of vitamin D_3 to 25-hydroxyvitamin D_3 [$25(OH)D_3$] by 25-hydroxylase, which predominantly occurs in the liver (21) and several other tissues. The second step is activation of $25(OH)D_3$ to 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], by a 1α-hydroxylase mainly in the kidney mitochondria (71, 134), and macrophage and skin (71). Degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$ is initiated with side-chain hydroxylation at C-23 and C-24 by 23/24-hydroxylases mainly in the kidney (150, 204). Vitamin D_2 (ergocalciferol), which is another dietary source of vitamin D, is converted from ergosterol in plants. The metabolic pathways as well as bioactivities of vitamin D_2 are same as vitamin D_3 in animal body.

The key enzyme to synthesis $1,25(OH)_2D$ from 25-hydoroxyvitamin D, 1α-hydroxylase, is regulated by PTH, cAMP and vitamin D restriction (29). In absence of

PTH stimulation to 1α-hydroxylase, 24-hydroxylase is increased and catalyzes 1,25-$(OH)_2D$ to an inactive form, calcitoic acid (77). Thus, the kidney is critical for the normal vitamin D metabolism.

Hyp mice have exhibited abnormalities in both $1,25(OH)_2D$ synthesis and catabolism in the kidney. Pi deficiency stimulates the hormone synthesis in the normal mice but decreases the production in *Hyp* mice (54, 278). Neither vitamin D deficiency nor calcium restriction enhances renal 1α-hydroxylase in *Hyp* mice (241, 242). In addition, the renal 1α-hydroxylase response to infusion of PTH, cAMP and PTH-related peptide is also reduced in *Hyp* mice. Although the 24-hydroxylase does not appear to be affected by Pi deficiency in normal mice, downregulation of $1,25(OH)_2D$ catabolism is associated with low plasma P_i. Furthermore, upregulation of $1,25(OH)_2D$ catabolism and enhanced 24-hydroxylase mRNA abundance are associated with high plasma Pi (246). Those abnormalities are considered as results of defective PHEX protein mediating active phosphatonin (possibly FGF-23) and its receptor interaction.

Abnormal Intestinal P_i Absorption in XHL

In addition to the kidney abnormality, impaired intestinal P_i uptake was observed in young XLH patients (50), and was suggested due to be defective transport of P_i in intestinal mucosa (217). Furthermore, juvenile *Hyp* mice exhibit a significant impairment in jejunal P_i absorption and lower plasma levels of $1,25(OH)_2D$ relative to normal mice (30). Since the intestinal abnormality was improved by $1,25(OH)_2D$ administration in juvenile *Hyp* mice, the abnormal P_i absorption in the intestine may be secondary to

abnormal renal vitamin D metabolism (30). Although no molecular study has investigated the defective intestinal P_i absorption in XLH patient or *Hyp* mouse, potential scheme is illustrated in Figure 2. Current therapy for XLH patients is a combination of the oral administration of P_i and 1,25-$(OH)_2$D (244). However, it is far from ideal so that the novel therapeutic options are necessary.

Autosomal Dominant Hypophosphatemic Rickets (ADHR)

Similar to XLH, ADHR is characterized by low serum Pi concentrations, rickets, osteomalacia, leg deformities, short stature, bone pain, and dental abscesses. The ADHR Consortium (2000) described a positional cloning approach used to identify the ADHR gene encoding a member of the fibroblast growth factor family, Fibroblast growth factor 23 (FGF-23). The missense mutations identified in FGF-23 gene of ADHR patients affect two arginines located in cleavage site for degradation (1). ADHR phenotype is caused by the gain-of-function mutations that block FGF-23 degradation.

Discovery of Phosphatonin Candidate, Fibroblast Growth Factor 23 (FGF-23)

Tumor-induced osteomalacia is one of the paraneoplastic disorders characterized by hypophosphatemia associated with renal phosphate wasting. The fact that removal of responsible tumors normalizes phosphate metabolism is evidence that a humoral phosphaturic factor is responsible for tumor-induced osteomalacia. Shimada et al. (2000) cloned the cDNA, which turned out to encode FGF-23, as a causative factor of tumor-induced osteomalacia. Administration of recombinant FGF-23 decreased serum

phosphate in mice within 12 hours and furthermore, hypophosphatemia with typical phenotypic characters was observed when Chinese hamster ovary (CHO) cells stably expressing FGF-23 were subcutaneously implanted into nude mice. Thus, overproduction of FGF-23 causes tumor-induced osteomalacia, whereas mutations in the FGF-23 gene result in ADHR possibly by preventing proteolytic cleavage, which enhances the biologic activity of FGF-23. Recent study has identified that FGF-23 is a secreted protein, which is abundantly expressed by several different tumors that induce osteomalacia (268). Therefore, FGF-23 may be the candidate phosphate-wasting factor, phosphatonin.

FGF-23 may be an important humeral factor to regulate the renal and intestinal P_i transport systems. The anionic P_i uses Na-dependent active transport systems to be transferred into the cytosol against its electrochemical gradients. The Na/P_i cotransporters expressed in the intestine and kidney are reviewed below.

Sodium Dependent Phosphate Cotransporters

Three Na/P_i cotransporters, type I, type II and type III, have been molecularly identified. They have no significant homology at the level of amino acid sequences, and localize to different chromosomes. However, hydrophobicity analyses of their amino acid sequences revealed that they might be similar transmembrane proteins with six to ten transmembrane segments. Characteristic comparison of these three Na/P_i cotransporters is depicted in Table 1.

Table 1. Comparison of Na/Pi transporters. This table is compiled data described in references listed in text.

		Type II		
Type	Type I	Type IIa	Type IIb	Type III
	NaPi-I	Na/Pi-IIa	Na/Pi-IIb	Glvr-1, Ram-1
Gene locus	chr.6	chr.5	chr.4	chr.2 & 8
Amino acids	465	635	697	679 & 656
Transmembrane	6-8	8	8	10
N-Glycosylation site	+	+	+	+
Km	0.2-0.3mM	0.1-0.2mM	0.05mM	0.025mM
Substrate	Pi, Organic cation	Pi	Pi	Pi
pH dependency	None	Increased at high pH	Increased at low pH	Increased at low pH
Tissue expression	Kidney, Liver	Kidney	S. intestine, Lung and other organs	Ubiquitous
Expressed location	BBM	BBM	BBM	BLM
Regulatory factors	insulin, glucagons	dietary Pi, GH PTH, VD3, GC, EGF, T4	dietary Pi, VD3 No Effect by PTH EGF, GC	dietary Pi, VD3
Affected by mut PHEX	?	Decreased transcription	?	?

Type I Na/P_i Cotransporter (NPT1)

A cDNA (GenBank accession number J05048) encoding type I Na/P_i cotransporter was initially identified by screening a rabbit kidney cortex library for expression of P_i transport activity in *X. laevis* oocytes (266). The apparent K_m for P_i was ~0.3 mM for expression of the human and ~1 mM for the rabbit NPT1. The apparent K_m value for Na^+ interaction was ~50 mM, and no pH-dependence was observed in oocytes (36, 167). In electrophysiological studies in oocytes, evidence was obtained that the type I transporter protein might be a multifunctional anion channel for chloride and organic anions (37). Furthermore, characteristics of NPT1 do not resemble the characteristics of Na dependent P_i uptake observed in the renal BBM vesicles. Therefore, NPT1 is not a major Na/P_i cotransporter in the renal BBM.

Type IIa Na/P_i Cotransporter

Na/P_i-IIa is expressed in apical membranes of epithelial cells in the renal proximal tubules, and it represents the major Na/P_i cotransporter in the kidney (42, 149). Unlike NPT1, the properties of Na/Pi-IIa are similar to those of previously characterized Na dependent P_i cotransport activity in BBM vesicles isolated from proximal tubule (42, 44). Additionally, Tenenhouse et al. (1998) demonstrated that NPT1, Na/P_i-IIa, Glvr-1 and Ram-1 account for approximately 15%, 84%, 0.5 % and 0.5%, respectively, of total Na/Pi cotransporter mRNA in the kidney (245). In Na/P_i-IIa null mice, the renal BBM Na/Pi cotransport activity was reduced by 70% of normal mice (14, 247). Furthermore, Na/P_i-IIa null mice exhibit similar phenotype of a Mendelian disorder of renal phosphate

absorption, hereditary hypophosphatemic rickets with hypercalciuria (HHRH) (14). The phenotype includes increased urinary excretion of P_i, decreased Na/P_i cotransport in isolated BBM vesicles. Na/P_i-IIa associated Na/Pi cotransport exhibited an apparent K_m for P_i of 0.1 mM, a K_m for Na^+ of 40-60 mM, pH-dependence (higher activity at more basic pH), and an inwardly directed current that was compatible with a transport stoichiometry of three Na ions being transported together with one P_i ($H_2PO_4^{2-}$) ion (42, 70, 149). The stoichiometry (Na^+:P_i) determined by voltage-clamp conditions in oocytes was also 3:1 (Figure 5) (42, 70, 149)

The promoter regions of three Na/P_i-IIa genes have been identified from human (87, 235), opossum kidney (OK) cells (99), and mouse (87). The human promoter (GenBank accession # D89927) was shown to be responsive to vitamin D_3 and the vitamin D_3 responsive *cis*-element was identified (236). Further, molecular investigations with the human Na/P_i-IIa promoter revealed that dietary P_i restriction in mice leads to increased binding of a transcription factor (called TFE3) to 6 sites in the promoter region which increases Na/Pi-IIa gene transcription rates (118). Studies with the OK cell Na/P_i-IIa gene promoter (GenBank accession number AJ003021) showed that OK cell Na/P_i-IIa gene expression is transcriptionally enhanced in response to lowered bicarbonate/carbon dioxide conditions (109). Although the murine promoter region (GenBank Accession # U57491) was also identified (87), its regulatory mechanism has not been studied (87).

Type III Na/P_i Cotransporters

Receptors for gibbon ape leukemia (Glvr-1) and mouse amphotropic (Ram-1) retroviruses have been shown to exhibit Na/dependent Pi transport (116, 117, 183), and were named as PiT-1 and PiT-2, respectively. The type III Na/P_i cotransporters are widely expressed in many tissues such as kidney, small intestine, bone, heart, and liver. Thus, type III cotransporters are considered as general housekeeping proteins. In mouse kidney, transcripts of type III cotransporters are found in BLM of the proximal tubule. Based on the mRNA levels, type III Na/P_i cotransporters are two orders of magnitude less abundant than type IIa transporters (247). Its role in the proximal tubule does not seem to be P_i export, but P_i import when luminal P_i entry is insufficient for cell metabolic functions.

Type III transport is characterized by an apparent K_m for P_i in the order of 20-30 μM and an apparent K_m for Na of 40-50 mM. The pH dependence of type III Na/P_i cotransporters is the opposite of the type IIa cotransporter (type III decreases activity by increasing pH). Similar to the type IIa, type III-mediated transport of P_i is electrogenic with a net influx of a positive charge during the transport cycle, with a 3:1 stoichiometry.

The Na/P_i transport mechanism has been also described in the basolateral membrane (BLM) of the intestinal epithelium. Studies using the BLM vesicles suggest an existence of higher-affinity Na/P_i cotransporter, with a K_m of 14-93 μM (79, 120, 178). Recently, a cDNA that encodes a type III Na-dependent phosphate cotransporter from mouse small intestine (mPit-2) has been cloned and suggested as the basolateral Na/P_i cotransporter (10). However, basolateral Na/P_i cotransporter is unlikely important in control of plasma

P_i levels because tight junction prevents dietary P_i from accessing BLM directly, but may have a role to maintain intracellular P_i levels.

Type IIb Na/P_i (Na/P_i–IIb) Cotransporter

Na/P_i–IIb Cloning

The cDNA encoding type IIb Na/P_i-cotransporter was originally cloned by screening an expressed sequence tag (EST) clone derived from a cDNA library of murine embryonic cells, and was determined as follows (100). Amino acid comparisons revealed that the type IIb protein is 57 % homologous to the renal type IIa Na/P_i-cotransporter (Figure 3). There is no homology between type IIb and type I or type III Na/P_i-cotransporters. Highest homologies between type II Na/P_i-cotransporters are seen in regions that also have been proposed to represent transmembrane regions (172), whereas the most striking difference between mouse type IIa and type IIb Na/P_i-cotransporter was found in the C-terminal region. Compared to type IIa sub-family, type IIb proteins have a longer C-terminal tail containing a stretch of 6-10 cysteine residues. Therefore, the cysteine clusters are used as the hallmark of type IIb sub-family. In addition to mouse Na/P_i-IIb cotransporter, genes encoding type IIb sub-family have been cloned in rat (AAF76291), human (AAF31328), bovine (S49228), chicken (AAG35801), carp (AAG35803), dogfish (AAG35795), flounder (AAB16821), rainbow trout (AAG35798), zebrafish (AAG35356), and little skate (AAG35797).

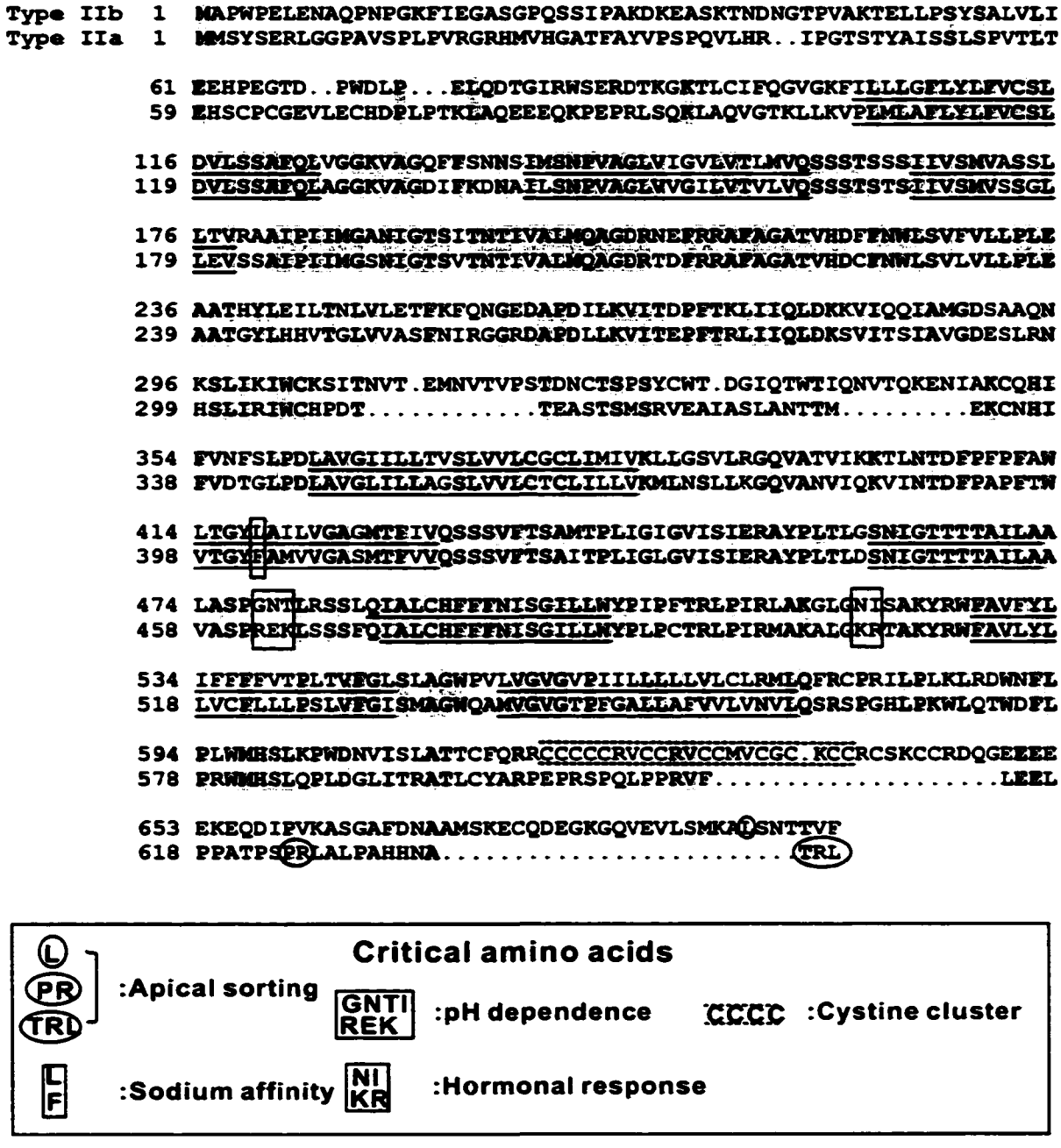

Figure 3. Sequence Comparisons Between Type IIa and Type IIb Na/P_i Cotransporters. The conserved amino acids are shadowed and predicted transmembrane domains are underlined. Critical amino acids shown in the figure are explained in the text (42, 55, 56, 100, 112-114, 149, 171).

Tissue Localization

Expression of type IIb mRNA was found in a variety of tissues including the BBMs of the upper small intestinal epithelium where it is thought to be the major Na/P_i cotransporter (100). By immunohistochemistry of mouse duodenum, expression of the type IIb protein was localized at the BBM of enterocytes (100). In addition to the intestine, Na/P_i-IIb cotransporter is highly expressed in type II alveolar cells in the lung, and is likely involved in surfactant synthesis (88).

Characterization of Na/P_i-IIb Cotransporter

The calculated molecular weight of type IIb protein is 78 kDa, although a single band of ~108 kDa was observed on western blot using type IIb specific antiserum. Because multiple potential *N*-glycosylation sites are contained in a suggested extracellular loop and because *N*-glycosylation sites have been identified in the extracellular loop of the renal type II cotransporter (92), the molecular mass of 108 kDa likely represents the glycosylated form of the type IIb protein.

Kinetic parameters of Na/P_i-IIb cotransporter are determined in *X. laevis* oocytes injected with Na/P_i-IIb cRNA (100). An apparent K_m for P_i of ~50 μM and an apparent K_m for Na of ~30 mM were determined by either isotope flux or by electrophysiological measurements. Its transport activity was slightly higher at more acid pH-values but not significantly pH-dependent in this study. Additionally, significant acidic pH-dependence was reported using human Na/P_i-IIb cotransporter (64). Therefore, pH-dependence of type IIb cotransporter is consistent with results found in intestinal BBMVs (23, 137), but

is opposite to that of the renal type IIa Na/P_i-cotransporter (higher at basic pH). Na/P_i transport mediated by Na/P_i-IIb protein is electrogenic. Transport characteristics of type IIb-mediated Na/Pi cotransport in oocytes are similar to those of Na/P_i cotransport in BBM vesicles isolated from rodent small intestine (176). To date, it is known that vitamin D_3 administration and low phosphate diet increase the intestinal Na/P_i-IIb transport activity and protein levels but do not alter mRNA levels in rodents (90, 115). In addition, another study has suggested EGF treatment transcriptionally regulates Na/P_i-IIb gene expression (277).

Type IIa and type IIb transporters are 57% homologous in amino acids (100), are predicted to contain eight transmembrane domains with the N- and C-terminal tails facing the cytoplasm (149), and are both *N*-glycosylated. Despite the overall structural similarity, these isoforms have striking difference in their C-terminal regions. In addition, the isoforms are different in many aspects such as tissue specificity, sodium-affinity, pH-dependence, and hormonal responses. In order to address possible structural effects on the characteristics and function, the following studies were performed using several mouse IIa-IIb chimeras and site-directed mutagenized cotransporters expressed in *Xenopus* oocytes and other cells. Currently identified amino acids involved in structural effects in these two isoforms are indicated in Figure 3.

Function and Structure Comparison between Na/P_i-IIa and Na/P_i-IIb

Apical Sorting

Type IIa and IIb cotransporters are located in BBMs of the proximal tubule and small intestine, respectively. Na/P_i-IIb protein is also widely found in other tissues. A series of site-directed mutagenesis studies using mouse IIa-IIb chimeras identified that a leucine residue (L691) within the C-terminal domain is necessary for Na/P_i-IIb apical expression in Caco-2 and OK cells (114). The C-terminal of Na/P_i-IIa protein contains PR-residues and TRL-residues, which work for apical sorting specifically in OK cells but not in Caco-2 cells (113). These results support different tissue specificity of two isoforms.

pH Dependence

The isoforms differ in pH dependence. The activity of type IIa increases at higher pH, whereas, type IIb activity slightly decreases at higher pH. A study using mouse IIa-IIb chimeras identified three amino acid residues between fourth and fifth transmembrane domains (charged residues [REK] in IIa and uncharged residues [GNT] in IIb) are critical for pH dependence (56). Indeed, exchange of REK and GNT residues created opposite pH dependence in type IIa and IIb proteins.

Na^+ Affinity

The affinity for Na^+ and P_i of Na/P_i-IIa is lower than those of Na/P_i-IIb. Studies with chimeras determined that part of the second intracellular loop, the fifth transmembrane domain and part of the third extracellular loop determine Na^+ affinities in

both proteins (55). Site-directed mutagenesis of F_{402} residue to L in Na/P_i-IIa, and L_{418} residue to F in Na/P_i-IIb resulted in the type IIa cotransporter with a Na^+ affinity similar to type IIb, and type IIb cotransporter with a Na^+ affinity similar to type IIa, respectively. Therefore, F_{402} residue of IIa and L_{418} residue of IIb are important to determine Na^+ affinity for both proteins.

Hormonal Response

Injection of PTH in rats and mice induced rapid retrieval of Na/P_i-IIa protein. Similar acute reduction was observed in intestinal Na/P_i-IIb protein of mice fed high P_i diet. The structural effects on PTH-induced retrieval of these proteins were studied with mouse IIa-IIb chimeras and site-directed mutants fused to the enhanced green fluorescent protein in OK cells (112). In this study, type IIa that encodes two charged amino acids, K_{503} and R_{504}, was endocytosed in response to PTH whereas type IIb, encoding uncharged residues, N_{520} and I_{521}, was not. In addition, mutant Na/P_i-IIb possessing K_{503} and R_{504} instead of N_{520} and I_{521} was internalized, but mutant Na/P_i-IIa in which K_{503} and R_{504} were replaced to N_{520} and I_{521} failed to be retrieved by PTH treatment. The results indicated that two charged amino acids, K_{503} and R_{504}, of type IIa cotransporter are essential for PTH-induced internalization.

Disulfate Bonds

Sequence comparison between type IIa and type IIb proteins found that both cotransporters contained several cysteine residues in the predicted second extracellular

loop. When rat intestinal BBM protein was exposed to reducing agents, Na/P_i-IIa cotransport activity was decreased and the 80-90 kDa protein was cleaved into two bands of 45-50 kDa (15). A site-directed mutagenesis study suggested two S-S bonds between C_{306} and C_{334} and C_{225} and C_{520} in type IIa proteins (132). The cleavage of S-S bonds in Na/P_i-IIa protein are proposed to induce conformational change in Na/P_i-IIa protein, resulting in inhibition of transport activity and internalization and subsequent lysosomal degradation. Although there are S-S bonds within Na/P_i-IIb protein, reducing agents had no effects on its molecular mass.

N-linked Glycosylation

The Na/P_i-IIa cotransporter has multiple potential *N*-glycosylation sites within the second extracellular loop. Site-directed mutagenesis studies using *X. laevis oocytes* identified two *N*-glycosylation sites that were likely important for effective cell surface expression but not involved in its functional activity (92). The Na/P_i-IIb protein was also suggested to be an *N*-glycoprotein because its molecular weight (~108 kDa) was larger than the predicted size (~78 kDa) and there was putative *N*-glycosylation consensus in its amino acid sequence (100). We found that the mouse Na/P_i-IIb protein is an *N*-glycoprotein using enzymatic deglycosylation study (chapter 3) (5). In addition, we observed potential age- and GC- dependent glycosylation in type IIb protein. The amino acid sequences of mouse type IIa and type IIb cotransporters found in several species are compared in Figure 12.

Further investigation is necessary to elucidate the roles and developmental regulation of *N*-glycosylation in Na/P_i-IIb protein. The following review of glycosylation mechanisms will help to decipher glycosylation mechanisms.

Glycosylation

N-glycosylation is one of the most common post-translational modifications in eukaryotic cells. Glycosylation increases diversity of proteins without modulating genomic information, and has potential to respond to environmental changes. Unlike *O*-glycoproteins, *N*-glycoproteins are cotranslationally synthesized. Highly conserved core formation of N-glycosylation is necessary process to fold newly synthesized proteins properly and transported correctly to final destinations. Terminal oligosaccharides of glycoproteins are diverse in tissues and species, and are varied by environmental changes. Diverse roles of glycosylation include protein folding, quality check, trafficking from the ER to the Golgi, endosome sorting, apical sorting and overall function through its structural role.

Synthesis of Homogenous Core Glycans

An *N*-linked glycosylation is one of the most common post-translational modifications. An *N*-glycan binds to the polypeptide chain through an *N*-glycosidic bond with the side chain of an asparagine (Asn) that is part of the Asn-X-Ser/Thr consensus sequence (124, 186). Although *N*-glycans are highly diverse, core glycans, which is initially added to growing nascent peptides in endoplasmic reticulum (ER), are

homogenous. Synthesis of core glycans starts on the cytosolic surface of the ER membrane by the addition of sugars, one by one, to lipid carrier, dolichylphosphate. When two N-acetylglucosamines and five mannoses have been added, the oligosaccharide is flipped to the lumenal side of the membrane (93). When seven further mannoses and three terminal glucoses are added, the core glycan (GlciMan9GlcNAc2) is ready to be transferred to peptides (35). The coupling of the glycan and peptides is catalyzed by the oligosaccharyltransferase enzyme complex, which recognizes Asn residues in the Asn-X-Ser/Thr consensus sequences and transfers the core oligosaccharide to Asn residues (212). The terminal glucoses are trimmed away by glucosidase I and II, and terminal mannoses by one or more different ER mannosidases. Further trimming and addition occur in Golgi apparatus.

Protein Folding

Cotranslational *N*-linked glycosylation is essential for proper folding for *N*-glycoproteins (86). This process allows cells to secrete large and complex proteins at high levels. Inhibition of glycosylation induced misfolded, aggregated proteins, resulting in failure to reach a functional locations (35, 190). In vitro studies with glycosylated and nonglycosylated proteins have confirmed positive effects of oligosaccharide moieties on the folding processes although glycans are not essential to maintain the folded structure after proteins are properly folded (93, 106).

Calnexin-Calreticulin Cycle

The calnexin-calreticulin cycle is a central process to fold protein properly and allow only correctly folded protein to leave from ER (221, 281). An *N*-glycans work as recognition tags to get onto this process. Membrane-bound calnexin (CNX) and lumenal calreticulin (CRT) are homologous ER lectins that bind transiently to virtually all newly synthesized glycoproteins after two terminal glucoses have been trimmed away from N-linked core gleans glycosidase I and II (91, 184, 237, 283). The protein complex is thereby exposed to another folding factor, ERp57, a thiol oxidoreductase that binds to both CNX and CRT (159). When the remaining third glucose residue is trimmed by glucosidase II, the complexes dissociate. If the glycoprotein is not folded at this time, the oligosaccharides are reglucosylated by an ER glucosyltransferase, and the protein reassociates with CNX and CRT. The cycle is repeated until the protein is either folded or degraded. The CNX- CRT cycle promotes correct folding, inhibits aggregation of folding intermediates, blocks premature oligomerization, regulates ER degradation, and provides quality control by preventing incompletely folded glycoproteins from exiting to the Golgi complex (93, 191, 280). Miss-folded mutant proteins and proteins that fail to reach their native conformation are selectively degraded in the ER (269).

Sorting in the Golgi

Golgi apparatus consists of the cis-Golgi compartment, the medial compartment and the trans-Golgi network (TGN), where glycosylation is completed. Although sorting of glycoproteins occurs all along the cis-trans axis, the TGN serves as a major sorting

station where cargo molecules are packed into secretary vesicles, regulated secretary granules, or vesicles destined for the endosomal/lysosomal system (157). *N*-glycans are important for proper sorting lysosomal enzymes and apical membrane proteins in the TGN. Mannose-6-phosphate receptors (M-6-P receptors) in the TGN plays an important role to sort lysosomal enzymes in the TGN (125). The roles M-6-P receptors escorts lysosomal enzymes via clathrin-coated vesicles to endosomes, and returning empty to the TGN. The pH-dependent binding abilities of M-P-6 receptors help them to load cargo efficiently in the TGN (pH 6.3-pH6.5) and release in endosome (pH 5.0) (141).

Sorting of Plasma Membrane Proteins

Epithelial cell surface consist of functionally and compositionally distinct apical and basolateral membranes, which are separated by tight junctions. To maintain right polarity of epithelial cell surface, the correct sorting mechanisms that segregate apical and basolateral proteins and the proper transport systems are critical. The strict sorting was observed in filter-grown MDCK cells that express very few common proteins in apical and basolateral membranes (BLMs) (202).

Basolateral Sorting

Basolateral targeting seems to be dominant over apical sorting. When basolateral targeting signals were disrupted, many BLM proteins were targeted efficiently to the apical membrane (156). Furthermore, influenza virus HA, an apically sorted *N*-glycosylated protein possessing a transmembrane domain able to associate with

glycolipid-cholesterol rafts (211), was sorted to the BLM when basolateral targeting signals were introduced into its cytoplasmic domain (28). Basolateral targeting signals that have been identified are a tyrosine motif (156), a double leucine motif (103), or hydrophobic/aromatic amino acids (8). These signals are localized in the cytoplasmic domain of transmembrane proteins such as potassium channel, Kir2.3, (136), LDL receptor (156), and hNGF receptor (135).

Apical Sorting

Two, direct and indirect, pathways have been identified to deliver apical membrane proteins in filter-grown Caco-2 cells (135, 155). In the direct pathway, proteins are sorted into vesicles by apical sorting signals in the TGN, and are targeted directly to the correct destination. In the indirect pathway, all membrane proteins are first delivered to the basolateral membrane. From there, apical proteins are endocytosed and transported to the apical domain by transcytosis. Alternatively, proteins can reach the plasma membrane by random sorting, in which they are first transported randomly to both membrane surfaces. Those proteins that belong to the opposite surface are then either degraded, or transcytosed to their final destination (281).

Carbohydrate-Mediated Apical Sorting in TGN

In the direct pathway, some *N*-glycans can act as apical targeting signals although the mechanisms of carbohydrate-mediated cell surface transport has not been fully understood. Gut et al. (1998) demonstrated that *N*-linked carbohydrates of reporter

proteins with no cytoplasmic basolateral targeting signals were transported to apical membrane via an apical sorting pathway (85). As apical targeting signals, *N*-linked carbohydrates seem to utilize forms called glycolipid-cholesterol rafts for mediated-mediated cell surface transport (221). In the TGN, the glycolipid-cholesterol rafts are formed in detergent-insoluble fraction including apical membrane proteins, glycosylphosphatidylinositol (GPI)-anchored proteins, cholesterol, and glycosphingolipids, and move within the fluid bilayer (80). *N*-linked glycans are considered to interact with glycolipid-cholesterol rafts by binding to lectin-like molecules such as VIP-36, an *N*-acetyl galactosamine-binding protein of apical and basolateral transport vesicles (67), or annexin XIIb, an isoform of the epithelial specific phospholipid-binding protein (66, 131). As a result of association of lectins with rafts, *N*-linked glycoproteins are segregated in the TGN-subdomains, which are incorporated into apical transport vesicles.

Overall Roles of Glycosylation

All mutations discovered in congenital disorders of glycosylation (CDG) are critical for protein folding, sorting and transportation in the ER and the Golgi (14). Thus, correct *N*-glycosylations are critical as the recognition tags to be transferred from the ER and the Golgi to the final destinations. Additionally, *in vitro* studies have indicated that glycosylation is involved in protein stability (273), function (143, 154, 202, 249, 271) and clearance (186).

In the small intestine, roles of glycans of glycoproteins are poorly understood although many proteins located in the BBMs are glycoproteins, and developmental differences in the glycans are frequently used as a marker for intestinal maturation (81). One example to imply a potential role of glycosylation in the intestine is seen in the glycans of mammalian intestinal mucins, large-molecular-weight glycoproteins, which are also developmentally regulated (223). Newborn mucin differs from adult mucin in carbohydrate composition of their glycan chains that provide potential binding sites for luminal organisms analogous to intestinal glycoprotein receptors (220). Therefore, the developmental differences in mucin glycans may cause the different susceptibility to infectious agents between adults and newborns.

CHAPTER II

Molecular Cloning of Murine Sodium-Phosphate Cotransporter Type IIb (Na/P_i-IIb) Gene Promoter and Characterization of Gene Structure[1]

Introduction

Dietary inorganic phosphate (P_i) is absorbed primarily in the jejunum by a sodium-dependent phosphate transport mechanism (173, 178) in the brush-border membrane. P_i reabsorption occurs in the renal proximal tubule mainly through the type II sodium-dependent phosphate cotransporter (Na/P_i-IIa) (13, 42). Biochemical and functional studies have revealed that the intestinal P_i transport system is pH dependent (23), and is regulated by dietary P_i (177) and vitamin D_3 (58) but is not responsive to PTH (137). Recently, an apically expressed type II Na/P_i cotransporter cDNA was cloned from the mouse small intestine (called Na/P_i-IIb) (100). This transport protein was suggested to be the major small intestinal Na/P_i transporter based on the observation that it shares nucleotide sequence similarity, kinetic character and membrane localization with the Na/P_i-IIa cotransporter (100). Meanwhile, these transporters differ in pH dependency (100), and response to dietary P_i status and vitamin D_3 treatment (90, 115). To date, the effects of other physiological factors on gene expression have not been examined to more extensively address Na/P_i-IIb gene regulation. The following investigation was

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[1] Arima, K., J. F. Collins, E. R. Hines, L. Bai, and F. K. Ghishan. (2000) *Biochim Biophys Acta* 1494: 149-154.

undertaken to elucidate the genetic regulation of the Na/P_i-IIb cotransporter in the mammalian intestine. We further sought to compare the Na/Pi-IIb gene structure as well as 5'-flanking region with those of the Na/Pi-IIa gene to clarify the genetic relationship between these two cotransporters (87).

Methods, Results and Discussion

A mouse genomic BAC library (Research Genetics, Inc.; Huntsville, AL) was screened with a radiolabeled 371 bp PCR amplified probe corresponding to nt 37 to 407 of the Na/P_i-IIb cDNA (100). A positive clone was confirmed by slot blot analysis with probes corresponding to nt 1 to 100 at the 5'-end and nt 3975 to 4024 at the 3'-end of the cDNA (100) as previously described (169).

DNA was purified from positive clones using the P1/BAC isolation method as described extensively elsewhere (http://www-seq.wi.mit.edu/protocols/BAC.html, Whitehead Institute; Cambridge, MA). The promoter region was sequenced by primer walking on both strands (W.M. Keck Biotechnology Laboratory at Yale University; New Haven, CT). The 5'-flanking DNA sequence (≈1000 nucleotides are shown in Figure 4) was deposited in the Genbank database (accession # 211863) and analyzed for putative *trans*-acting factor binding sites using the Omiga Sequence Analysis software (version 1.1, Oxford Molecular; Oxford, England) and WWW Signal Scan IMD Search Service (http://bimas.dcrt.nih.gov/molbio/matrixs).

The following regulatory elements were detected: AP2 (AP2 binding site), AP4-GT2 (AP4/GT2A/GT2B binding site), CAC (CACCC binding site), gluco (glucocorticoid

response element), MoLV (Moloney leukemia virus binding site), NFY (NF-Y binding site), T_3R-β (ligand-activated thyroid hormone receptor response element) and Tant (T-protein binding site) (Figure 4). This promoter lacked a TATA box, but several potential GC boxes were found.

In addition, we searched for conserved regions between the IIb promoter region and the known murine Na/P_i-IIa promoter (GenBank accession # U57491). Two conserved motifs, which we called motif A and motif B, were detected by the MEME system (Multiple EM for Motif Elicitation version 2.2, http://www.sdsc.edu/MEME/meme/website/intro.html). This program finds common, ungapped patterns in a set of sequences by the expectation maximization algorithm (11). Although their relative locations are not similar (motif A: nt –956 to –985 in the type IIb vs. nt –184 to –213 in the type IIa; motif B: nt –792 to –814 in the type IIb vs. nt –73 to –95 in the type IIa; Figure 4), motif A is 80 % conserved and motif B is 74 % conserved between the two genes. Interestingly, motif B is 61 % conserved among murine IIa, IIb and human IIa genes and furthermore, 70% conserved in human IIb promoter (unpublished communication in our lab). These data imply that motif A and B may play a functional role in the mammalian Na/P_i-II gene promoters. On the other hand, the Na/P_i-IIb gene promoter is more G/C-rich than that of the Na/P_i-IIa gene within the first 200 bp 5' of the transcriptional initiation site (72% vs. 46%). Proximal promoters high in G/C content are often regulated in part by the SP1 family of transcription factors, so it would seem plausible that Na/P_i-IIb promoter regulation may involve these nuclear proteins.

Figure 4. Nucleotide Sequence of the 5'-Flanking Region of the Murine Na/P_i-IIb Gene. 999 bp of the putative promoter region (Genbank Accession #AF211863), exon 1 (nt +1 to +104), and part of intron 1 of the Na/P_i-IIb gene are shown in this figure. Part of exon 1, previously published (100), is underlined by a wavy line and the site of the extra G in the cDNA but not in the BAC clone is indicated by ⇑. Part of intron 1 is shown in lowercase letters. The transcription initiation site (+1) is indicated by a circle. Potential consensus sites recognized by various transcriptional factors (for an explanation of these, see the text) are indicated by gray boxes and restriction enzyme sites (*Nhe* I, *Bst98* I, *BssH* II and *Xho* I) used for creation of promoter constructs are underlined. The asterisks indicate that the putative *cis*-elements and *Xho* I site are on the complementary strand. The site of the PE primer used for primer extension is underlined by a double line. Highly conserved regions between the murine type IIb and type IIa promoters are boxed (motif A and motif B) and identical nucleotides in these regions are shown in bold, italicized letters. Motif A is 80 % conserved and motif B is 74 % conserved between the two genes.

While no perfect SP1 binding sites were noted, several atypical SP1 binding sites (i.e. 5/6 bases match) are present. Furthermore, no more conserved motifs were detected in the upstream region between the IIb and IIa genes.

Overall, these observations suggest potential differential transcriptional regulation of the two Na/P_i-II genes. In fact, Hattenhauer et al (1999) reported differential effects of acute P_i deprivation on the two isoforms. Acute P_i deprivation increased only Na/P_i-IIa protein levels, although a chronic low P_i diet enhanced both Na/Pi-IIa and Na/Pi-IIb protein levels (with no change being seen in Na/Pi-IIb mRNA). Additionally, recent studies by Katai et al (1999) have confirmed that the intestinal Na/Pi-IIb gene is not responsive to low P_i diet. Furthermore, it was shown that the intestinal IIb gene is unresponsive to treatment with vitamin D_3, unlike the renal IIa isoform (10, 11).

Additionally, one G shown at the 5'-end of the previously published cDNA sequence (9) was not observed in this genomic clone, so that G was removed from the numbering scheme associated with promoter analysis. We assume this observation may be due to mouse strain differences or normal DNA sequence heterogeneity between individuals. Note however, that we have not altered the numbering scheme with reference to the Na/P_i-IIb cDNA.

Intron-exon boundaries of the Na/P_i-IIb gene were determined by the following procedure. BAC DNA was digested with ten restriction enzymes, size fractionated in a 0.8% agarose gel, denatured and then transferred to a nitrocellulose membrane as previously described (169). The membrane was hybridized with the end-labeled 371-bp Na/P_i-IIb cDNA probe. Two positive hybridization fragments, a ~3.8 kb *Pst* I band and a

~2.2 kb *Msp* I band, were gel-purified and ligated into predigested pGEM-T vector (Promega; Madison, WI). Sequence analysis showed that part of intron 1 and all of introns 2 and 3 were contained within the ~3.8 kb *Pst*I fragment and the overlapping ~2.2 kb *Msp*I fragment. The exon 1/intron 1 5' boundary and the junctions between exon/intron pairs 4 through 12 were determined by sequencing subcloned PCR products amplified from BAC DNA and the junctions between exon 6/intron 6 and intron 6/exon 7 were determined by direct BAC DNA sequencing. The PCR primer sets and sequencing primers are shown in Table 2. Additionally, PCR amplification using primer sets 12 and 13 indicated that the Na/P_i-IIb gene did not contain an intron downstream of nt 1502. Intron/exon boundaries and sizes of the exons and introns are shown schematically in Figure 5. In addition, donor/acceptor sites, interrupted codons and intron positions found in the Na/P_i-IIb gene are shown in Table 3.

The gene was shown to span more than 18 kb and it contains 12 introns (93 bp to ~5.4kb in size) and 13 exons (41 to 2537 bp in size). The methionine initiation codon, ATG, is located within exon 2, and the termination codon, TAG, is within exon 13. All identified boundaries are consistent with the GT-AG-rule (27). The nucleotide sequence of all introns (or partial introns) that were determined on both strands has been deposited in the GenBank (accession #s AF230467-AF230480). These data were utilized to then compare the murine Na/P_i-IIa and Na/P_i-IIb gene structures, which were found to be remarkably similar (Figure 5).

	Primer Set	Sequence (5' to 3')	5'end Location
1	Sense	GGTGCCTCCTGCTGTC	3
	Antisense	CATCCTGACTGACTGTGAGG	-3000^{I-1}
2	Sense	CCGTCTGGAATAGTCCTCTC	524^{I-4}
	Antisense	CAGCCACAGGATTAGACATG	480
3	Sense	CATGTCTAATCCTGTGGC	461
	Antisense	CTCATTTCTGTCTCCTGC	671
4	Sense	ACCTCCATCACCAACAC	618
5	Antisense	AGAGCACGAACACAGAGA	738
6	Sense	TCTCTGTGTTCGTGCTCTTG	721
	Antisense	ACACCAGATCTTGATCAGGC	953
7	Sense	CCAGAACAAGAGCCTGATCA	923
	Antisense	GGTAACGTTCTGGATGGTCC	1070
8	Sense	TCGACTGATAACTGCACCTC	996
	Antisense	TCATGATCAGACAGCCACAG	1191
9	Sense	CTTCGTGAACTTCAGTCTCC	1103
	Antisense	CAATACCGATGAGTGGAGTC	1383
10	Sense	GACTCCACTCATCGGTATTG	1364
	Antisense	GAGAACTCCTCAAGGTGTTG	1497
11	Sense	CAACACCTTGAGGAGTTCTC	1478
	Antisense	GATAGAAGACTGCGAACCAG	1641
12	Sense	ATCTCTGCCAAGTATCGCTG	1605
	Antisense	CCAACCATGTGTCTCTCCAT	2759
13	Sense	ATGGAGAGACACATGGTTGG	2740
	Antisense	ACAAGACAGAAGATGGCCTG	3924

Table 2. Primers Used to Determine Exon-Intron Structure. This table shows the locations of primers used to determine the exon-intron structure of the murine Na/P_i-IIb gene. All the paired primers were used for PCR amplification, while sense primer 5 and antisense primer 6 were used for BAC DNA sequencing. The asterisk indicates that the number is counted from +1 in relation to the transcription initiation site described in the text. $^{I-1}$ and $^{I-4}$ indicate that the primers are within intron 1 or intron 4, and the numbers are counted from the 5' end of the respective intron. Sense primer 2 was designed as a 16 mer to match its T_m to that of paired antisense primer 2. All other numbers are in relation to the previously described Na/P_i-IIb cDNA (100).

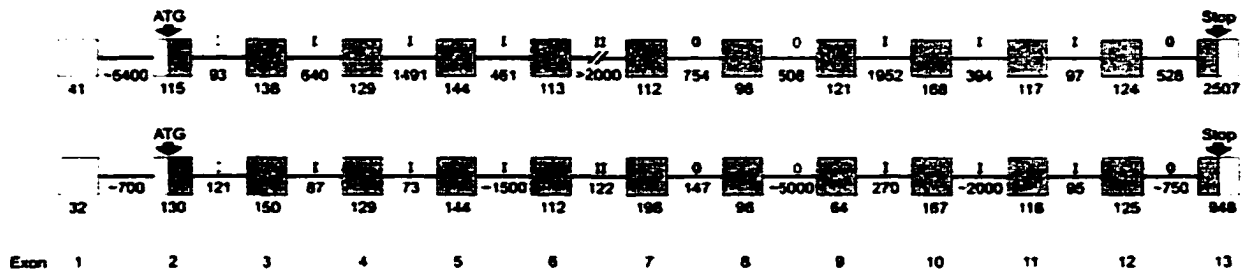

Figure 5. Comparison of the Intron-Exon Organizations of the Murine Na/P_i-IIa and Na/P_i-IIb Genes. The upper figure represents the murine Na/P_i-IIb gene structure and the lower figure shows that of the murine Na/P_i-IIa gene (12). Exons are shown as boxes (yellow- noncoding region; blue- coding region) and introns are shown as intervening lines. Numbers directly below boxes and lines indicate the sizes of exons and introns, respectively. Roman numerals above the lines indicate splicing positions within codon triplets interrupted by introns (0- no split codon; I- splicing after the first base; II- splicing after the second base). The translation initiation and termination sites are indicated as ATG and Stop. Exon numbers are shown at the bottom.

Intron No.	5'-splice donor	3'-splice donor	amino acid	Intron No.	5'-splice donor	3'-splice donor	amino Acid
1	CGCTG/gtgag	tgcag/ATCAT	5'-UTR	7	TCCAG/gtaat	ctcag/CTGGA	Gln
2	CAAAA/gtagg	tccag/CAATG	Thr	8	ATGTG/gtaat	tttag/ACTGA	Val
3	GTCAG/gtaag	cacag/AGAGA	Glu	9	TAAAT/gtgag	ttcag/GTCAG	Cys
4	TGGAG/gtaag	tctag/GCAAA	Gly	10	TACTG/gtagg	tccag/ATTTC	Asp
5	TTCAT/gtgag	ttcag/TGCTG	Leu	11	CATCG/gtatt	aacag/GTATT	Gly
6	AGAAG/gtagg	catag/GGCAT	Arg	12	TCCAG/gtaat	ttcag/ATTGC	Gln

Table 3. Donor/Acceptor Sites and Interrupted Codons Found in the Na/P_i-IIb Gene. This table shows the sequence of the donor/acceptor sites, and amino acids encoded by the interrupted codons found in the Na/P_i-IIb gene. The codon positions are relative to the previously described the Na/P_i-IIb cDNA (100). Exonic sequences are shown in capital letters; intronic sequences are in lowercase. Bold letters indicate the codons interrupted by introns. Intron locations are as follows: intron 1 (I-1), nt 41; I-2, nt 156; I-3, nt 294; I-4, nt 423; I-5, nt 567; I-6, nt 679; I-7, nt 875; I-8, nt 971; I-9, nt 1092; I-10, nt 1260; I-11, nt 1377; I-12, nt 1502.

The murine Na/P_i-IIa (87) and the Na/P_i-IIb genes both have 12 introns and 13 exons, and the exon-intron boundaries are highly conserved in both splicing positions within the codon triplets as well as in the amino acids encoded by these interrupted codons.

The interrupted amino acids were identical between the 2 genes with the exception of intron 1 which had a Thr codon in IIb and a Val codon in IIa, and intron 7 which had a Val codon in IIb and a Glu codon in IIa. In addition, the sizes of corresponding exons within the open reading frames of the two genes are strikingly similar. These observations suggest that the two Na/P_i cotransporter isoforms likely evolved from a common ancestral gene.

Primer extension was performed using total RNA isolated from mouse small intestine or rat intestinal epithelial (RIE-1) cells (1) by a previously described method (13) with slight modifications (described in Figure legend 3). Additionally, a PCR based, double-stranded dideoxy sequencing reaction was performed with the *fmol* DNA Sequencing System (Promega) with pGEM-3Zf (+) plasmid DNA and pUCM13 forward primer (Promega). The primer extension product and sequencing ladder were fractionated together on a 6% polyacrylamide gel to identify the transcription initiation site. The entire experiment was repeated 2 times with intestinal RNA purified from different groups of mice and once with RNA isolated from RIE-1 cells.

Primer extension analysis generated a predominant band from mouse intestinal (Figure 6A) and RIE-1 cell (not shown) total RNA, and comparison with a sequencing ladder prepared with pGEM-3Zf (+) and pUCM13 forward primer indicated that the transcriptional initiation site was 95 nucleotides from 5' end of the pUCM13 forward

primer (Figure 6B). This finding allowed us to determine that the transcriptional initiation site for the Na/P_i-IIb gene is 64 bp upstream of the previously identified cDNA. This method was used because of the relatively high G/C content and small size of exon 1. The resulting high T_m of the PE primer (used for the primer extension reaction) made it unsuitable as a sequencing primer with the BAC clone or a plasmid template. In this fashion, we used the pGEM-3Zf (+) sequence as a 1 bp ladder and we were able to determine the exact size of our primer extension product. The transcription initiation site was designated bp +1 and all subsequent nucleotide numbering concerning promoter analysis utilizes this numbering scheme. Both repetitions with mouse intestinal RNA showed the exact same result and the single repetition with RIE-1 cell RNA showed an identical result.

Three reporter gene plasmid constructs containing various lengths of the 5'-flanking region of the murine Na/P_i-IIb gene were made. A 1072 bp fragment was PCR amplified from BAC DNA using mutagenic primers corresponding to nt –946 to –965 (5'-CTGTAGCTAGCATTGGCACC-3') and nt +66 to +82 (5'-GGACAGCTCGAGGCACC-3') of the Na/P_i-IIb gene, which created internal *Nhe* I and *Xho* I restriction enzyme sites for subcloning (mutated bases are underlined). PCR was performed using *pfu* DNA polymerase (Promega) by standard methods. The amplified fragment was subcloned into pPCR-Script Amp SK (+) cloning vector (Stratagene; San Diego, CA) as per the company's protocol. The insert was then excised by *Nhe* I and *Xho* I digestion and subcloned into predigested pGL3-Basic luciferase reporter vector (Promega).

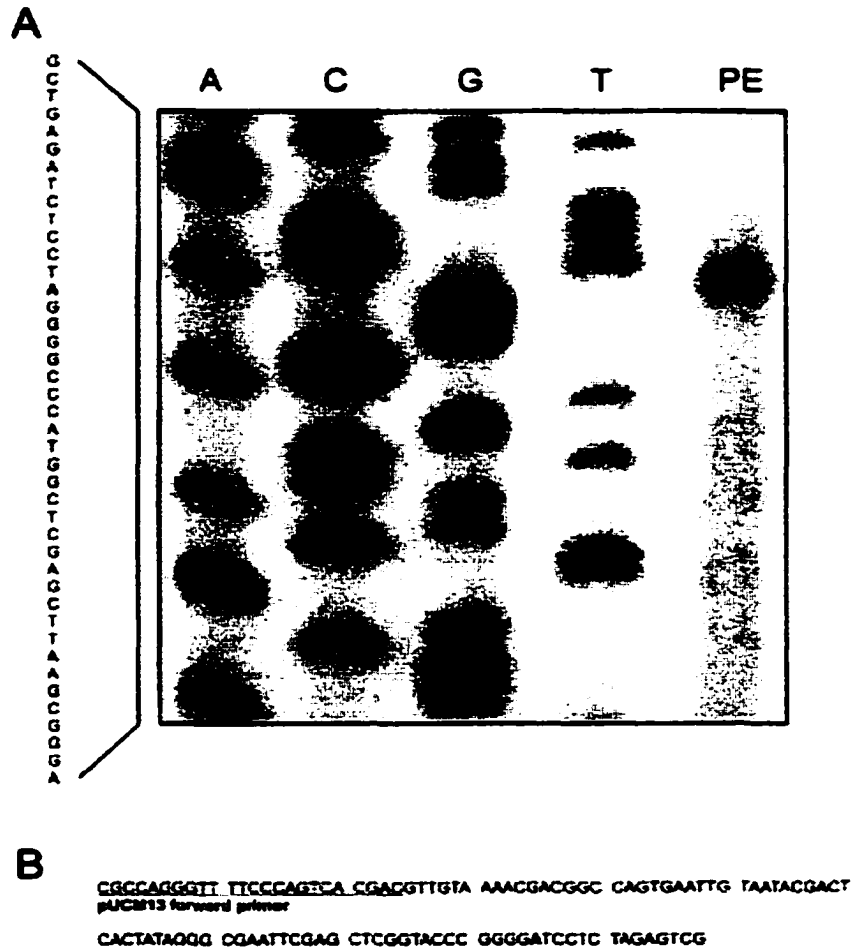

Figure 6. Identification of the Transcriptional Initiation Site of the Murine Na/P_i-IIb Gene. Panel A: This panel shows the results of the primer extension with mouse small intestinal RNA and sequencing reactions. The transcription initiation site was determined by primer extension using the PE primer described in the text and shown in Figure 1. A synthetic oligonucleotide complementary to nt 13-32 of the published Na/P_i-IIb cDNA was PAGE-purified and 5'-end labeled with [γ-^{32}P]-ATP (ICN; Costa Mesa, CA). The labeled primer was annealed with 40 µg of total RNA by heating at 90° C for 2 min and then cooling to 55° C over 45 min. Extension reactions were carried out as previously described. A PCR based double-stranded DNA sequencing reaction was run in parallel with the primer extension product on a 6% sequencing gel. From left to right, the lanes of the gel are A, C, G, T, and the primer extension product. **Panel B:** This panel shows a partial sequence of the pGEM-3Zf(+) plasmid and the location of pUCM13 forward primer (underlined). The primer extension product aligned with the A indicated by bold type in both panels.

This construct contained nt –954 to +73 of the Na/P_i-IIb gene. To make 2 shorter constructs, this DNA was digested with the restriction enzymes *Nhe* I (nt –955 to -960), plus *Bst981* (nt -425 to -430) or *BssH II* (nt –159 to –164) [see Figure 4], and the 5' and 3' single-stranded overhangs were then polished using *pfu* DNA polymerase. The blunt-ended plasmid fragments were then gel purified and religated upon themselves to create –429 to +73 and –159 to +73 constructs. All three constructs were confirmed by sequence analysis.

The constructs were then tested for their functional abilities as promoters in transient transfection assays in rat intestinal epithelial (RIE-1) cells (20). Prior to selecting RIE-1 cells for transfections, expression of the type IIb Na/P_i gene was confirmed by RT-PCR (data not shown). Promoter construct DNA was transfected into the RIE-1 cells, which were maintained under standard conditions, by liposome-mediated transfection as previously described (169). Parallel transfections were also carried out with a negative control vector (promoterless firefly luciferase reporter, pGL3-Basic). As a control for transfection efficiency, pRL plasmid vector that encodes renilla luciferase under the control of the HSV-TK promoter was cotransfected in all experiments at a 1:30 dilution. Dual-luciferase reporter assays were performed by a standard protocol (Promega). Firefly luciferase activity in each cell lysate was normalized for renilla luciferase activity. Each experiment was repeated in quadruplicate with separate cell populations and results were averaged from three observations per repetition. The promoter activities of the three reporter gene constructs are shown in Figure 7. Cells transfected with –159/+73, –429/+73 and –954/+73 constructs showed significant luciferase activity as compared to

pGL3 Basic vector without a promoter insert (1.65 ± 0.70 for –159/+73 vs. 0.038 ± 0.01 for pGL3 Basic vector, n=4, $P< 0.01$; 3.13 ± 0.46 for –429/+73 vs. 0.038 ± 0.01 for pGL3 Basic vector, n=4, $P< 0.001$; 0.84 ± 0.19 for –954/+73 vs. 0.038 ± 0.01 for pGL3 Basic vector, n = 4, $P< 0.01$). The –429/+73 construct exhibited higher promoter activity than that of the –954/+73 construct ($P< 0.01$), but the –159/+73 construct showed no difference. These data exemplified the presence of basal *cis*-elements within the –159/+73 region and they further suggested the presence of a negatively-acting *cis*-element(s) between bp –429 and bp –954.

In conclusion, we cloned the 5'-flanking region of the murine Na/P_i-IIb cotransporter gene, which encodes what is considered to be a major P_i transport system in the mammalian intestine. *In vitro* reporter gene assays clearly demonstrated that the 5'-flanking region of the Na/P_i-IIb gene functions as a promoter of gene transcription. However, the exact physiological role and regulation of this gene in the mammalian intestine are not presently known. The studies presented in this communication will now make it possible to decipher the transcriptional regulation of this important gene. Overall, the current studies described here and future investigations will further contribute to our understanding of mammalian P_i homeostasis.

This study was supported by NIH grant #R37DK33209 and W. M. Keck foundation.

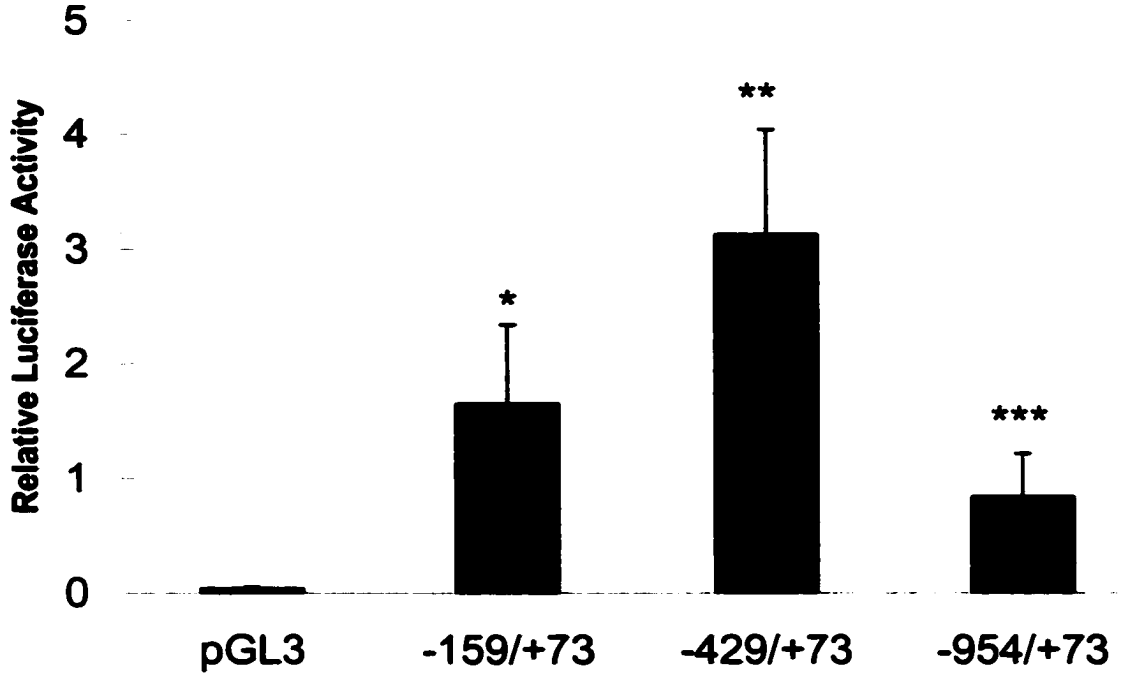

Figure 7. Luciferase Activity of Na/P_i-IIb Promoter Constructs in RIE-1 Cells. Dual luciferase reporter gene assays were performed 48 hr after transient transfection of the cells. Firefly luciferase activities were normalized to cotransfected renilla luciferase activities and are represented as relative activity on the Y-axis. The –159/+73, –429/+73 and –954/+73 constructs are indicated on the X-axis along with pGL3, which indicates a negative control (promoterless) vector. * $P< 0.01$ vs. pGL3, ** $P< 0.001$ vs. pGL3; *** $P< 0.01$ vs. pGL3 and $P< 0.01$ vs. +73/–429.

CHAPTER III

Glucocorticoid Regulation and Glycosylation of the Mouse Intestinal Type IIb Na/P_i Cotransporter During Ontogeny[1]

Introduction

Inorganic phosphate (P_i) homeostasis is partially maintained by type II sodium-dependent phosphate cotransporters, which include type IIa (Na/P_i-IIa), and type IIb (Na/P_i-IIb). Na/P_i-IIa is expressed in apical membranes of epithelial cells in the renal proximal tubules, and it represents the major Na/P_i cotransporter in the kidney. The Na/P_i-IIa cotransporter is regulated by several physiological effectors including glucocorticoids (83), epidermal growth factor (EGF) (3, 4), parathyroid hormone (108, 174, 194), thyroid hormone (2, 225), vitamin D_3 (236), and dietary phosphate (41, 46, 49, 101, 118, 174, 239, 265). Meanwhile, the Na/P_i-IIb cotransporter is expressed in several tissues including the brush-border membranes (BBMs) of the small intestinal epithelium where it is thought to be the major Na/P_i cotransporter (90, 100). Similar to Na/P_i-IIa, intestinal Na/P_i-IIb is also regulated by EGF (277), and by vitamin D_3 and dietary phosphate (90, 115). Both isoforms have similar functional properties, and they show striking homology in their amino acid sequences with eight predicted transmembrane domains and a large hydrophilic loop between the third and forth transmembrane

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[1] Arima, K., Hines, E.R., Kiela, P.R., Drees, J.B., Collins, J.F., and Ghishan, F.K. (2001) *Am J Physiol Gastrointest Liver Physiol* In press.

domains. The hydrophilic loop in the renal type IIa protein contains two *N*-linked glycosylation sites located at Asn-298 and Asn-328, and glycosylation of these sites is likely important for plasma membrane expression (92).

The genes encoding these proteins have very distinct 5'-flanking regions that include functional promoters (6). Thus, their gene expression is likely controlled by different transcriptional mechanisms. For example, renal Na/P_i-IIa gene expression is enhanced by vitamin D_3 administration and low phosphate diet (118, 236). In contrast, these treatments post-transcriptionally increase intestinal Na/P_i-IIb protein abundance and Na/P_i transport activity, but do not alter Na/P_i-IIb mRNA expression (90, 115).

Age-related plasma P_i levels may result from decreased renal and intestinal P_i absorption (7). In intestine, sodium-dependent P_i (Na/P_i) absorption across the apical and endoplasmic reticulum membranes of enterocytes is higher in suckling animals than in young adult animals (22, 25, 78). Conversely, renal Na/P_i cotransport is lowest in suckling rats, highest in weanling rats and declines with age (238). Furthermore, the age-related decline in renal P_i absorption is likely due to lower Na/P_i-IIa expression (2, 83, 238). However, a correlation between ontogeny of intestinal Na/P_i cotransport and expression of the Na/P_i-IIb cotransporter has not yet been documented.

During the suckling/weaning transition in rats and mice, the intestinal mucosa rapidly matures in conjunction with the dietary change from milk to carbohydrate-based solid food. Skeletal growth is also most rapid in this period. Thus, it is not surprising that the suckling rat intestine shows elevated absorptive capacities for a number of nutrients including phosphate (94).

Among physiological factors involved in intestinal maturation, glucocorticoids (GCs) are the most potent regulators (94). Indeed, direct measurement of rat plasma concentration of endogenous GCs demonstrated that total plasma corticosterone, the principal GC in rats and mice, was less than 0.5 μg/ml on days 6-12 (corresponding to the suckling period), and it rose to 5 μg/ml on days 17-24 (corresponding to the weaning period) and then it gradually decreased into adulthood (95).

Exogenous GC administration induces precocious intestinal maturation in the first and second postnatal weeks by modulating gene expression, membrane fluidity and patterns of protein glycosylation (17, 40, 72, 98, 180, 260). Meanwhile, adrenalectomy and GC antagonists delay intestinal maturation (94). Clinical observations showed that chronic GC administration enhances urinary P_i excretion and reduces plasma P_i levels in patients (122, 130). Pharmacological doses of GCs have also been reported to reduce renal and intestinal P_i absorption, and renal Na/P_i-IIa expression (25, 83). Furthermore, long-term GC treatment increases the risk of bone demineralizaion in children and is known to increase the risk of developing osteoporosis in adults (72, 192). Although GCs appear to play a crucial role in regulating intestinal P_i absorption, the effects of GCs on Na/P_i-IIb expression have not been addressed.

The purpose of the current studies was to investigate molecular mechanisms involved in intestinal Na/P_i-IIb expression during postnatal development, and to look at the effect of GC administration. Our results showed that Na/P_i-IIb is differentially expressed during mouse ontogeny. We further demonstrate that Na/P_i-IIb expression is decreased by GC treatment, with the effect being most pronounced in the suckling period.

Additionally, we provide evidence that Na/P_i-IIb is an *N*-linked glycoprotein and that glycosylation occurs around the time of the suckling/weaning transition.

Materials and Methods

Animals

Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used in groups of four to six animals for all experiments. The following ages groups were used: suckling mice at 14 days of age (14 d), weanling mice at 21 days of age (21 d), young adult mice at 8 weeks of age (8 wk), and old adult mice at 8-9 months of age (8-9 m). Animals were subcutaneously injected with methylprednisolone (MP; Solu-Medrol, Upjohn; Kalamazoo, MI) at a dose of 30 μg/g body wt or equal volumes of saline once every 12 h for a total of four injections. The animals were sacrificed 3 h after the last injection by cervical dislocation after CO_2 narcosis. Animals were supplied with food and water *ad libitum*. The proximal two-third of the small intestine was used for all experiments. All animal procedures were approved by the University of Arizona Institutional Animal Care and Use Committee.

Chemicals and Reagents

Total RNA was isolated from tissues using TRIZOL Reagent (GIBCO BRL, Bethesda, MD). $KH_2[^{32}P]O_4$ (1 Ci/nmol) for uptake studies and [α-^{32}P]dATP (3000 Ci/mmol) for Northern blot analyses were purchased from NEN (Boston, MA). Strip-EZ PCR kit, which was used to generate antisense, radioactive probes for Northern blot

analyses, and Ultrahyb buffer were from Ambion (Austin, TX). Nitrocellulose membranes (Nitroplus) were from Osmonics (Westboro, MA). SuperSignal, Western Blot Stripping buffer and X-ray film were from Pierce (Rockford, IL). SDS-PAGE precast gels and protein molecular weight standards were from Bio-Rad (Hercules, CA). Horseradish peroxidase-linked secondary antibodies (anti-rabbit IgG) were from Amersham (Piscataway, NJ). Mouse anti-β-actin monoclonal antibody was purchased from Sigma Chemical (St. Louis, MO). Plasmid DNA was isolated from bacterial cultures with the QIAfilter Plasmid Maxi Prep Kit and DNA fragments were gel-purified utilizing Qiaquick gel extraction kit from Qiagen (Valencia, CA). Protein was quantitated utilizing Biorad protein assay reagent. Peptide *N*-glycosidase F (PNGase F) and Endoglycosidase H (Endo H) were from New England BioLabs (Beverly, MA). Herculase proof-reading, high temperature DNA polymerase was from Stratagene (San Diego, CA). *Taq* polymerase and restriction enzymes were from Promega (Madison, WI). All other chemicals and reagents were purchased from Fisher Biotechnology (Pittsburgh, PA) or Sigma Chemical.

BBMV and BLMV Preparations

BBMVs were prepared from the intestinal mucosa from groups of 5-6 animals for 14 d and 21 d old mice and from groups of 4-5 animals for 8 wk and 8-9 m old mice, by the $MgCl_2$ precipitation technique as previously described (176, 178). Additionally, BLM vesicles were isolated from 8 wk-old mice by a well-established method in our lab (48). The final BBM and BLM vesicle pellets were resuspended in resuspension buffer (280

mM mannitol, 20 mM HEPES-Tris, pH 7.4), homogenized with a 26-gauge needle and protein was quantitated. For uptake analyses, BBM preps were used on the day of purification and were never frozen.

Uptake Analysis of Intestinal BBMVs

$^{32}PO_4^{2-}$ transport was measured by a rapid filtration technique as described previously (41, 45, 47, 48, 83). The Na-dependent component of P_i uptake was obtained by subtracting uptake values in the presence of KCl from uptake values in the presence of NaCl. Values are means ± SE for each age group and represent the results of three separate experiments with samples isolated from different groups of animals.

Northern Blot Analysis

Total RNA was isolated from at least 4 mice per group. To generate Na/P_i-IIb specific probes, the Na/P_i-IIb open reading frame (ORF) was amplified from mouse intestinal RNA by RT-PCR with sense primer at bp 15 (15L; 5'-TGTCCCTTACCTGCGA-3') and antisense primer at bp 2264 (2264R; 5'-TAGGAGAGCTAGAGTTGGTG-3') in mouse Na/P_i-IIb cDNA (GenBank accession number AF081499) using a high-fidelity DNA polymerase. PCR products were A-tailed, subcloned into the pCR 3.1-Uni vector (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* DH-5α. The entire amplicon was confirmed by sequence analysis. Then, plasmid DNA was digested with *Nhe*I and *Eco*RI, and the fragment containing the entire Na/P_i-IIb ORF was gel-purified and used as template to make mouse Na/P_i-IIb-specific

probes. GAPDH template was also amplified from mouse intestinal RNA by RT-PCR with sense primer at bp 563 (563L; 5'-ATGACCACAGTCCATGCCAT-3') and antisense primer at bp 832 (832R; 5'-CTGCTTCACCACCTTCTTGA-3') in mouse GAPDH cDNA (GenBank accession number NM 008084). The PCR fragment was gel-purified and confirmed by sequence analysis.

Antisense probes were synthesized utilizing [α-^{32}P] dATP, and Na/P_i-IIb template and 2264R primer, or mouse GAPDH template with 763R primer (5'-CAGTGAGCTTCCCGTTCA-3'). Both radioactive probes were purified using G-50 columns (Bio-Rad). Northern blots were carried out according to the manufacturers' protocol (Ambion). In preliminary experiments, it was noted that expression of Na/Pi-IIb mRNA in 14 d old mice was much higher than in older mice and was severely reduced with MP treatment. Furthermore, Na/Pi-IIb hybridization bands in older mice (i.e. 8 week and 9 months) were not visible when 10 μg of total RNA was loaded, while those in suckling mice exceeded the linear range of phosphorimage analysis. Therefore, in order to exemplify the band in each age group, it was necessary to load variable amounts of total RNA (10-40 μg) per lane, resulting in different apparent intensities of the reference mRNA band (GAPDH). This unequal loading was corrected for by taking ratios between NaP_i-IIb and GAPDH hybridization band intensities. Quantitation of hybridization signals was done by phosphorimage analysis utilizing volume integration (FX Molecular Image, Bio-Rad) or by scanning densitometry (GS-700 Imaging Densitometer, Bio-Rad). The Northern blot experiment was performed three times with total RNA samples

isolated from different groups of animals. Na/P_i-IIb hybridization intensities were normalized for GAPDH levels on the same blot and the ratios were averaged.

Production of Na/P_i-IIb-Specific Antiserum

A multiple antigen peptide (MAP) corresponding to the C-terminal region of the mouse Na/P_i-IIb protein was synthesized by Research Genetics (Huntsville, AL). The antigenic peptide is 95% homologous to the rat Na/P_i-IIb protein (GenBank accession number AAF76291). This region of the molecule showed no amino acid sequence homology to any other proteins in the GenBank. The peptide was injected into rabbits for polyclonal antibody production (Research Genetics). Antibody specificity was assessed by western blots using antiserum, preimmune serum or immunogenic peptide-pretreated serum, and also by immunohistochemical analyses (as described below).

Western Blot Analysis of Mouse Intestinal BBM Proteins with Na/P_i-IIb Specific Antiserum

Intestinal BBM and BLM proteins were purified from mice and rats as described. Twenty to sixty micrograms of protein were placed in a two-fold excess of Laemmli solubilization buffer (2% SDS, 10% glycerol, 1 mM EDTA, and 2 mM β-mercaptoethanol, pH 6.8), boiled for 4 min, and placed on ice. Protein samples were fractionated by 7.5% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in PBS with 0.05% Tween 20 (PBST) and 10% nonfat dry milk, rinsed with PBST-0.1% milk and incubated overnight at 4°C with a 1:500

dilution of Na/P_i-IIb-specific antiserum in PBST-0.1% milk. Additionally, some blots were reacted with preimmune serum, and serum that was pretreated with antigenic peptide (1000 μg/ml at 4°C overnight) at 1:500 dilution. The membranes were washed with PBST-0.1% milk two times for 5 min each and then incubated with the secondary antibody (anti-rabbit IgG) at 1:20,000 dilution for 40 min. Finally, the membranes were washed with PBST-0.1% milk four times for 15 min each, reacted with chemiluminescence reagent for 5 min, and then exposed to film. Membranes were stripped and subsequently reacted with β-actin antiserum at a 1:5,000 dilution. Na/P_i-IIb-specific band intensities were determined by densitometric analysis and were normalized for β-actin band intensities on the same blot. This experiment was repeated three times with protein samples isolated from different groups of animals.

Immunohistochemical Analysis of Rat Intestine with Na/P_i-IIb-Specific Antiserum

Proximal intestinal tissue was harvested from 3-wk-old rat, fixed in paraformaldehyde, embedded in paraffin, and sections were cut and affixed to slides. After deparrafinization, slides were blocked by overnight incubation with 5% normal goat serum (Vector Labs, Burlingame, CA) at room temperature in a humidified chamber. Na/P_i-IIb antiserum was reacted with sections for 30 min at a 1:250 dilution in PBS. Some sections were reacted with secondary antibody only at a 1:400 dilution. Slides were subsequently reacted with secondary antiserum (Alexa Fluor 568 goat anti-rabbit IgG; Molecular Probes, Eugene, Oregon) at a 1:400 dilution of the company stock solution (2 mg/ml) and visualized by confocal microscopy (LSC-1024ES laser scanning confocal

[Bio-Rad] equipped with a Nikon TE-300 research grade microscope) using the HQ 598 40 emission filter and an excitation wavelength of 568 nm. All slides were visualized with the exact same confocal settings.

Prediction of Glycosylation Sites in Na/P_i-IIb

A database search for *N*-linked (N-X-S/T) and *O*-linked glycosylation sites (GGS/T) was performed with the mouse Na/P_i-IIb protein sequence. Also, the largest extracellular loop of mouse Na/P_i-IIb was aligned with mouse Na/P_i-IIa (GenBank accession number AAC42026) and Na/P_i-IIb cloned from other species including rat (AAF76291), human (AAF31328), bovine (S49228), chicken (AAG35801), carp (AAG35803), dogfish (AAG35795), flounder (AAB16821), rainbow trout (AAG35798), zebrafish (AAG35356), and little skate (AAG35797). Single and multiple amino acid alignments were performed with the GenBank BLASTP (http://www.ncbi.nlm.nih.gov/BLAST), DNAMAN (Lynnon BioSoft, Vaudreuil, Quebec, Canada), and LALIGN (http://fasta.bioch.virginia.edu/fasta/lalign.htm) computer programs.

Treatment of Mouse Intestinal BBMs with Glycosidases

Twenty to sixty micrograms of mouse intestinal BBM protein isolated from 2 wk-old and 8 wk-old mice was mixed with 1 µl of denaturing buffer (5% SDS, 10% β-mercaptoethanol) and boiled for 10 min. Samples were subsequently incubated with

500U of PNGase F or Endo H for 2 h at 37 °C. Digested proteins were fractionated by 7.5% SDS-PAGE and analyzed by immunoblotting with Na/P_i-IIb-specific antiserum.

Statistical Analysis of Results

Data were analyzed for statistical significance by Student's *t*-tests or ANOVA followed by Fisher's protected least significant difference post hoc test by using the Statview software package (version 4.53, SAS Institute, Cary, NC). Data are expressed as means ± SE.

Results

Uptake analysis of Intestinal BBM Vesicles

Intestinal BBM vesicles were prepared from animals treated with MP or saline at various ages. Uptake of radioactive phosphate was measured by a rapid filtration technique with or without sodium at pH 7.4. In control animals (saline treated), Na-dependent P_i uptake (in nmol· mg protein^{-1}· 10 s^{-1}) was highest in 14 d old mice (1.63 ± 0.43; n=3 for all groups) as compared to the other three age groups (P <0.0001 for 14 d vs. other ages). Uptake levels diminished with age until adulthood as indicated by the higher level in 21 d animals (0.24 ± 0.02) than in 8 wk (0.09 ± 0.05, P <0.001) and 8-9 m (0.04 ± 0.03, P <0.001) mice. No significant difference in uptake was present between the 8 wk and 8-9 m mice. To determine the age-specific response to GCs on intestinal Na-dependent P_i uptake, MP injected mice were utilized for uptake analysis and compared to saline injected (control) animals. MP administration significantly decreased

intestinal Na-dependent P_i uptake in 14 d (1.63 ± 0.43 control and 0.48 ± 0.1 MP; P <0.05) and 21 d (0.24 ± 0.02 control and 0.04 ± 0.02 MP; P <0.05) mice but had no effect in older animals (0.09 ± 0.05 control and 0.05 ± 0.04 MP in 8 wk; 0.04 ± 0.03 control and 0.10 ± 0.03 MP in 8-9 m). Data are expressed graphically in Figure 8.

Northern Blot Analysis

Total RNA was isolated from intestine of MP or saline injected mice from each age group, fractionated on denaturing agarose gels and transferred to nylon membranes. Blots were hybridized with radiolabeled antisense Na/P_i-IIb and GAPDH-specific probes. Na/P_i-IIb hybridization levels were quantitated by densitometry and normalized for GAPDH levels (Figure 9). Intestinal Na/P_i-IIb hybridization levels in 14 d suckling mice (7.35 ± 1.66 Na/P_i-IIb/GAPDH ratio) were ~ 3-fold higher than in 21 d weanling (2.53 ± 1.15) and older mice (8 wk, 2.22 ± 1.28; 8-9 m, 1.71 ± 0.24; P <0.0001 for 14 d vs. other groups). MP administration significantly decreased Na/P_i-IIb hybridization levels in 14 d (7.35 ± 1.66 control vs. 2.02 ± 1.55 MP; P <0.05), 21 d (2.53 ± 1.15 control vs. 0.44 ± 0.09 MP; P < 0.05), and 8-9 m (1.71 ± 0.24 control vs. 0.71 ± 0.26 MP; P < 0.05) animals. The difference in 8 wk mice however was not significant (2.22 ± 1.28 control vs. 1.00 ± 0.42 MP).

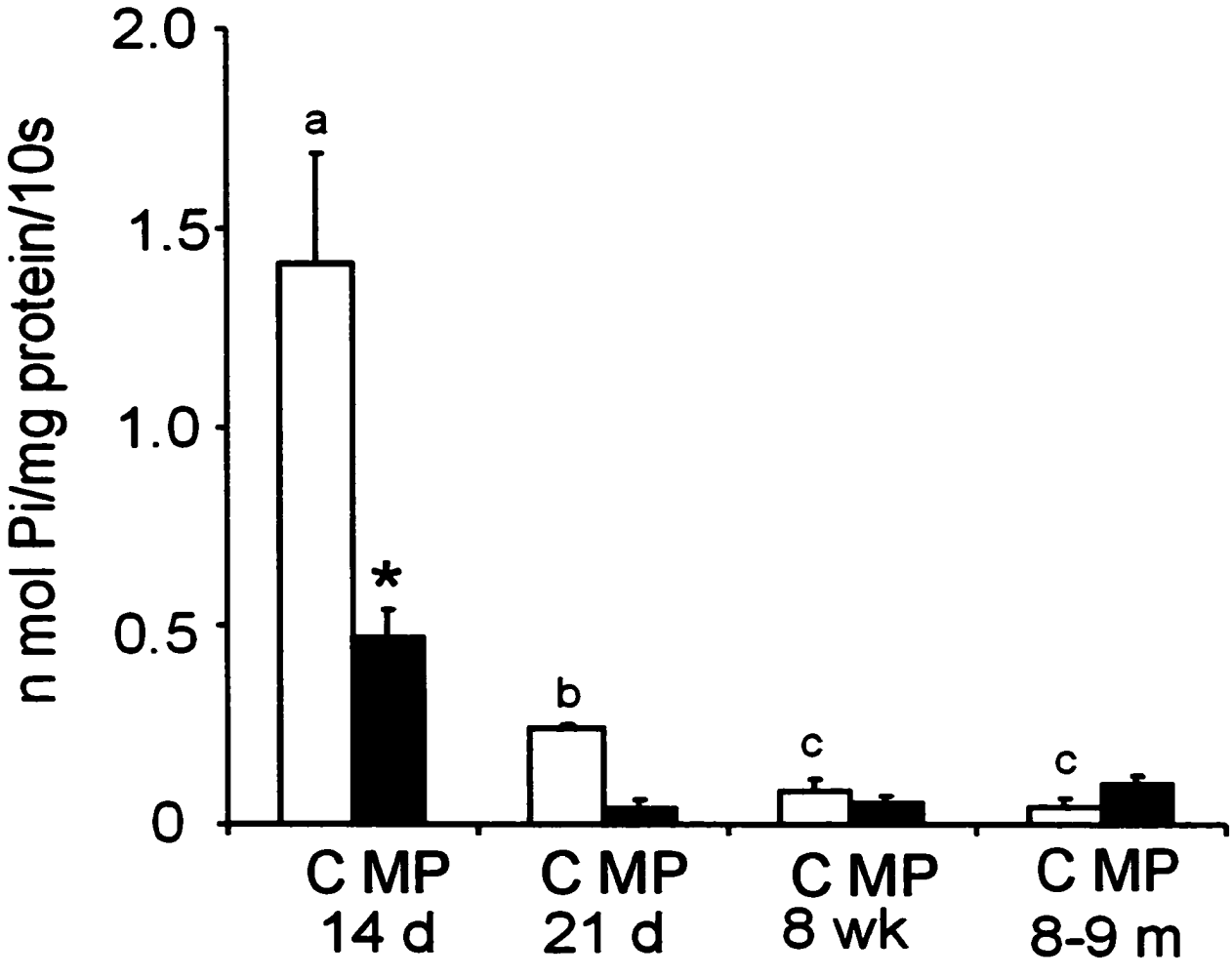


Figure 8. Effect of MP on Na-Dependent P_i Uptake. Brush border membrane vesicles (BBMVs) were isolated from intestinal mucosa of suckling (14d), weanling (21d), young (8 wk) and old (8-9 m) adult mice and assayed for Na-dependent P_i uptake at 10 s (initial rate conditions). The Na-dependent component of P_i uptake was obtained by subtracting uptake in the presence of KCl from uptake in the presence of NaCl. Values are means ± SE of 3 experiments. C, control (saline injected); MP, methylprednisolone treated. P <0.0001 for a vs. b and a vs. c; P <0. 001 for b vs. c. * P <0.05 between control and MP treated mice at that age.

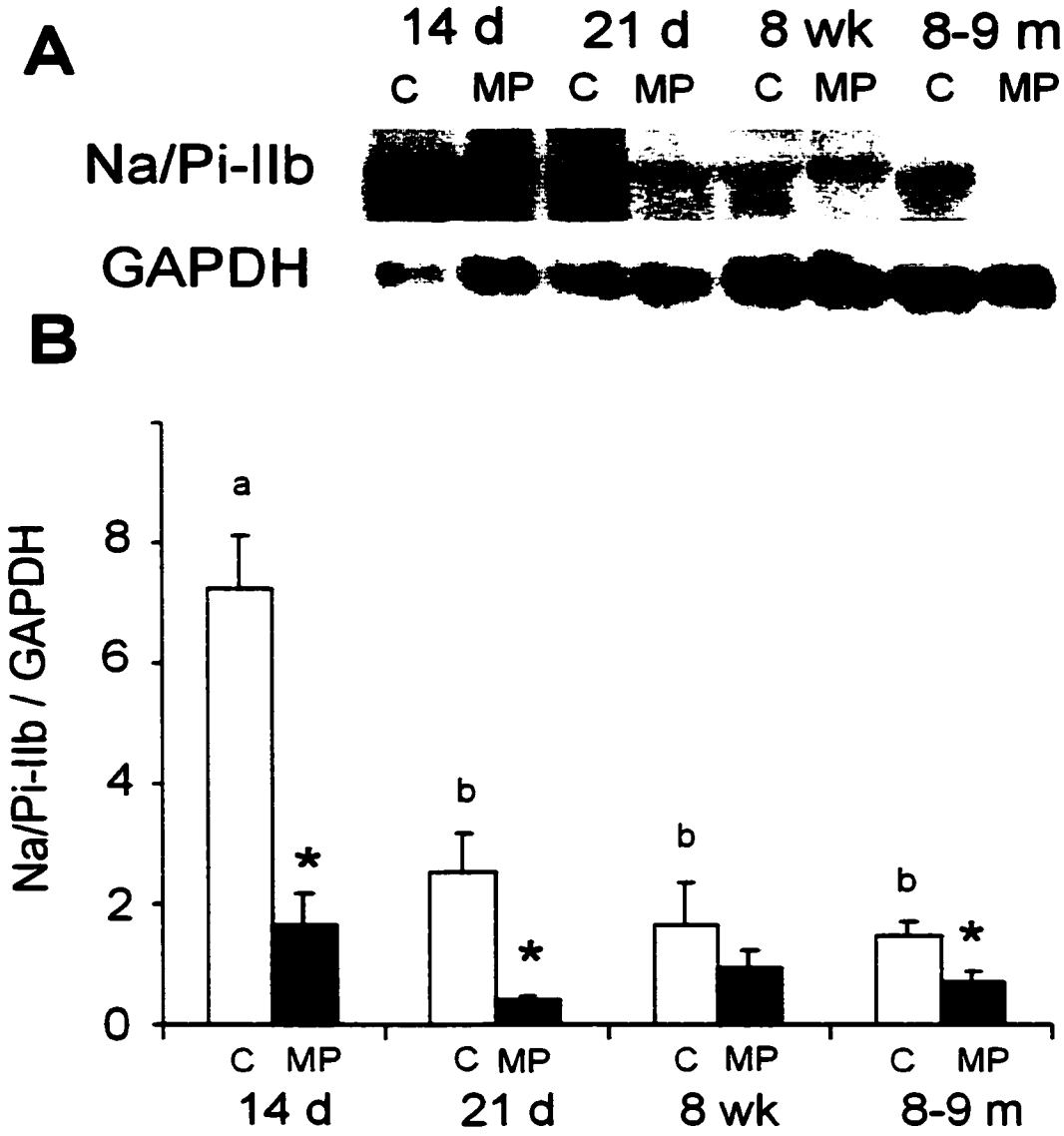
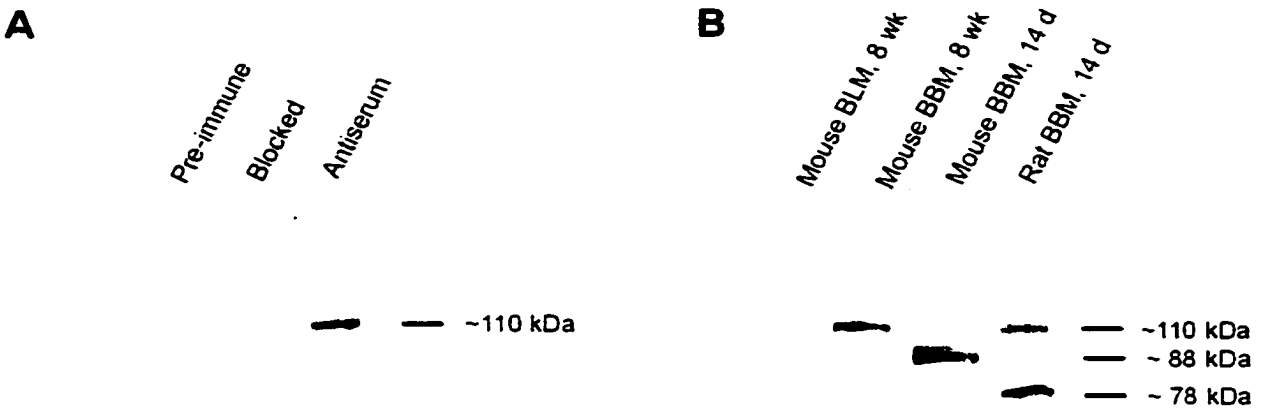


Figure 9. Effect of Methylprednisolone (MP) on Na/P_i-IIb mRNA Expression in Suckling (14 d), Weaning (21 d), Young (8 wk) and Old (8-9 m) Adult Mice. Total RNA was isolated from the intestine of groups of mice, fractionated by denaturing agarose gel electrophoresis and transferred onto nitrocellulose membranes. Different amounts of total RNA (between 10 and 40 μg) were loaded per lane since band intensities were highly variable between groups (see Methods). Panel A: Na/P_i-IIb hybridization band is shown at ~4 kb (*above*) and GAPDH hybridization band is shown at ~1.2 kb (*below*). Panel B: Graphical representation of data obtained from 3 independent Northern blot experiments. Values are means ± SE. P <0.0001 between a and b; * P <0.05 between control and MP treated mice. In both panels, C denotes control (saline injected) and MP denotes methylprednisolone treated.

To further exemplify the specificity of the immune serum, proximal intestinal tissue was reacted with Na/P_i-IIb specific antiserum. Results showed specific staining of the apical membranes of enterocytes (Figure 10, Panel C), while secondary antibody alone showed no staining.

Western Blot Analyses of Intestinal BBM Proteins with Na/P_i-IIb Specific Antiserum

To determine the pattern of Na/P_i-IIb protein expression during ontogeny, 20-60 μg of BBM protein from MP or saline injected mice was used for western blot analyses with Na/P_i-IIb-specific antiserum (Figure 11). Na/P_i-IIb protein levels (presented as a Na/P_i-IIb/β-actin ratio) were markedly higher in suckling animals (1.44 ± 0.29) as compared to the other age groups (21 d, 0.26 ± 0.16 8 wk, 0.29 ± 0.09; 8-9 m, 0.15 ± 0.11; P <0.0001 for 2 wk vs. other groups). MP administration decreased Na/P_i-IIb protein levels more than 3-fold in 14 d mice (1.44 ± 0.29 control vs. 0.38 ± 0.17 MP; P <0.05) but had no effect in 8 wk (0.29 ± 0.09 control vs. 0.29 ± 0.06 MP), and 8-9 m-old animals (0.15 ± 0.11 control vs. 0.05 ± 0.03 MP). Although the data was not statistically significant, Na/P_i-IIb protein levels in 21 d appeared to be also downregulated by MP (0.26 ± 0.16 control vs. 0.11 ± 0.10 MP). Additionally, the molecular weight of the immunogenic band in 14 d old mice was smaller than in the other age groups (~88kDa vs. ~110kDa), and more than one band was present in MP treated 14 d suckling mice.



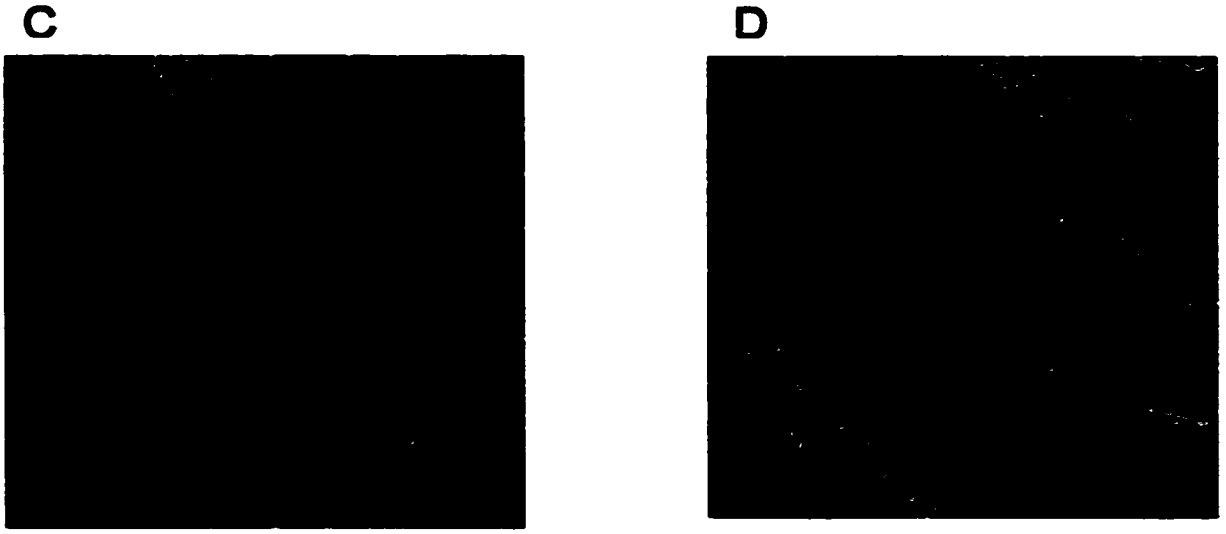


Figure 10. Na/P_i-IIb Antiserum Characterization. Brush-border membrane (BBM) or basolateral membrane (BLM) proteins were isolated from intestinal mucosa in mice and rats. For immunohistochemistry, proximal intestinal tissue was harvested from 3-wk-old rat. Panel A: Western blot analysis of BBM proteins from 8 wk-old mice with Na/P_i-IIb-specific antiserum (Antiserum), preimmune serum (Pre-immune), and antigenic protein-pretreated serum (Blocked). Panel B: Western blot analysis of BBM or BML proteins from mice and rats with Na/P_i-IIb-specific antiserum. Panel C & D: Immunohistochemical analysis of proximal intestine from a 3-wk-old rat. Magnification of both C and D is ×40. C: Na/P_i-IIb antiserum reaction. D: Secondary antibody only.

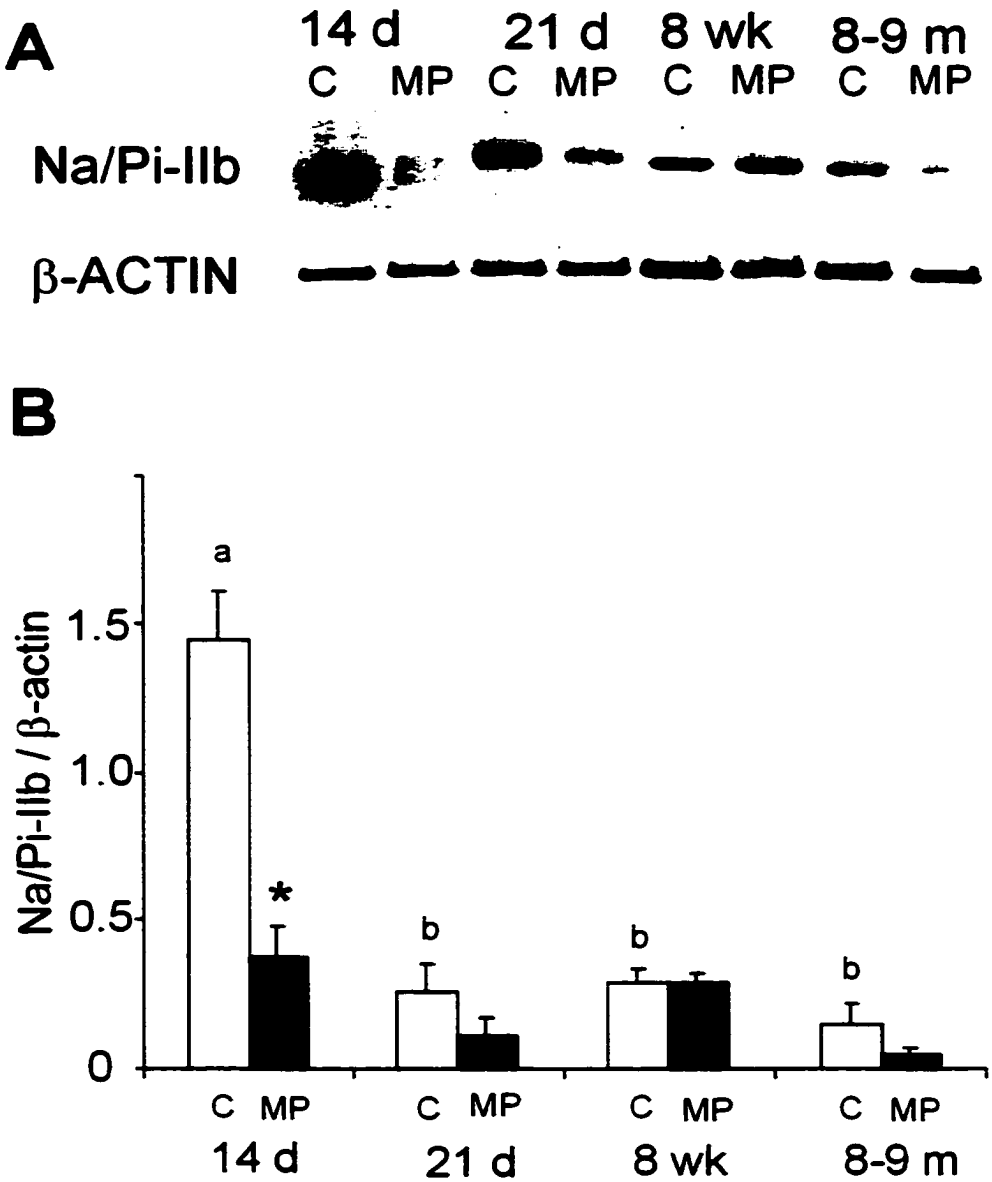


Figure 11. Effect of Methylprednisolone (MP) on Na/P_i-IIb Protein Abundance in Suckling (14 d), Weanling (21 d), Young (8 wk) and Old (8-9m) Adult Mice. BBM proteins were purified from groups of mice, fractionated by SDS-PAGE, and transferred to nitrocellulose membranes. Blots were reacted with Na/P_i-IIb-specific antiserum at a 1:500 dilution. Panel A: The Na/P_i-IIb bands are shown at ~110 kDa or ~88 kDa, and the β-actin band is shown below at ~42 kDa. Panel B: Graphical representation of data obtained from 3 independent western blot experiments. Values are means ± SE. P <0.0001 between a and b; * P <0.05 between control and MP treated mice at the age. In both panel, C denotes control (saline injected) and MP denotes methylprednisolone treated.

Treatment of Mouse Intestinal BBMs with Glycosidases

Different molecular weights of the immunoreactive bands of 14-day-old mice versus older mice suggested that the NaP_i-IIb protein is differentially modified with age. Analysis of the mouse Na/P_i-IIb amino acid sequence identified six potential consensus sequences for *N*-linked glycosylation sites (N-X-S/T located at Asn-295, -308, -313, -321, -340 and -356) within a large putative extracellular loop. There were no putative *O*-linked glycosylation (G-G-S/T) identified. A GenBank BLASTP search found that the Na/P_i-IIb cDNA had been cloned from eleven species. Amino acid alignments indicated that three *N*-linked glycosylation sites at Asn-295, -313, and -340 are conserved in all known Na/P_i-IIb sequences and the site at Asn-321 is present in 9 of 11 species (Figure 12). Furthermore, the putative sites at Asn-295 and -340 are conserved with the identified *N*-linked glycosylation sites in mouse Na/P_i-IIa (92).

In order to investigate the variation in molecular weight of the Na/P_i-IIb protein, we performed enzymatic deglycosylation studies. Treatment of BBM proteins with peptide N-glycosidase F (PNGase F) decreased the molecular weight of immunoreactive Na/P_i-IIb from both 14 d (~88 kDa) and 8 wk mice (~110 kDa) to ~78 kDa, which is the calculated molecular weight based on the predicted amino acid sequence (Figure 13). Digestion with Endoglycosidase (Endo H) had no apparent effect on the molecular weight of the protein.

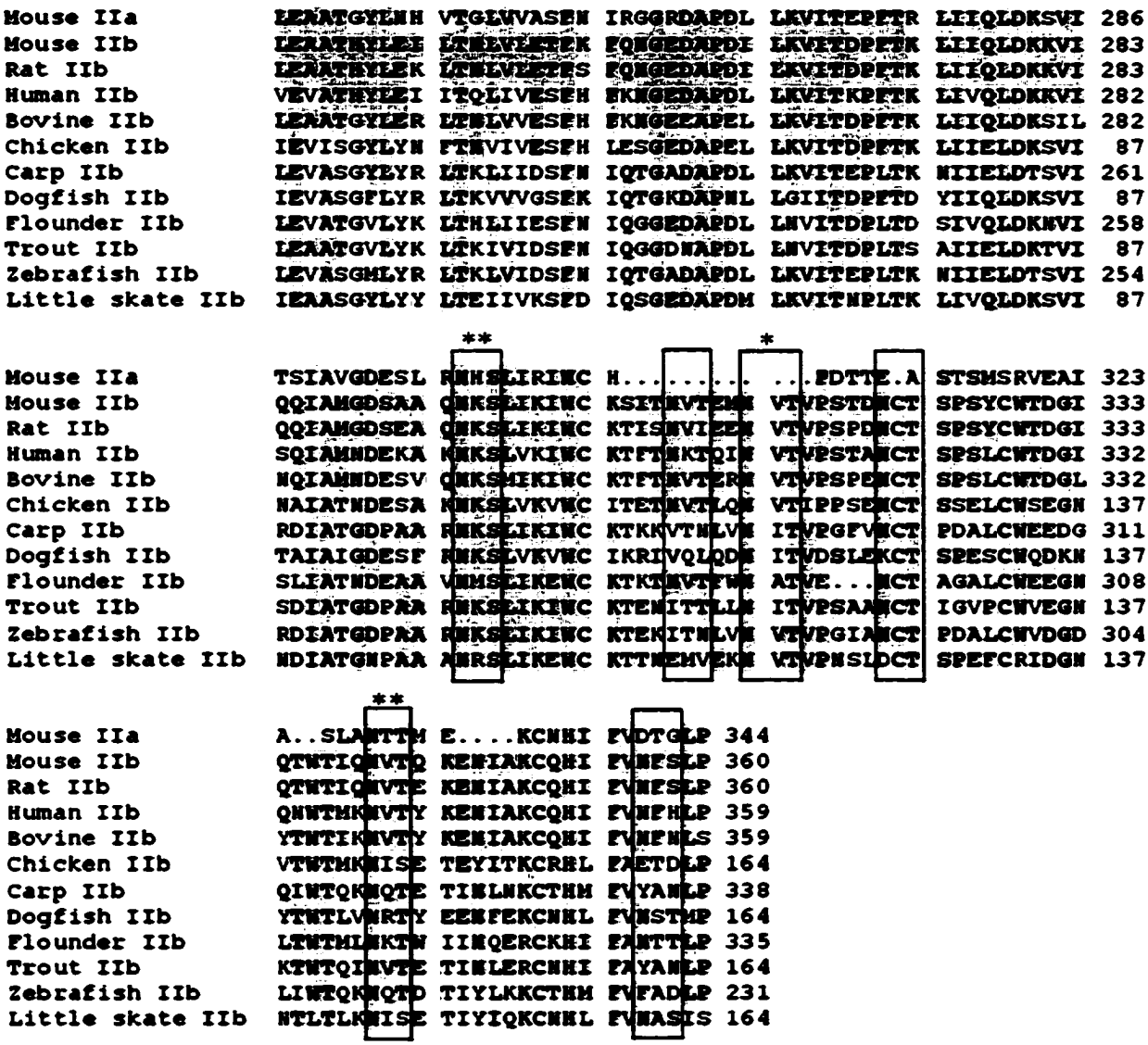


Figure 12. Multiple Sequence Alignment of Mouse Na/P_i-IIa (GenBank accession number AAC 42026) and Na/P_i-IIb Isoforms from Several Species. The amino acids associated with putative *N*-linked glycosylation sites are boxed and the conserved amino acids are shadowed. ** Conserved potential *N*-linked glycosylation sites in the Na/P_i-IIb family and in mouse Na/P_i-IIa. * Conserved potential *N*-linked glycosylation sites in the Na/P_i-IIb family.

Figure 13. Analyses of *N*-Glycosylation of the Na/P_i-IIb Protein with PNGase F and Endo H Using BBMs Isolated from Suckling (14 d) and Young Adult (8 wk) Mice. Forty to sixty μg of BBMs were incubated without enzyme (−) or with PNGase F or Endo H for 2 h at 37°C. The proteins were then analyzed by western blot analysis with Na/P_i-IIb-specific antiserum.

Discussion

The intestinal Na/P_i-IIb cotransporter likely plays a major role in the regulation of P_i homeostasis. Although P_i homeostasis is a tightly controlled process, lower plasma P_i levels have been observed in old age and in the case of chronic GC treatment (7, 130, 206). In both cases, decreased renal P_i absorption was reported and was associated with reduced Na/P_i-IIa expression, (2, 83, 238). To date, the downregulation of intestinal P_i absorption has not been investigated with respect to possible alterations in Na/P_i-IIb cotransporter expression although reduced intestinal P_i absorption has been reported in old animals. We hypothesized that the age-related and GC-induced reductions of total intestinal Na/P_i transport would correlate with decreases in intestinal Na/P_i-IIb expression. Therefore, the present work focused on the ontogeny of intestinal Na/P_i uptake and Na/P_i-IIb expression at distinct periods throughout the lifespan, and how pharmacological doses of GCs affect the same processes.

In laboratory animals, sodium-dependent P_i (Na/P_i) absorption across jejunal, apical and endoplasmic reticular membranes of enterocytes was significantly greater in suckling (2 wk) rats as compared to adolescent (6 wk) rats (22, 78). A study with rabbits at various ages (2 wk, 4 wk, 6 wk and 3 m) demonstrated similar results in which duodenal and jejunal Na/P_i uptake was greatest in 2 wk-old animals and severely decreased in older (3 m) animals (25). Conversely, renal Na/P_i cotransport increases during the suckling/weaning transition and decreases with age. It is unclear why changes in intestinal and renal P_i (re)absorption during ontogeny are opposite during the suckling/weaning transition. Data from our laboratory previously demonstrated that renal

Na/P_i cotransport was lowest in 2 wk-old rats, highest in 3 wk-old rats and declined in 6 wk and 4 m-old rats (238). They further demonstrated that age-related decreases in Na/P_i uptake were paralleled by changes in Na/P_i-IIa protein levels but no changes were seen in Na/P_i-IIa mRNA levels. Another study compared young (3 m) and old (24 m) adult rats and found that serum Pi concentrations, renal Na/P_i cotransport, and Na/P_i-IIa protein and mRNA abundance were markedly higher in younger than in older rats (2). Furthermore, age-related decreases in plasma P_i levels may result from decreased renal and intestinal P_i absorption (7). In the kidney, the age-related decline in renal P_i absorption is likely due to lower Na/P_i-IIa expression (2, 238), however, a correlation between ontogeny of intestinal Na/P_i cotransport and expression of the Na/P_i-IIb cotransporter has not yet been documented.

Total intestinal Na/P_i uptake in the current studies was highest in 14 d suckling mice, lower in 21 d weanlings and lowest in older age groups. This observation agreed with previous reports that intestinal Na/P_i transport was higher in suckling than in adult rats and rabbits (22, 25, 78). Moreover, in the current studies, the decline in uptake activity was not constant with age, instead was rapid (7-fold) from 14 d to 21 d mice and then it gradually decreased (3-fold) from weanling to young adult mice (8 wk). No difference was seen between the younger (8 wk) and older adult animals (8-9 m). Therefore, the suckling/weaning transition seems to be a critical period for changes in intestinal Na/P_i transport activity.

Similar patterns of decline were observed in Na/P_i-IIb protein levels during the suckling/weaning transition. Na/P_i-IIb protein levels in the suckling mouse intestine were

6-fold higher than in the weanling and adult groups. Thus, the decrease in total intestinal Na/P_i uptake seems to parallel the decrease in Na/P_i-IIb protein expression. These results support the previous suggestion that Na/P_i-IIb protein is the major intestinal Na/P_i cotransporter. In addition to decreases in protein abundance, Na/P_i-IIb mRNA levels were also altered with age. Na/P_i-IIb mRNA in suckling mouse intestine was markedly higher than in the older groups, but the difference between suckling and the older animals was only 3-fold. Since mRNA levels did not decrease as severely as uptake and protein levels, it is conceivable that part of the observed decreases in functional protein expression may be due to post-transcriptional mechanisms. Alternatively, the discrepancies may be due to the different methodologies used and their differential sensitivities.

To characterize Na/P_i-IIb-specific antiserum, we performed western blots and immunohistochemical analyses. The antiserum detected Na/P_i-IIb protein in mouse BBM as a single band of ~110 kDa, which was consistent with a previous report (100). Prior to immunohistochemistry studies, rat Na/P_i-IIb protein was confirmed to be detectable with the antiserum in western blots. Proximal intestine from a 3 wk-old rat was alternatively used for immunohistochemistry because of technical difficulties with obtaining intact tissue samples from young mouse intestine. Immunohistochemical analysis exemplified the specificity of the antiserum as it only reacted with the intestinal apical membranes.

In western blot analyses from different aged mice, the molecular weight of Na/P_i-IIb in suckling mice was lower (~88 kDa) than the protein in older animals (~110 kDa). We found three conserved *N*-linked glycosylation sites among the known Na/P_i-IIb cotransporter family members, and two of the three sites were conserved with sites

confirmed to be glycosylated in the mouse renal Na/P_i-IIa cotransporter (92). Since these results suggested that mouse Na/P_i-IIb cotransporter may also be an *N*-glycoprotein, we performed enzymatic deglycosylation studies with PNGase F and Endo H. PNGase F cleaves between the innermost *N*-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides of *N*-linked glycoproteins (151), whereas Endo H cleaves the chitobiose-core of high mannose and some hybrid oligossacharides of *N*-linked glycoproteins (207). Treatment with PNGase F decreased the molecular weight of both Na/P_i-IIb specific bands (~88 kDa and ~110 kDa) to its predicted size (~78 kDa), while treatment with Endo H had no apparent effect. Therefore, our findings strongly suggest that the type IIb Na/P_i transporter is an *N*-linked glycoprotein containing complex oligosaccharides, and that glycosylation occurs during the suckling/weaning transition. To date, the effects of GCs on Na/P_i-IIb expression have not been addressed, although GC-induced downregulation of intestinal Na/P_i transport has been reported (24).

In the present study, MP injection reduced Na/P_i uptake (3.4-fold), and Na/P_i-IIb protein levels (3.8-fold) and mRNA (3.7-fold) in suckling animals. Therefore, our results indicate that the GC-induced decrease in intestinal Na/P_i transport correlates well with Na/P_i-IIb protein and mRNA level reductions in suckling animals. The parallel decline in mRNA abundance implicates a possible genomic effect of GCs. Indeed, several studies have suggested maturation of intestinal proteins by genomic action of GCs (98, 224, 260). However, one previous study suggested that GCs modulate patterns of glycosylation in suckling rat intestine through enhanced fucosyl-transferase activity (17), and thus, we cannot eliminate the possibility that Na/P_i-IIb glycosylation is enhanced by

nongenomic actions of GCs during suckling period. Furthermore, the 21 d animals showed MP responsiveness in regards to intestinal Na/P_i uptake and Na/P_i-IIb mRNA expression, although no significant alteration was seen in Na/P_i-IIb protein levels.

There are clear differences between Na/P_i-IIb and its renal homologue, Na/P_i-IIa in expression during ontogeny and hormonal response. Our findings indicate that 1) intestinal Na/P_i uptake activity, and Na/P_i-IIb protein and mRNA levels are all highest during the suckling period, 2) Na/P_i-IIb GC-responsiveness is also highest during the suckling period, and 3) the Na/P_i-IIb protein is not fully processed (glycosylated) until weaning. Accumulated evidence regarding renal Na/P_i-IIa have indicated that 1) renal Na/P_i uptake activity and Na/P_i-IIa protein are lower during the suckling period than during weaning, 2) Na/P_i-IIa GC-responsiveness is not seen during the suckling period but is observed in adults, and 3) there is no evidence of unglycosylated Na/P_i-IIa at any age in rodents.

Because the intestine matures very rapidly during the suckling/weaning transition (94), it is not surprising that suckling animals have a higher capacity to absorb nutrients than older animals. The rapid decline in Na/P_i uptake activity and Na/P_i-IIb expression between the suckling and weaning periods suggests that Na/P_i transport system matures early in postnatal life. In conclusion, we found that Na/P_i-IIb mRNA expression rapidly decreases during the suckling/weaning transition and that mRNA expression is regulated by GCs in the suckling period. This decrease in mRNA expression is likely responsible for the observed decrease in Na/P_i-IIb protein expression and the concomitant decrease in intestinal Na/P_i transport in early life. Further investigation is necessary to decipher

maturational mechanisms of Na/P_i-IIb cotransporter expression and function during the suckling/weaning transition.

CHAPTER IV

Summary of Chapter II results

The type IIb sodium-phosphate cotransporter (Na/P_i-IIb) is expressed in the brush-border membrane of mouse enterocytes and is involved in phosphate (P_i) homeostasis (100). We have cloned, sequenced (GenBank accession number AF211863) and characterized the 5'-flanking region, and determined the gene structure of the Na/Pi-IIb gene. The gene spans more than 18 kb and consists of 12 introns and 13 exons. The exon-intron boundaries of this gene are similar to those of the Na/Pi-IIa gene in both splicing positions within the codon triplets as well as in the amino acids encoded by these interrupted codons. The major transcription initiation site, as determined by primer extension, was shown to be 64 bp upstream of the previously identified cDNA (GenBank accession number AF081499). Three reporter gene constructs, –159/+73, –429/+73 and –954/+73, showed significant luciferase activity 22-82 fold over background, respectively) in RIE-1 cells. These observations will allow functional characterization of these important gene regulatory regions and will further contribute to our understanding of mammalian P_i homeostasis.

Summary of Chapter III results

We studied the effects of development and GCs on an apically expressed intestinal sodium-phosphate cotransporter (Na/P_i-IIb) in mice. Mice at ages of 14 days, 21 days, 8 weeks, and 8-9 months were injected with methylprednisolone (MP) or saline once every 12 hr for a total of four times. Na/P_i uptake activities in intestinal brush-border membrane (BBM) isolated from control groups were highest in 14-d (1.63 ± 0.43, $P < 0.0001$ for 14 d vs. other ages), lower in 21-d (0.24 ± 0.02) and further reduced in 8-w (0.09 ± 0.05, $P < 0.001$) and 8-9-m-old mice (0.04 ± 0.03, $P < 0.001$). MP treatment significantly decreased Na/P_i uptake activity in 14-d-old mice (1.63 ± 0.43 control and 0.48 ± 0.1 MP; $P < 0.05$) and 21-d-old mice (0.24 ± 0.02 control and 0.04 ± 0.02 MP; $P < 0.05$). Northern blot analyses demonstrated that Na/P_i-IIb mRNA levels in 14-d-old mice (7.35 ± 1.66 Na/P_i-IIb/GAPDH ratio) were markedly higher than other age groups (21-d, 2.53 ± 1.15; 8-wk, 2.22 ± 1.28; 8-9-m, 1.71 ± 0.24; $P < 0.0001$ for 14 d vs. other groups) and MP-treated groups showed a drastic decrease in the mRNA levels in 14-d (7.35 ± 1.66 control vs. 2.02 ± 1.55 MP; $P < 0.05$), 21-d (2.53 ± 1.15 control vs. 0.44 ± 0.09 MP; $P < 0.05$), and 8-9-m (1.71 ± 0.24 control vs. 0.71 ± 0.26 MP; $P < 0.05$) animals, but 8-w-old mice (2.22 ± 1.28 control vs. 1.00 ± 0.42 MP). Na/P_i-IIb specific antiserum was produced in rabbits against a C-terminal peptide, and characterized by western blot and immunohistochemistry analyses. In western blots, antigen blocking and the use of preimmune serum showed no immunoreactivity with mouse BBM proteins. The antiserum recognized a single band in BBM proteins isolated from 14-d-old mice (at ~88 kDa) and 8-wk-old mice (at ~110 kDa). Immunohistochemistry also showed that the

antiserum reacted with a BBM protein in rat intestinal epithelium, while the preimmune serum had no reactivity. Western blots demonstrated that Na/P_i-IIb protein levels (presented as a Na/P_i-IIb/β-actin ratio) were markedly higher in suckling animals (1.44 ± 0.29) as compared to the other age groups (21 d, 0.26 ± 0.16 8 wk, 0.29 ± 0.09; 8-9 m, 0.15 ± 0.11; P <0.0001 for 2 wk vs. other groups). In addition, MP treatment significantly decreased the protein abundance in 14-d-old mice (1.44 ± 0.29 control vs. 0.38 ± 0.17 MP; P <0.05) and slightly decreased the abundance in 21-d (0.26 ± 0.16 control vs. 0.11 ± 0.10 MP) and 8-9-m-old (0.15 ± 0.11 control vs. 0.05 ± 0.03 MP) mice. An additional study was performed to investigate the age-dependent size difference of Na/P_i-IIb protein in14-d and 8-wk-old mice. Deglycosylation using PNGase F reduced the molecular weight of the proteins (~88 kDa and ~110 kDa) to the predicted size (~78 kDa). In conclusion, we demonstrated that mouse intestinal Na/P_i-IIb mRNA levels decreased during the suckling/weaning transition and also that the mRNA expression was regulated by GCs in suckling animals. The decrease in the mRNA coincides with the decrease in Na/P_i-IIb protein levels and concomitant decrease in intestinal Na/P_i cotransport activities in early life. We also found that Na/P_i-IIb is an *N*-glycoprotein and that its glycosylation is developmentally regulated during the suckling/weaning transition.

Implications

The overall aim of my project was to better understand the physiological role of the recently cloned intestinal Na/P_i-IIb cotransporter because it is considered to be a major sodium-dependent phosphate (Na/P_i) cotransporter in the mammalian intestine (100). In general, P_i homeostasis is tightly regulated by intestinal Na/P_i absorption and renal Na/P_i reabsorption. However, P_i homeostasis in neonates mainly relies on dietary P_i absorption via the intestinal Na/P_i uptake. In addition, P_i demand in neonates is so high that P_i homeostasis maintains higher plasma P_i levels than in adults. Therefore, adequate dietary supply of P_i and active intestinal Na/P_i cotransport systems are necessary to prevent infants from P_i deficiency.

Rickets and osteopenia are commonly observed complications in low-birth-weight infants and preterm infants receiving GC-treatment. In addition, GCs are well known risk factors to induce abnormal bone mineralization and osteoporosis. However, the molecular aspects of these complications in prematurity have never been elucidated. Studying developmental and hormonal effects on Na/P_i-IIb cotransporter expression helps to understand how preterms develop hypophosphatemia leading to rickets and osteopenia during infancy, and osteoporosis in later life.

We found that developmental and GC regulation of Na/P_i-IIb cotransporter occurs at both the mRNA level and protein level. The parallel decline in protein and function is further evidence that the Na/Pi-IIb cotransporter is the predominant Na-dependent P_i cotransporter in the intestine.

A developmental decrease in mouse intestinal Na/Pi-IIb mRNA levels and GC-dependent reduction of the mRNA levels in suckling mice may be regulated at transcriptional level. During the suckling/weaning transition, age-dependent downregulation of lactase activity is not regulated by dietary lactose (69, 209) but seems to be genetically programmed (63, 227, 253, 262). Sucrase-isomaltase activity is also upregulated at transcription level during the suckling/weaning transition (255). Cdx1, Cdx2, NF-LPH1 and HNF-1 have been reported as potential *trans*-elements that transcriptionally modulate these enzyme activities (see Developmental effects on intestinal enzymes, In Introduction). Among them, Cdx2 is believed to a transcription factor involved in regulating development and differentiation of intestinal epithelial cells (63). Meanwhile, decrease in mRNA level of NF-LPH1 coincides mRNA level of Na/Pi-IIb cotransporter during the suckling/weaning transition (253). These nuclear factors may have broader roles in intestinal development and morphogenesis, and may be involved in Na/Pi-IIb cotransporter gene regulation.

GCs are well known anti-inflammatory and immunosuppressive drugs. GCs are also the most potent hormonal factor to regulate the intestinal maturation. The precocious decrease in Na/Pi-IIb mRNA and protein levels in suckling animals strongly supports the hypothesis that the administration of exogenous GCs during the first and second postnatal week causes precocious maturation of numerous aspects of intestinal structure and function (94). To examine the effects of GCs on Na/P_i-IIb gene expression, we cloned the functional promoter of Na/P_i-IIb gene and found putative regulatory elements, including GC response element (GRE). Therapeutic effects of GCs are mostly receptor-mediated

genomic effects. Thus, GRE in Na/P_i-IIb gene promoter region can be important for GC-mediated downregulation in Na/P_i-IIb mRNA.

We found that Na/P_i-IIb is an *N*-linked glycoprotein and that the glycosylation is age-related. In addition, GC-treatment increased the molecular weight of type IIb protein in 14-day-old mice. In the small intestine, α-2,6-sialyltransferase is the predominant during the suckling period while α-1,2-fucosyltransferase becomes predominant during the weaning period. Thus, high fucosylation is more commonly observed in intestinal glycoproteins in adult rats than in suckling rats.

GC treatment induces precocious change in patterns of glycosylation by decreasing α-2,6-sialyltransferase activity and increasing α-1,2-fucosyltransferase activity during the first two postnatal weeks. Based on these previous observations, we speculate that the shift from sialylation to fucosylation in *N*-glycans of the Na/P_i-IIb protein causes the molecular weight increase during the suckling/weaning period, and the shift can be induced precociously by GC administration in suckling animals.

The roles of the *N*-glycan in Na/P_i-IIb protein remain unknown. Proper cotranslational *N*-linked glycosylation is essential for protein to be exported from the ER to Golgi apparatus, where *N*-glycans are further modified (see Glycosylation, In Introduction). Unique *N*-glycans work as signals for proper sorting of lysosomal enzymes and apical membrane proteins in trans-Golgi network. We observed Na/P_i-IIb protein expression and Na/P_i cotransport activities in intestinal apical membrane of suckling, weaning and adult mice. These observations suggest that the *N*-glycosylated Na/P_i-IIb protein is successfully folded in the ER and delivered to the right membrane in all age

groups. Meanwhile, the changes in pattern of glcosylation coincide with changes in Na/P_i-IIb protein abundances in intestinal brush-border membrane. One possibility is that oligosaccharide differences between *N*-glycans found in suckling and older mice may be critical for apical sorting efficiency in the trans-Golgi network. Another possibility is that the adult form of *N*-glycan may be more susceptible for protein degradation or internalization in apical membrane. Some intestinal proteins such as lactase and sucrase-isomaltase also change oligosaccharide in their *N*-glycans during the suckling/weaning period. Therefore, understanding the roles of *N*-glycan in Na/P_i-IIb protein may elucidate general roles of *N*-glycosylation during development.

Future Direction of Investigation

Significant intestinal maturation must occur during the suckling/weaning transition. Accumulated evidence suggests that exogenous GC can precociously induce the intestinal maturation in suckling animals. Based on the previous findings, further investigation is proposed to elicit the hormonal and developmental downregulation of Na/P_i-IIb cotransporter at both transcriptional and posttranslational levels.

Determine Glycosylation Sites and Their Roles in Na/P_i-IIb Cotransporter Protein

Mouse intestinal Na/P_i-IIb cotransporter is an *N*–glycoprotein and its molecular weight varies depending on age and plasma GC-levels. The smaller molecular weight found in suckling animals is associated with the highest levels of Na/P_i-IIb protein and Na/P_i uptake activity during ontogeny. In sucking animals, GC treatment clearly reduced

Na/P_i-IIb protein levels and Na/P_i uptake. Furthermore, the molecular weights of multiple bands shown in MP-treated suckling mice were intermediate sizes between ~88 kDa and ~110 kDa. These results imply that glycosylation of Na/P_i-IIb protein is developmentally and hormonally regulated in mice. The reported plasma GC levels are very low in suckling rats, and administration of GCs in suckling animals induced precocious glycosylation by enhancing α-1,2 fucosyltransferase activity in the intestine (17, 94). Therefore, we hypothesize that glycosylation of Na/P_i-IIb protein is likely age-dependent and that the glycosylation event may be enhanced by exogenous GCs that modulate enzyme activities involved in *N*-glycosylation process, such as α-1,2 fucosyltransferase. We further hypothesize that glycosylation of Na/P_i-IIb protein may be involved in age-dependent and/or GC-mediated reduction of Na/P_i transport function, possibly by modulating protein stability, cell surface expression and/or intracellular trafficking (92, 105, 106, 153, 186, 256).

The system that we will use to test our hypotheses is cRNA injected *Xenopus laevis* oocytes and Caco-2 cells. The *X. laevis* oocyte system has often been used to study intestinal glycosylated transporters because it provides the benefits of utilizing oocytes for functional assays (92, 153, 256). Other model systems frequently used to study intracellular traffic of newly synthesized apical membrane proteins, are Madin-Darby canine kidney (MDCK) cells and Caco-2 cells, which form a well-polarized monolayer that can be manipulated from both apical and basolateral sides when these cells grow on permeable filters (85, 105, 107, 187, 256). RT-PCR analysis showed expression of Na/P_i-

IIb mRNA in Caco-2 cells (276). Therefore, Caco-2 cells may also represent an appropriate model to study the role of *N*-glycosylation in Na/P_i-IIb protein.

Intestinal BBM proteins will be prepared from MP or saline treated 14-day and 21-day mice for subsequent examination of α-2,6-sialyltransferase and α-1,2-fucosyltransferase enzyme activities. In order to determine if Na/P_i-IIb protein is an α-1,2-fucoprotein, α-1,2-fucosylation will also be assayed. Furthermore, the degree of fucosylation will be compared between BBM proteins isolated from MP (or saline as a control) treated 14-d-mice, and from 14-d and 8-wk-old mice. These results may provide an explanation for the different molecular weights in Na/P_i-IIb protein expressed in suckling and weanling mice.

Glycosylation at specific sites will be examined by using series of mutations will be introduced to Na/P_i-IIb protein to disrupt potential glycosylation sites on the protein. The effects of these mutations will be studied in *X. laevis* oocytes and also Caco-2 cells as an alternative and corroboration system. Glycosylation is often critical for apical sorting. Karim-Jimenez et al. (2000) identified a critical leucine residue (L691) for apical sorting but did not investigate a potential role for the *N*-glycan as an apical sorting signal. The effects of *N*-linked glycosylation on cell surface expression of Na/P_i-IIb transporter will be evaluated by immunoprecipitation of biotinylated cell surface proteins. Cellular localization of the wild-type and mutant IIb proteins will be determined by immunocytochemistry. To determine whether the glycosylation-caused decrease in Na/P_i-IIb accumulation in BBM is due to a change in the rate of Na/P_i-IIb protein turnover, pulse-chase studies of the injected oocytes will also be performed.

Alternatively, Caco-2 cells will be transient transfected with the Na/P_i-IIb wild-type construct and various mutant constructs, and utilized to define the role of *N*-linked glycosylation as described above.

Determine the Hormonal and Developmental Regulation of Na/P_i-IIb Cotransporter Gene

Our results showed that the Na/P_i-IIb mRNA as well as protein levels in suckling mice were significantly higher than levels found in older mice. Additionally, GC treatment decreased Na/P_i-IIb mRNA (3.7-fold) and protein levels (3.8-fold) in suckling animals. We cloned the 5'-flanking region of Na/P_i-IIb gene and subsequent promoter assays in RIE-1 cells confirmed that three reporter-gene constructs containing various lengths of the promoter (-159 to +73, -429 to +73 and –954 to +73, relative to transcriptional initiation site) contained functional basal promoter activity. Because alterations in mRNA levels are often the result of alterations in gene-promoter activity, we hypothesized that the decrease in Na/P_i-IIb mRNA levels was due to reductions in Na/P_i-IIb promoter activities. Therefore, we will further characterize the 5'-flanking region of mouse Na/P_i-IIb gene.

Prior to the studies, we will perform nuclear run-on transcription assays to determine whether the decrease in Na/P_i-IIb mRNA is due to an altered transcription level of Na/P_i-IIb gene. In order to map GC response element(s) (GRE) or identify *cis*-elements involved in GC-mediated inhibition of Na/P_i-IIb transcription, RIE-1 cells will

be transient transfected with the three promoter constructs and cultured in the absence or presence of GCs for 24 hr before luciferase assays.

Alternatively, we will use ~2.5 kb of 5'-flanking region of mouse Na/P_i-IIb gene (unpublished data) to design additional reporter constructs. The longer constructs will allow us to identify any potential upstream GRE(s) or *cis*-elements, which are involved in GC-mediated transcriptional inhibition.

A comparison of luciferase activities of various length constructs will allow us to localize the region(s) that includes the *cis*-elements. The random and/or PCR-based deletion mutation approach will be used to narrow down the large region by making a shorter construct that still maintains responsiveness to GC. The precise transcriptional regulatory sequences will be mapped by PCR-based site-directed mutagenesis, which abolish GC-responsiveness of the reporter gene in transfected cells.

The series of deletions and mutations of the 5'flanking region of Na/P_i-IIb gene in reporter gene constructs followed by luciferase assays will determine GC responsive sequence(s) in the promoter. We will further confirm the *cis*-element(s) by electrophoretic mobility shift assay (EMSA) and DNase I footprinting because transcriptional regulation is affected by the direct interaction of *cis*-elements with transcriptional factors. These techniques have been commonly used to define DNA-protein interactions. The outcome will define the location and extent of the binding region of the transcriptional factor that is involved in GC-mediated inhibition of Na/P_i-IIb transcription. If the *cis*-element(s) identified by these studies is known sequence such as GRE or AP-1 sites, commercially available antibodies will be used to look for

supershifting of the DNA-protein complex band in an attempt to identify the bound protein.

The mechanisms of GC-mediated Na/P_i-IIb transcriptional inhibition will be characterized by the *in vitro* techniques described above. However, our work as well as that of others suggests that endogenous GCs are involved in intestinal maturation. Because the age-related effects on Na/P_i-IIb gene expression may include physiological factors other than GC, the developmental down-regulation of Na/P_i-IIb transcription will also be studied *in vivo* using transgenic mice. The age-dependent effects on Na/P_i-IIb transcriptional activities would be supported by an observation of higher luciferase activities in 2-wk suckling than in 8-wk adult mice.

In addition, the Na/P_i-IIb/Luc transgenic line will be also utilized to further examine GC-mediated transcriptional regulation of Na/P_i-IIb gene. A decrease in luciferase activities in MP-treated animals would confirm physiological significance of 5'-flanking region including specific *cis*-element(s) that would have or could be identified by promoter, DNase I footprinting and EMSA assays *in vitro*.

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