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Characterization and regulation of type II rat renal sodium-dependent phosphate cotransporter NaPi-2

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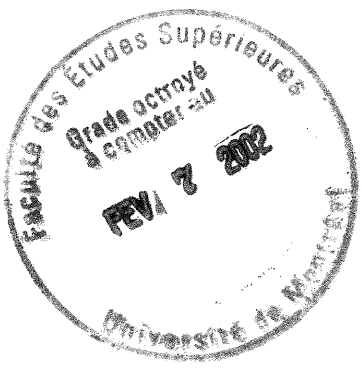

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SUMMARY

Homeostasis of inorganic phosphate (Pi) is maintained mainly by a sodium-dependent Pi (Na^+/Pi) reabsorbing process at the proximal tubules of kidneys. Recently the molecular bases of the major transport systems have been identified. To understand the underlying mechanisms of renal proximal tubule Na^+/Pi cotransport, this research project characterized the structure-function relationship of the cotransporters with emphasis on NaPi-2, the rat type II Na^+/Pi cotransporter. With the aid of biochemical methods including SDS-PAGE, Western blot, radiation inactivation, deglycosylation, amino acid sequencing and vesicular uptake assay, the native size of NaPi-2 was determined, the enzymatic cleavage of NaPi-2 protein was examined, the site of cleavage was identified, and the functional implications of the cleavage were analyzed. The regulation of NaPi-2 proteins as well as the Na^+/Pi cotransport activity under different physiological and pathophysiological conditions were also investigated.

Two polyclonal antibodies were raised in rabbits, one (anti-C) against the C-terminal while the other (anti-N) against the N-terminal 14-amino acid segments of NaPi-2 protein, which has a calculated molecular weight of 68 kDa. Immunoblot analysis under non-reducing conditions of proximal tubular brush border membrane (BBM) with either antibodies revealed proteins of approximately 180 kDa (p180) and 70 kDa (p70). When separated under reducing conditions, proteins of about 70 kDa and 40 kDa (p40) were detected with anti-C, while proteins of about 70 kDa and 45 kDa (p45) were detected with anti-N. These results suggest that 1) NaPi-2 proteins in the BBM exist both as oligomers (p180) and as monomers (p70), and the oligomeric form is likely composed of two monomers linked together via disulfide bond(s); 2) the monomers are of two types: one is intact p70, as detected in the presence of reducing agent, while the other is cleaved p70 with the C-terminal product being p40 and the N-terminal product being p45. In their native form, some the monomers are composed of p40 and p45 linked via disulfide bonds. Dose-dependency analysis on the effect of reducing agent on the abundance of p180 and Na^+/Pi cotransport activity of the BBM revealed that p180 is an active form of NaPi-2. Much higher concentration of reducing agent (ß-mercaptoethanol) was required to reduce NaPi-2 in intact membrane vesicles than in solubilized BBM preparations, indicating that the affected disulfide bonds are not exposed to the extracellular compartment. The target sizes estimated from the radiation-induced loss of intensity of p40, p70 and p180 were all around 190 kDa, suggesting that NaPi-2 exists as an oligomer in which the subunits

are close to one another. When protein samples were pretreated with reducing agent before irradiation, target sizes estimated from the radiation-induced loss of intensity of p40 and p70 were 74 and 92 kDa respectively, showing the presence of disulfide bond in the molecular structure of NaPi-2.

N-deglycosylation experiments revealed that both p40 and p45 are glycosylated, with deglycosylated sizes of 30 kDa and 35 kDa, respectively. This indicates that 1) p40 and p45 are cleavage products, as the sum of the deglycosylated sizes is close to the predicted size of 68 kDa; 2) the cleavage site is located between Asn 298 and Asn 328, as site-directed mutagenesis has demonstrated that these two sites are the only ones used for NaPi-2 N-glycosylation. The precise site of this cleavage was analyzed by N-terminal amino acid microsequencing of the purified p40 polypeptide. Results indicated that the N-terminal sequence of p40 is VEIAG. Therefore, the cleavage site should be between residues R319 and V320 of NaPi-2.

To determine the relationships among different forms of NaPi-2 during the modulation of transport activity, the profile of NaPi-2 protein expression was analyzed in three different situations affecting Na/Pi cotransport, including dietary Pi, developmental age, and localization within the kidney cortex. Higher transport activity was observed in BBM of adult rats fed a low phosphate diet, in BBM of adults as compared to suckling rats, and in BBM of juxtamedullary compared to superficial kidney cortex of adult animals. Higher activity was always associated with a higher expression of monomers and dimers by Western blotting, suggesting that both species are involved in modulating Pi transport. Changes in protein expression were greater than those in activity, being 3 to 4-fold higher than activity for age and localization, and 13-fold higher for diet. Moreover, the contribution of intact and cleaved p70 polypeptides to the level of monomers and dimers varied from one situation to another and variations can even be in opposite direction. These findings indicate that supramolecular assemblys of monomers and dimers are necessary for activity, and that there is not a unique expression pattern controlling the transport of Pi by NaPi-2.

Acute renal ischemia/reperfusion is a major cause of renal failure. To understand the mechanisms underlying the ischemia/reperfusion-induced renal proximal tubule damage, we analyzed the expression of Na^+/Pi cotransporter NaPi-2 in BBM isolated from rats subjected to 30 min renal ischemia followed by 60 min reperfusion. Transport activity of the BBM vesicles was also determined. Ischemia caused a significant decrease (about 40%, $p < 0.05$) in

the expression of all forms of NaPi-2 in the BBM, despite a significant increase ($31 \pm 3\%$, $p < 0.05$) in the Na^+/Pi cotransport activity. After reperfusion, both NaPi-2 expression and Na^+/Pi cotransport activity returned to control level. In contrast to Na^+/Pi cotransport, ischemia decreased Na^+/D-glucose cotransport by $45 \pm 4\%$ ($p < 0.01$), while reperfusion caused further decrease in both Na^+/D-glucose (by 60%) and Na^+/L-proline (by 33%) cotransports. NaPi-2 abundance in cortex homogenate is less affected than that in BBM, suggesting a relocalization of NaPi-2 to the BBM after reperfusion. These data indicate that NaPi-2 protein and the BBM Na^+/Pi cotransport activity respond uniquely to reversible renal ischemia and reperfusion, and thus may play an important role in maintaining and restoring the function of the proximal tubule during these processes.

RÉSUMÉ

L'homéostasie du phosphate inorganique (Pi) est maintenue principalement par le processus de réabsorption du phosphate due aux cotransporteurs de phosphate sous dépendance sodique (Na^+/Pi) au niveau des tubes proximaux du rein. Récemment, les bases moléculaires des systèmes majeurs de transport ont été identifiées. Pour comprendre les mécanismes impliqués dans le cotransport Na^+/Pi au niveau des tubes proximaux, ce projet de recherche avait pour but de caractériser la relation entre la structure et la fonction des cotransporteurs de phosphate avec une emphase sur le cotransporteur Na^+/Pi de type II (NaPi-2) chez le rat. Les méthodes biochimiques telles que l'électrophorèse, l'immunobuvardage, l'inactivation par radiation, la déglycosylation, le séquençage en acides aminés et le transport vésiculaire d'isotope, nous ont permis de déterminer la taille de la protéine NaPi-2, d'identifier le site de clivage enzymatique de NaPi-2 et d'analyser son implication fonctionnelle. Enfin, la régulation de l'expression de NaPi-2 et de l'activité du cotransporteur sous diverses conditions physiologiques et physiopathologiques a été étudiée.

Nous avons produit chez le lapin deux anticorps polyclonaux; l'un (anti-C) est dirigé vers l'extrémité C-terminale et l'autre (anti-N) est dirigé contre l'extrémité N-terminale de la protéine NaPi-2. Le poids moléculaire de NaPi-2 déduit de la séquence du gène est de 68 kDa. Grâce à ces anticorps, nous avons détecté une quantité abondante de NaPi-2 dans les membranes en bordure en brosse (MBB) des tubes proximaux. Lorsque les protéines des MBB sont séparées par électrophorèse en conditions non réductrices, des protéines ayant des poids moléculaires respectifs de 180 kDa (p180) et de 70 kDa (p70) sont détectées avec les deux anticorps. Lorsque séparées en conditions réductrices, des protéines de 70 kDa et 40 kDa (p40) sont détectées avec l'anticorps anti-C alors que des protéines de 70 kDa et 45 kDa (p45) sont détectées avec l'anticorps anti-N. Ces résultats suggèrent que 1) la protéine NaPi-2 existe sous forme oligomérique (p180) et sous forme monomérique (p70) dans les MBB, la forme oligomérique pourrait être composée de deux monomères liés par un pont disulfure; 2) il y a deux types de monomères, une première forme intacte p70 détectée en présence de l'agent réducteur et deux formes issues de la p70, donnant un produit C-terminal de 40 kDa et un produit N-terminal de 45 kDa. Dans sa forme native, une partie des monomères serait composée de p40 et p45 liées par des ponts disulfures. Une analyse de l'effet d'une dose croissante de l'agent réducteur 2-mercaptoéthanol sur l'abondance de p180 et l'activité du cotransporteur Na^+/Pi des MBB révèle que la forme p180 est la forme active de NaPi-2. Des

concentrations très élevées en agent réducteur sont requises pour dissocier les sous-unités de NaPi-2 dans des vésicules membranaires intactes comparativement aux préparations de MBB solubilisées avec un détergent. Ces résultats indiquent que les ponts disulfures impliqués ne sont pas exposés à la surface du cotransporteur. Les tailles fonctionnelles des protéines p40, p70 et p180 obtenues par inactivation aux radiations sont estimées à 190 kDa. Ceci suggère que la protéine NaPi-2 existe sous une forme oligomérique dans laquelle les sous-unités sont très proches l'une des autres. Lorsque les MBB ont été prétraitées avec un agent réducteur avant l'irradiation, les tailles fonctionnelles de p40 et p70 sont estimées à 74 et 92 kDa respectivement. Ceci démontre la présence de ponts disulfures dans la structure moléculaire de NaPi-2.

Les expériences de déglycosylation des MBB sur des résidus asparagines (Asn) démontrent que la p40 et la p45 sont glycosylées. Le fait que les tailles des protéines déglycosylées sont de 30 et 35 kDa, respectivement, indique que 1) la p40 et la p45 sont des produits de clivage puisque la somme de leurs tailles lorsque déglycosylée est très proche de la taille prédite de 68 kDa à partir de la séquence du gène; 2) le site de clivage se situe entre l'Asn 298 et l'Asn 328 puisque la mutagenèse dirigée a démontré que ce sont les seuls sites de N-glycosylation de NaPi-2. Le site précis de clivage a été identifié par microséquençage en N-terminal du polypeptide p40 que nous avons purifié. Les résultats montrent que la séquence en N-terminal de p40 est V320EIAG, permettant de localiser le site de clivage entre R319 et V320 de NaPi-2.

Pour identifier les différentes formes de NaPi-2 impliquées dans la modulation de son activité, les profils de leur expression ont été analysés dans trois différentes situations où le cotransporteur Na^+/Pi est modulé soit: par la quantité de Pi dans l'alimentation, au cours du développement et la localisation des néphrons dans le cortex rénal. Une induction importante de l'activité du transporteur est observée dans les MBB de rats adultes exposés à une alimentation pauvre en phosphate versus une alimentation normale, dans les MBB de rats adultes versus les rats nouveau-nés et dans les MBB des néphrons juxtamédullaires versus ceux du cortex rénal. Cette activité élevée est toujours associée avec une expression élevée du momomère (p70) et du dimère (p180), indiquant que chaque forme module l'activité de transport. Les changements dans l'expression des différentes formes de NaPi-2 sont cependant plus marqués que ceux observés pour l'activité. L'expression de NaPi-2 augmente de 3 à 4 fois plus rapidement que l'activité avec l'âge alors que la différence dans la diète est

de 13 fois supérieure. De plus, la contribution des polypeptides p70, intact ou clivé, dans l'expression du monomère et du dimère varie d'une situation à l'autre et parfois dans des directions opposées. Ces résultats indiquent que l'assemblage supramoléculaire du monomère et du dimère est nécessaire pour l'activité.

L'ischémie et la reperfusion sont des causes majeures des dysfonctions rénales. Pour comprendre les mécanismes sous-jacents responsables des dommages induits aux tubes proximaux, l'analyse de l'expression et de l'activité de NaPi-2 a été effectuée sur MBB isolées de reins ayant subit soit 30 min d'ischémie, soit 30 min d'ischémie suivi de 60 min de reperfusion. L'ischémie seule réduit significativement (40% $p< 0.05$) l'expression de toutes les formes de NaPi-2, mais entraîne une augmentation significative de l'activité du cotransporteur ($31 \pm 3\%$, $p < 0.05$). Le niveau d'expression de NaPi-2 dans les MBB est plus réduit que dans les homogénats suggérant que le transporteur dans les membranes apicales est très vulnérable à l'ischémie. Après 60 min de reperfusion, l'expression et l'activité sont identiques à celles observées en condition témoin. En comparaison, l'activité du transporteur Na^+/D-glucose est réduite de $45 \pm 4\%$ ($p < 0.01$) après l'ischémie et de 60% après la reperfusion. L'activitié du transporteur Na^+/L-proline est réduite de 33% en reperfusion. Donc, le cotransporteur NaPi-2 des MBB pourrait jouer un role dans le maintien et la restoration des fonctions des tubes proximaux lors de l'ischémie et de la reperfusion.

CONTENTS

LIST OF FIGURES AND TABLES

Thesis — Molecular mechanism of rat renal sodium-dependent phosphate cotransport

Manuscript 1 — Involvement of disulphide bonds in the renal sodium/phosphate cotransporter NaPi-2

Manuscript 3 Different expression patterns of NaPi-2 transporter modulate sodium-phosphate cotransport by rat kidney

Manuscript 4 Effect of ischemia/reperfusion on renal brush-border membrane sodium-dependent phosphate cotransporter NaPi-2

LIST OF ABBREVIATIONS

AC	adenylyl cyclase
ADF	actin deploymerizing factor
BBM	brush-border membrane
BBMV	BBM vesicles
BLM	basolateral membrane
cAMP	cyclic adenosine 5'-monophosphate
Cd	Cadmium
cDNA	complementary deoxyribonucleic acid
DAG	diacylglycerol
DTT	dithiothreitol
ECL	enhanced chemoluminescence
β-EtSH	β-mercaptoethanol
FPLC	fast performance liquid chromatography
Hg	hydrargyrum (mercury)
Ip	phosphate induced-current
GH	growth hormone
Glvr-1	cell surface receptor for gibbon ape leukemia virus
HPD	high phosphate diet
Hypo	animals subjected to a low phosphate diet
IP3	inositol-1,4,5-trisphosphate
IPAN	anti-sense transcript of NaPi-II-related mRNA
KLH	keyhole limpet hemocyanin
LPD	low phosphate diet
MBB	membranes en bordure en bross
mRNA	messenger ribonucleic acid
Na^+/Pi	sodium-dependent phosphate (transport)
NaPi-1	the first member of sodium-dependent phosphate cotransporter family
NaPi-2	the second member of sodium-dependent phosphate cotransporter family
NHE-3	Na^+/H^+ exchanger 3
OK	opossum kidney (cell line derived from renal proximal tubule)
p40	the 40 kDa C-terminal polypeptide of NaPi-2

p45	the 45 kDa N-terminal polypeptide of NaPi-2
p70	the 70 kDa NaPi-2 polypeptide
p180	the 180 kDa form of NaPi-2
PAGE	polyacrylamide gel electrophoresis
Pb	plumbum (lead)
PEX	Pi regulating gene with homology to endopeptidase on the X-chromosome
PFC	phosphonoformic acid
Pi	inorganic phosphate
PKA	protein kinase A
PKC	protein kinase C
PRE	Pi responsive element
PT	proximal tubule of kidney
PTH	parathyroid hormone
PTHrP	PTH related peptide
PVDF	polyvinylidene difluoride
Ram-1	cell surface receptor for amphotropic murine retrovirus
RIS	radiation inactivation size
SDS	sodium dodecyl sulfate
TCEP	tris-(2-carboxyethyl)phosphine
TEF3	transcription factor μE3
TM	transmembrane domain
TmP/GFR	tubular maximum for phosphate reabsorption /glomerular filtration rate
TS	target size
XLH	X chromosome-linked hypophosphotemia

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DEDICATION

For all my teachers, past and present

I. INTRODUCTION

Molecular mechanisms of rat renal sodium-dependent phosphate cotransport

I.1 General introduction

I.1.1 Biological function of phosphate

Phosphate is found in all organisms. It is a major anion in the extracellular matrix such as in the crystalline structure of the skeleton. It is involved in the maintenance of acid-base balance in the extracellular fluid. In the intracellular compartment, phosphate participates in the metabolism of organic substances and energy. It is an integral component of all glycolytic compounds from glucose-1-phosphate to phosphoenolpyruvate. It is a part of the structure of high-energy transfer compounds, such as ATP and creatine phosphate. It is also a part of the cofactors such as NAD, NADP, and thiamine pyrophosphate. It functions as a covalent modifier of numerous enzymes. Phosphate plays an important role in the structure and function of lipid membranes. Cell membranes are composed of lipids such as phosphatidylcholine. The phosphorylation of membrane receptors and other molecules is now believed to play a crucial role in transducing signals across the membrane. Thus, the phosphate ion is of critical importance to virtually all biological systems.

I.1.2 Turnover of phosphate in the body

The normal concentration of inorganic phosphate (Pi) in human plasma varies within a narrow range from 2.5 to 4.5 mg/dl (0.81 to 1.45 mM). The total plasma Pi consists of divalent or dibasic (HPO_4^{2-}) and monovalent or monobasic ($H_2PO_4^-$) forms. The valence of Pi changes with pH. At pH 7.4, the ratio of divalent Pi to monovalent Pi is 4:1 (Mizgala and Quamme, 1985; Genuth, 1988).

The turnover of Pi is controlled mainly by three organs: the intestine, the skeleton and the kidney, as illustrated in Figure 1. In the small intestine, Pi is absorbed from the diet at a relatively constant percentage. Thus, net intestinal absorption of Pi is linearly related to intake over a wide range, and adaptive regulation at this site is conventionally considered of minor importance. However, recent studies demonstrated that the intestinal Pi absorption systems share some common properties (the transporters, their regulations, etc.) with the renal Pi absorption systems (Field *et al.*, 1999). In contrast to intestinal Pi absorption, renal Pi absorption is saturable. Therefore, **renal handling of Pi provides the major mechanism for preserving Pi balance** (Genuth 1988; Murer and Biber 1992).

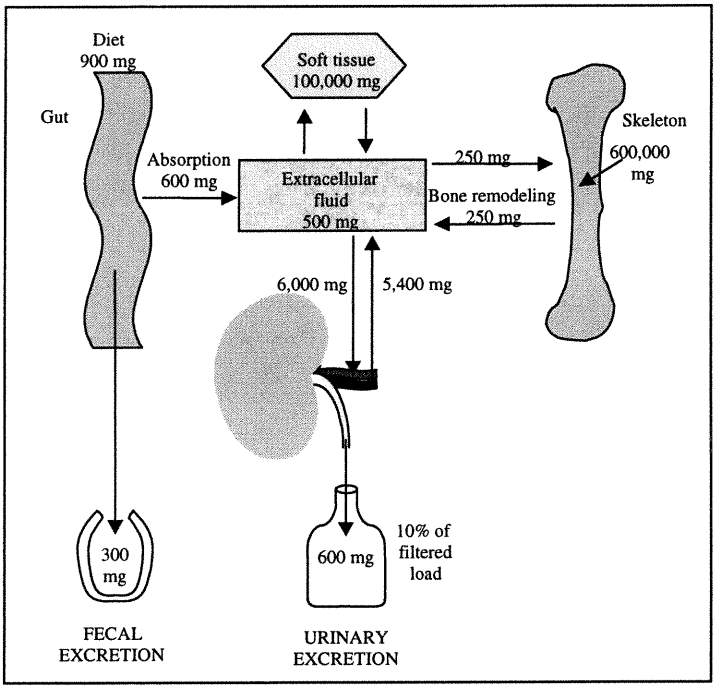

Fig. 1. Average daily phosphate turnover in human. Pi is absorbed in the gut, distributed mainly in the bone, soft tissues and extracellular fluid. The kidney reabsorbs most of the filtered Pi into blood, and is the most important organ for the maintenance of Pi balance. Adapted from Genuth 1988.

Of the daily filtered load of about 6000 mg (plasma concentration × glomerular filtration rate = 36 mg/L × 170 L/day), renal tubular reabsorption can vary from 70% to 100%, with an average of 90%. This provides the needed flexibility to compensate for large swings in dietary intake. Soft tissue stores of phosphate, such as in muscle, are quite large. Transfer between these stores and extracellular fluid is an important factor in minute-to-minute regulation of plasma phosphate concentration. About 250 mg, or half the total extracellular fluid pool of 500 mg, enters and leaves the bone mass daily in the process of bone remodeling (Genuth 1988). Because renal handling of Pi is so important in Pi balance, most of the clinical abnormalities related to Pi are due to over-excretion of Pi in the kidney. For example, in patients with X chromosome-Linked Hypophosphatemia (XLH) characterized by rachitic bone disease, growth retardation and hypophosphatemia, the reduced plasma Pi is a consequence of increased renal excretion of Pi (Rasmussen and Tenenhouse 1995).

To maintain a normal structure and function of all biological systems the Pi concentration in the extracellular fluid must be stabilized within a very narrow range. This

stabilization of Pi concentration is often called **Pi homeostasis**. Homeostasis is a term coined by the American physiologist Walter B. Cannon (1871-1945) to describe "the various physiologic arrangements which serve to restore the normal state, once it has been disturbed". Like those of many other extracellular components, Pi homeostasis is under the tight control of neuronal and humoral mechanisms. Although Pi homeostasis has been studied for nearly a century, the regulation of Pi at the molecular level began to become clear only recently, owing to the advances in molecular biology. In the following sections, we will give an overview of renal Pi transport and then concentrate on recent developments in this area.

I.2 Renal physiology of phosphate transport

I.2.1 Structure and function of the kidney

Mammalian kidneys are either unipapillary kidneys (as in rat or rabbit) or multilobar kidneys (as in humans) (Figure 2). The unipapillary kidney corresponds to a renculus of a multilobar kidney. A kidney has two major discernable areas: the cortex and the medulla. The medulla is divided into an outer medulla and an inner medulla with the papilla, which can be regarded as a dilation of the ureter (Schnabel and Kriz 1995).

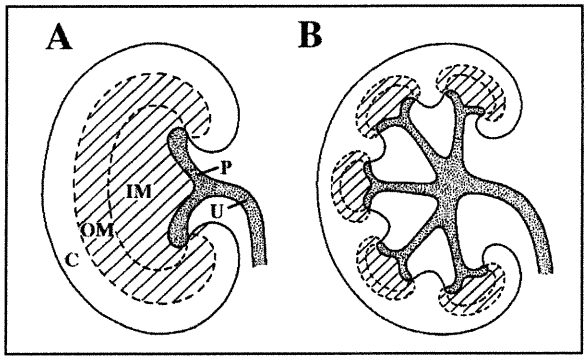

Figure 2. Different types of mammalian kidneys. **A)** Unipapillary kidney (rat) showing three major zone: cortex (C), outer medulla (OM), inner medulla (IM), pelvis (P) and ureter (U). **B)** Compound multilobar kidney (human). Individual medullary pyramids are shaded; calyces, pelvis, and ureter are stippled. In constrast to what is shown, the human kidney generally exihibits fusion of individual medullary pyramids terminating in a compound papilla. From Schnabel and Kriz, 1995

The functional unit of the kidney is a specific tubular structure called the nephron (Figure 3). A human kidney contains about 1 million nephrons. Each nephron starts with a renal corpuscle (Bowman's capsule and glomerulus), where the primary urine is filtered from the blood. This primary filtrate is then modified and concentrated within a long twisted tube

and finally drained through a collecting duct system into the renal pelvis. The tubular part of the nephron consists of a proximal and a distal convoluted tubule, which are connected by a loop (Henle's loop). The details of subdivisions are illustrated in Figure 3 (Schnabel and Kriz 1995).

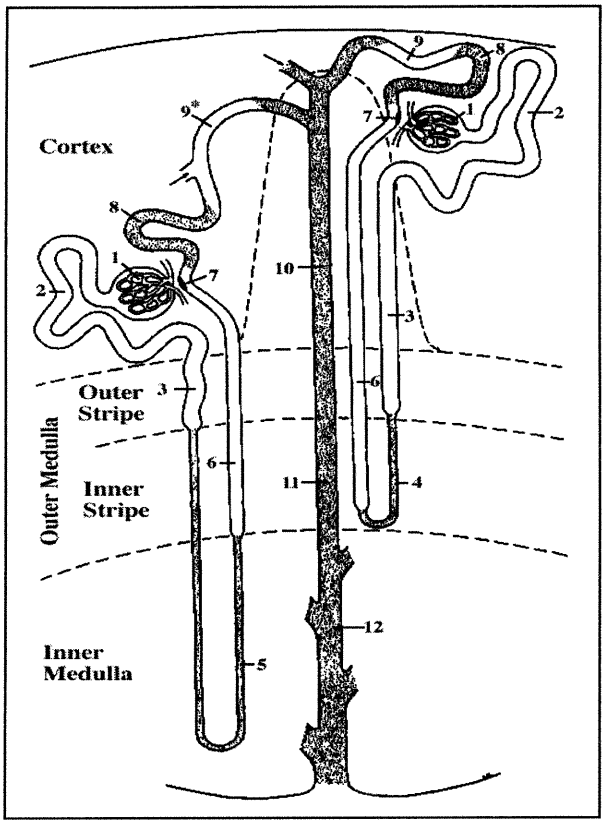

Figure 3. Schematic presentation of nephrons and collecting duct. This scheme depicts a short-looped and a long-looped nephron together with the collecting system (not drawn to scale). Within the cortex a medullary ray is delineated by a dashed line. **(1)** renal corpuscle including bowman's capsule and the glomerulus (glomerular tuft); **(2)** proximal convoluted tubule; **(3)** proximal straight tubule; **(4)** descending thin limb; **(5)** ascending thin limb; **(6)** distal straight tubule (thick ascending limb); **(7)** macula densa located within the final protion of the thick ascending limb; **(8)** distal concoluted tubule; **(9)**, connecting tubule; 9*, connecting tubule of the juxtamedullary nephron that forms an arcade; **(10)** cortical collecting duct; **(11)** outer medullary collecting duct; **(12)** inner medullary collecting duct. Schnabel and Kriz 1995

The proximal tubule is the crucial part of the nephron in Pi homeostasis. Histologically, the proximal tubule is divided into a straight part and a convoluted part. In its ultrastructure as seen under microscope, three segments (S1, S2, and S3) are generally distinguished. Within the second half of the convoluted part S1 cells are gradually replaced by S2 cells. The transition from S2 to S3 occurs along the straight part within the medullary rays and into the outer stripe. In humans, only two segments corresponding to S1 and S3 have been ultrastructurally characterized.

The major function of the kidney, i.e., reabsorption and excretion, is largely dependent on the specific ultrastructure of the proximal tubule. The most striking characteristic of the proximal tubule epithelium is the well-developed brush border composed of densely packed microvilli that greatly increases the luminal cell surface area (Figure 4). In the rat, the formation of the microvilli increases this area by a factor of 36! In renal physiology and biochemistry, the luminal microvilli-enriched membrane of the proximal tubule epithelia is usually called the brush border membrane (BBM). The BBM represents the morphologic basis of the proximal tubule to reabsorb the small compounds (peptides, organic solutes, glucose, Pi, etc.) that have been filtered through the glomerulus. The basolateral surface is also amplified by forming lateral cell processes extending radially from the central cell

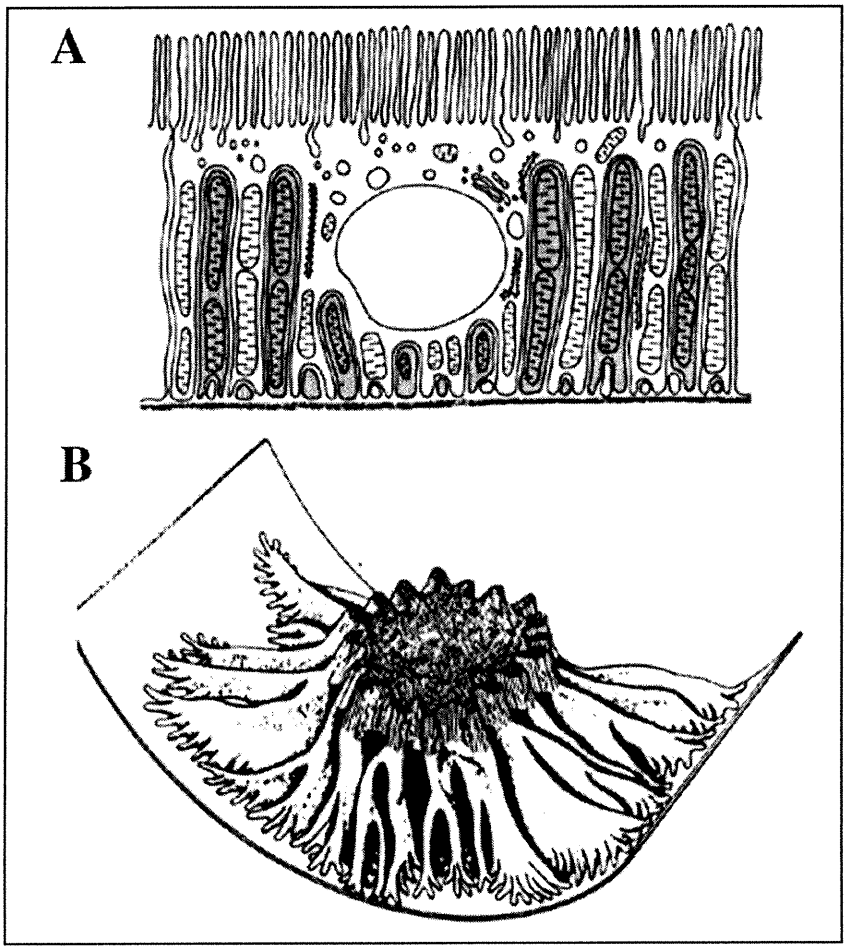

Figure 4. Schematics of proximal tubule epithelium. **(A)** Apicobasal section showing the apical brush border and the interdigitation of the cell bodies by large lateral processes. To demonstrate the cellular interdigitation, neighboring cells and their processes are stippled. The interdigitating processes are filled with mitochondria. **(B)** Three-dimensional model of a proximal tubule cell showing the large lateral processes. Schnabel and Kriz 1995

portion, interdigitating densely with those of neighboring cells (Figure 4). The intercellular spaces are separated from the luminal compartment by shallow tight junctions. Thus, the intercellular space is tightly sealed and is not permeable to most ions and molecules in the filtrate and the peritubular interstitium. These ions and molecules, e.g. Pi, can only be transported from one side of the cell membrane to the other through the transport systems located in the BBM or the basolateral membrane (BLM).

I.2.2 Pi reabsorption in the kidney

Because the kidney is the key organ in maintaining Pi homeostasis, numerous studies have been done to reveal the mechanisms underlying renal Pi handling (for reviews, see Mizgala and Quamme 1985; Murer and biber 1992). Earlier *in vivo* and *in vitro* micropuncture/microperfusion studies have demonstrated that the major site of renal Pi reabsorption is the proximal tubule, where 90% of the filtered Pi is reabsorbed, while the distal tubule reabsorbs only 5 to 10% (Amiel *et al.*, 1970; Greger *et al.*, 1977). In the proximal tubule, Pi in ultrafiltrate is transported unidirectionally from the lumen to the peritubular interstitium across the epithelial cell. Backflux of Pi from the interstitium to the lumen is negligible (Greger *et al.*, 1977a).

Proximal Pi transport is highly dependent on the presence of sodium ions (Na^+) in the tubular fluid (Baumann *et al.*, 1975). The electric potential inside the proximal tubule cell is 70 mV lower than that outside the cell, and the intracellular Pi concentration (2 mM) is higher than that in the extracellular fluid (1mM) (Rickard and Sheterline 1986). Studies using isolated BBM vesicles have established that the Na^+ electrochemical gradient across the BBM provides the driving force for active accumulation of Pi in the cell (Hoffmann *et al.*, 1976). The concensus is that Na^+ moves down its electrochemical gradient coupled to Pi, which moves against its chemical and electrical gradients, a process called secondary active transport. The driving force is the favorable electrochemical gradient for passive Na^+ movement that is ultimately maintained by the ATP-dependent active sodium pump located on the BLM. Exit of Pi from the cell into the peritubular interstitium across the BLM is by diffusion since the Pi moves down its electrochemical gradient. Therefore, **Na^+/Pi cotransport across the BBM is the rate-limiting step** in transcellular Pi movement at the proximal tubule. Of the two species of Pi, the divalent form is preferentially transported, although the monovalent Pi is also accepted by the same transport system in the BBM under

physiological conditions. Figure 5 summarizes the cellular mechanism of renal Na^+/Pi cotransport as established in earlier physiological studies.

The saturability of renal Na^+/Pi cotransport as observed in earlier studies suggests the existence of a specific cellular reabsorptive mechanism for Pi. However, the identity of the transport system has been understood only recently, owing to the development of molecular cloning techniques, as will be discussed next.

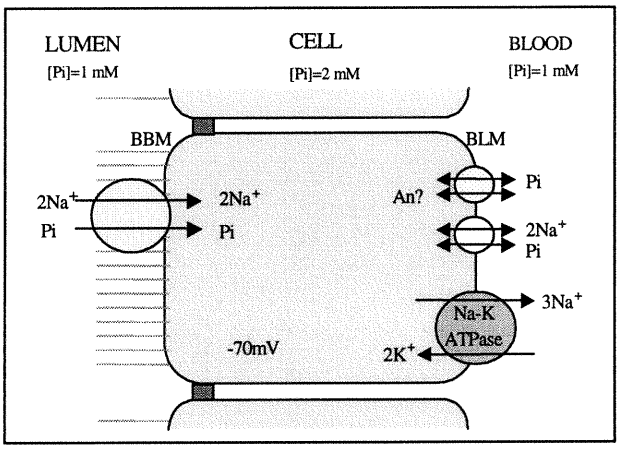

Figure 5. A schematic illustration of Pi transport across the proximal tubule. The major entry mechanism, which occurs at the BBM, is Na gradient dependent uptake, with a Na:Pi stoichiometry of 2:1. Pi exit at the BLM is mainly faciliated by different equilibrating transport systems with high capacity. Adapted from Murer *et al*, 1992 and Mizgala and Quamme 1985.

I.3 Approaches toward the identification of renal Na^+/Pi cotransporters

Many efforts have been made toward the molecular identification of the renal Na^+/Pi cotransport systems. Earlier studies used mainly indirect approaches, including reconstitution in liposomes of partially purified proteins (Debiec *et al.*, 1988; Schaeli *et al.*, 1986), labeling of gel electrophoresed proteins with competitive Na^+/Pi cotransport inhibitors such as N-acetylimidazole, phosphonoformic acid etc. (Al-Mahrouq *et al.*, 1991; Wuarin *et al.*, 1989), and molecular mass estimation based on radiation inactivation analysis on isolated renal brush border membrane vesicles (BBMV) (Béliveau *et al.*, 1988). Although these studies described the nature of the cotransporter as integral membrane proteins and their functional unit as oligomers, no further information about the Na^+/Pi cotransporter was available.

The identification of the small intestinal Na^+/D-glucose cotransporter by expression cloning (Hediger *et al.*, 1987) started a new era in the exploration of transmembrane transport

systems. Later, Murer and co-workers adapted this technique to identify the renal proximal tubule BBM Na^+/Pi cotransporters (Murer 1992). The essential steps of the expression cloning strategy are: (1) isolation of kidney cortical mRNAs. These mRNAs are expressed in *Xenopus laevis* occytes and the Na^+/Pi uptake activity is analyzed; (2) size fractionation of the mRNAs by gel electrophoresis or density gradient centrifugation. The fractions effective in Na^+/Pi cotransport are selected based on functional analysis after expression in oocytes; (3) construction of a cDNA (complementary DNA) library. The cDNA library is established by the reverse transcription of the size-fractionated mRNAs. This library is transcribed into cRNA for subsequent functional analysis in oocytes; (4) isolation of a single clone using sib selection protocols employing dilutions/replatings/replicas as well as growth/isolation of plasmids and *in vitro* transcription of cRNA at all steps. With this strategy, the renal proximal tubule BBM Na^+/Pi cotransporter in rabbit (named NaPi-1) was identified for the first time in 1991 (Werner *et al.*, 1991).

Shortly after the molecular identification of the rabbit renal Na^+/Pi cotransporter NaPi-1, many other renal Na^+/Pi cotransporters were identified. Using the rabbit kidney NaPi-1 cDNA as probe, the rat analogues of the Na^+/Pi cotransporters were cloned and designated RNaPi-1a and RNaPi-1b (Li and Xie 1995). By screening the cDNA libraries constructed from rat kidney cortex and human kidney cortex, the rat and human renal Na^+/Pi cotransporter were identified and designated as NaPi-2 and NaPi-3, respectively (Magagnin *et al.*, 1993). Additional Na^+/Pi cotransporters were identified by homology screening using a NaPi-2 cDNA probe: NaPi-4 from OK cells (Sorribas *et al.*, 1994), NaPi-5 from flounder kidney (Werner *et al.*, 1994), NaPi-6 from rabbit cortex (Verri *et al.*, 1995), NaPi-7 from mouse kidney cortex (Collins *et al.*, 1994; Hartmann *et al.*, 1995), and a Na^+/Pi cotransporter from bovine renal epithelial cell line NBL-1 (Helps *et al.*, 1995). So far, at least 17 renal Na^+/Pi cotransporters have been identified in different animals from fish to human. These cotransporters are identical neither in molecular structure nor in physiological function.

I.4. Classification of Na^+/Pi cotransporters

The general informations about the different types of renal Na^+/Pi cotransporters are summarized in Table I. It should be pointed out that there is no general standard for classification of Na^+/Pi cotransporters. The commonly used is structural classification based on a comparison of amino acid sequences derived from the nucleotide sequences of the

cDNAs. Functional classification with terms such as "regulated", "housekeeping", "constitutional" or "renal specific" has been used in the literature, but these terms are not clearly defined. Comparison of nucleotide sequences reveals that the Na^+/Pi cotransport systems NaPi-1 and NaPi-2/3 share only 20% overall homology, while NaPi-2 and NaPi-3 are 81% identical and 95% homologous (Magagnin *et al.*, 1993). Thus NaPi-1 and NaPi-2/3 represent structurally distinct types of Na^+/Pi cotransporters, and were classified as type I (NaPi-1-related) and type II (NaPi-2-related) Na^+/Pi cotransporters, respectively (Murer and Biber 1994).

Shortly after, several members of each type were identified. The type I cotransporters include NPT1 from human kidney cortex showing 81.5% homology to rabbit NaPi-1 (Chong *et al.*, 1993; Miyamoto *et al.*, 1995), Npt1 from mouse kidney showing high homology to NaPi-1 and NPT1 (Chong *et al.*, 1995), RNaPi-1a and RNaPi-1b from rat liver (Li and Xie 1995), and BNPI from rat brain (Ni *et al.*, 1994). In addition to NaPi-2 and NaPi-3, the other type II Na^+/Pi cotransporters include NaPi-4 from OK cell lines showing approximately 89% homology to NaPi-2 or NaPi-3 (Sorribas *et al.*, 1994), NaPi-5 from flounder kidney and intestine with 92% homology to NaPi-2 (Werner *et al.*, 1994), NaPi-6 from rabbit kidney cortex with 80-90% identity to NaPi-2/3/4 (Verri *et al.*, 1995), NaPi-7 from mouse with 98% identity to NaPi-2 (Collins and Ghishan, 1994), the Na^+/Pi cotransporter from bovine renal epithelial cell line (BNL-1) with 70% similarity to NaPi-2 and NaPi-3 (Helps *et al.*, 1995), a frog intestinal Na^+/Pi cotransporter with high degree homology to NaPi-3 (Ishizuya-Oka *et al.*, 1997), a murine intestinal Na^+/Pi cotransporter with 57 to 75% homology to mammalian and nonmammalian type II cotransporters (Hilfiker *et al.*, 1998), and human NaPi-3b from human intestine and lung with 78% identity to the murine intestinal Na^+/Pi cotransporter (Field *et al.*, 1999). Recently three isoforms for NaPi-2 have been discovered in rat kidney cortex, each representing only a part of the entire NaPi-2 polypeptide (Tatsumi *et al.*, 1998).

The proposal that the type II Na^+/Pi cotransporters be divided into two sub-groups has been raised because the highly homologous NaPi-5 and NBL-1 share amino acid sequences obviously different from that of NaPi-2, NaPi-3 or NaPi-4 (Helps *et al.*, 1995). Recently, the type II cotransporters were further divided into two subtypes according to the presence of clusters of cysteine residues in the C-terminal regions of their amino acid sequences.

Table I. Summary and classification of Na^+/Pi cotransporters

Name	Length of nucleotide (bp)	Length of polypeptide (aa)	Species or cell lines	Source of cDNA library	References
Type I cotransporters					
NaPi-1	1856	465	Rabbit	Kidney cortex	Werner *et al.*, 1991
PT1	1549	467	Human	Kidney cortex	Chong *et al.*. 1993
Npt1	1885	465	Mouse	Kidney cortex	Chong *et al.*, 1995
RNaPi-1a	2.3 kb 1.8 kb	465	Rat	Rat liver	Li and Xie, 1995
RNaPi-1b	1.8 kb	465	Rat	Rat liver	Li and Xie, 1995
BNPI	2.0 kb	560	Rat	Rat brain	Ni *et al.*, 1994
Type II cotransporters					
Type IIa					
NaPi-2	2464	637	Rat	Kidney cortex	Magagnin *et al.*., 1993
NaPi-2α	2389	337	Rat	Kidney cortex	Tatsumi *et al.*, 1998
NaPi-2β	1166	327	Rat	Kidney cortex	Tatsumi *et al.*, 1998
NaPi-2γ	1961	268	Rat	Kidney cortex	Tatsumi *et al.*, 1998
NaPi-3	2573	639	Human	Kidney cortex	Magagnin *et al.*, 1993
NaPi-4	2548	653	OK cell	Opossum kidney tubule	Sorribas *et al.*, 1994
NaPi-6	2462	642	Rabbit	Kidney cortex	Verri *et al.*, 1995
NaPi-7	2423	637	Mouse	Kidney cortex	Genbank gi:726321
P78-1	2401	637	Mouse	Kidney cortex	Collins and Ghishan, 1994
Type IIb					
NaPi-5	2424	637	Flounder	Kidney erythro-poietic tissue	Werner *et al.*, 1994
Bovine renal epitheial cell	2.2 kb	693	NBL-1 cell	Renal epithelial cells	Helps *et al.*, 1995
Human NaPi-3b	2073		Human	Human small intestine and lung	Field *et al.*, 1999
Xenopus intestine	2185/4660	674	Xenopus	Xenopus intestine	Ishizuya-Oka *et al.*, 1997
Murine intestinal Type IIb	4039	697	Mouse	Mammalian small intestine	Hilfiker *et al.*, 1998
Type III cotransporters					
Glvr-1 / PiT-1		679	Human	Human cDNA	O'Hara *et al.*, 1990
Ram-1 / PiT-2		656	Rat	Rat cDNA	Miller *et al.*, 1994

Those without the C-terminal cysteine residue clusters are sub-classified as type IIa, including NaPi-2, NaPi-3, NaPi-4, NaPi-6, and NaPi-7. Those with the C-terminal clusters are sub-classified as type IIb, including the ones identified in BNL-1 cell lines, *Xenopus* intestine, murine small intestine, as well as the flounder NaPi-5 (Hilfiker *et al.*, 1998). This classification could explain the different phosphate transport kinetics (*Vmax* and *Km*) observed in BNL-1 cell and OK cells (Helps *et al.*, 1995).

At the time the type I and type II cotransporters were described, the cell-surface receptors for gibbon ape leukemia virus (Glvr-1) and rat amphotropic virus (Ram-1) were demonstrated to be sodium-dependent phosphate transporters (Kavanaugh *et al.*, 1994; Olah *et al.*, 1994). Since they exhibit no significant overall sequence homology with the type I or type II Na^+/Pi cotransporters, they were classified as type III Na^+/Pi cotransporters (Kavanaugh and Kabat 1996).

I.5. Structures of different types of Na^+/Pi cotransporters

I.5.1 Structure of type I Na^+/Pi cotransporters

Type I Na^+/Pi cotransporters, e.g. NaPi-1, are about 465-amino acids in length, with a predicted unglycosylated mass of about 55 kDa (Werner *et al.*, 1991). Based on hydropathy analysis (Kyte and Doolittle 1982), the type I Na^+/Pi cotransporters have 6 to 9 transmembrane (TM) domains. The amino (N)-terminus is predicted to be located intracellularly, while the carboxyl (C)-terminus is located on the cell surface, i.e., facing the lumen of the proximal tubule (Werner *et al.*, 1991). There are two large extracellular loops, one is located between TM1 and TM2 and the other between TM5 and TM6. Three N-glycosylation sites (concensus sequence: NXS/T, where X ≠ proline. Kornfeld and Kornfeld 1985) are found in the first extracellular loop. Two potential protein kinase C (PKC) phosphorylation sites are conserved in the type I cotransporters.

The secondary structure of type I Na^+/Pi cotransporters is represented in Figure 6. The sodium-binding motif (SOB motif) is the concensus amino acid sequence (G-(L)-X20-40-A-XXXX-L-XXX-G-R-) involved in the binding of sodium in some mammalian and nonmammalian Na^+-dependent transport systems identified so far (Deguchi *et al.*, 1990). This SOB motif is also observed in NaPi-1 (Werner *et al.*, 1991).

I.5.2 Structure of type II Na^+/Pi cotransporters

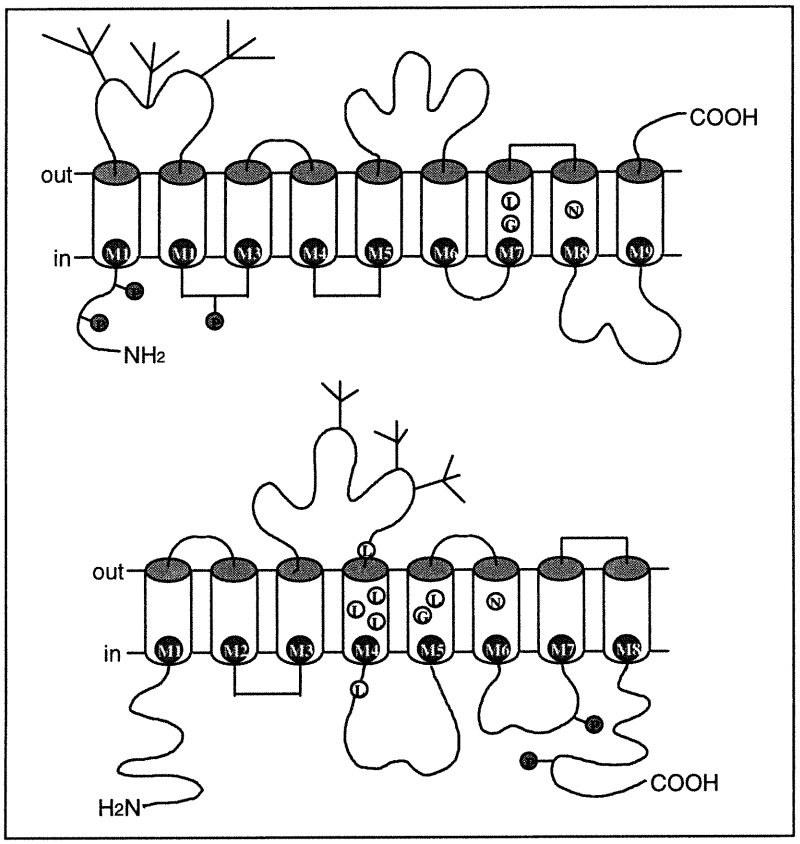

Figure 6. Proposed secondary structure of type I (upper schematic) and type II (lower schematic) renal Na^+/Pi cotransporters. G and L indicate the start of the SOB motif, N represents the N-glycosylation site residing in a hydrophobic environment and the circled P stands for concensus PKC phosphorylation sites. The second N-glycosylation site of the type II transporters is not found in the NaPi-2 system. The five Ls that reside in and close to the M4 of type II transporter represent the leucine zipper motif. The branched structures illustrate extracellular N-glycosylation sites. (Adapted from Biber and Murer 1994)

Type II Na^+/Pi cotransporters, NaPi-2, NaPi-3, and NaPi-4, for examples, are composed of about 640 amino acids, with unglycosylated molecular weight of about 70 kDa (Magagnin *et al.*, 1993; Sorribas *et al.*, 1994). Hydropathy analysis predicts 8 transmembrane domains within which the transporters share the highest similarity in their amino acid sequences (Figure 6). A leucine zipper motif is found in the type II cotransporters (TM4) but missing in the type I transporters (Sorribas *et al.*, 1994). Since leucine zippers have been proposed to facilitate protein dimerization (Landschulz *et al.*, 1988), the presence of this motif in the type II cotransporters suggests that these cotransporters may exist as oligomers. The SOB motives are also found in type II cotransporters and might explain the previous observation that the renal Pi transport is Na^+-dependent (Sorribas *et al.*, 1994). As described

earlier, the type IIb cotransporters differ from the type IIa cotransporters by possessing clusters of cystein residues in their C-terminal domains (Hilfiker *et al.*, 1998).

I.5.3 Structure of type III Na/Pi cotransporters

Hydropathy analysis predicts that Glvr-1 protein has 10 transmembrane domains with both N- and C-termini located intracellularly (O'Hara *et al.*, 1990; Boyer *et al.*, 1998). The hypothetical model of the secondary structure of this protein is shown in Figure 7.

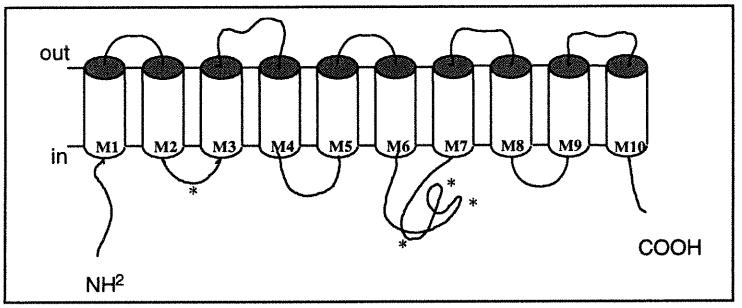

Figure 7. Hypothetical topographic representation of Glvr-1 in the membrane based on the secondary structure of Glvr-1 proposed by Johann et al. (Johann *et al.*, 1992) and on the analysis of the primary sequence using the MacVector software. According to this model, Glvr-1 could possess up to ten transmembrane domains with both N- and C-termini segments facing the cytoplasmic side of the membrane. Potential N-glycosylation sites are indicated by asterisks (*). Adapted from Boyer *et al*, 1998.

I.6 Tissue, cellular and subcellular distribution of Na^+/Pi cotransporters

I.6.1 Type I Na^+/Pi cotransporters

Northern blot analysis of various tissues from rat, rabbit, and human indicated that NaPi-1 is predominantly expressed in the kidney cortex, with a little in the liver (Biber and Murer 1994; Ghishan *et al.*, 1993; Werner *et al.*, 1991). Detailed analysis with microdissected single nephron segments and reverse transcription polymerase chain reaction (RT-PCR) demonstrated uniform expression of NaPi-1 mRNA throughout the rabbit proximal tubule. No differences in the expression of NaPi-1 mRNA between proximal tubules of superficial and juxtamedullary nephrons could be detected (Custer *et al.*, 1993). Immunohistochemical analysis using antibody raised against the C-terminal segment of NaPi-1 protein indicated that NaPi-1-immunoreactive protein is located predominantly at the BBM of the rabbit proximal tubule (Biber *et al.*, 1993). With Western blotting analysis, polyclonal antibodies raised against the 14-amino acid C-terminal segment of NaPi-1 recognized a 65 kDa protein in rat

kidney BBM, proteins of 50, 55, and 64 kDa in rabbit kidney BBM and major bands at 38 and 66 kDa in beef kidney BBM (Delisle *et al.*, 1994). In rat kidney, NaPi-1 is expressed only in the isolated BBM fraction, no immunoreactive signal was detected in BLM, cytosol, or microsomal compartments of the kidney cortex (Delisle *et al.*, 1994).

In mouse kidney, Npt1 mRNA was localized predominantly to the proximal tubule by both *in situ* hybridization and RT-PCR (Chong *et al.*, 1995). The distal tubule may be a minor site of Npt1 expression since its mRNA has also been detected in an immortalized mouse distal convoluted tubule cell line (MDCT) using RT-PCR (Tenenhouse *et al.*, 1998). Other members of type I cotransporters are mainly expressed extra-renally. RNaPi-1a/1b mRNAs have a broad pattern of tissue expression: they are mainly expressed in the liver, but are also found in kidney, heart, brain, and skeletal muscle (Li and Xie 1995). The rat brain-specific Na^+/Pi cotransporter BNPI is predominantly expressed in the brain, mostly in neurons of the cerebral cortex, hippocampus, and cerebellum, as well as in cultured cerebellar granule cells (Ni *et al.*, 1994).

I.6.2 Type II Na^+/Pi cotransporters

Northern blot analysis demonstrated that in rat the corresponding NaPi-2 mRNA species (approximately 2.7 kb) are expressed almost exclusively in kidney cortex. No hybridization with mRNAs isolated from a variety of other tissues (including intestinal segments, skeletal muscle, heart, brain, lung, liver) was observed (Magagnin *et al.*, 1993). In other species such as mouse, rabbit, pig, and human, NaPi-2 mRNA was also observed in kidney cortex. NaPi-3 mRNA has a similar tissue and species distribution, except that a 4.8 kb NaPi-3 mRNA was also found in the human lung (Magagnin *et al.*, 1993). RT-PCR and *in situ* hybridization analysis demonstrated uniform expression of NaPi-2 mRNA in the rat proximal tubule (Custer *et al.*, 1994). A small amount of NaPi-2 mRNA was also detected in collecting ducts, but immunohistochemical analysis did not show the presence of the type II protein there, suggesting that the protein is not expressed or the level of expression is very low (Custer *et al.*, 1994).

In contrast to the uniform expression of NaPi-1 throughout the kidney cortex (Custer *et al.*, 1993), NaPi-2 protein is distributed more heterogeneously. In terms of the cortex, the juxtamedullary section expresses more NaPi-2 than the superficial section. In terms of each nephron, the strongest NaPi-2-immunoreactive signal is observed in S1 segments of deep

nephrons, to a lesser extent in straight proximal tubules. Finally, in terms of the tubular epithelia, NaPi-2 expression is restricted to the BBM but not the BLM compartment (Custer *et al.*, 1994). In OK cells transfected with NaPi-2 cDNA, the expressed NaPi-2 proteins are localized only in the apical membrane, similar to the native Na^+/Pi cotransporter NaPi-4 of OK cell (Pfister *et al.*, 1997). Immunoelectron microscopic study demonstrated that NaPi-2 cotransporter is uniformly distributed along the microvilli (Murer and Biber 1996). Because of its highly restricted BBM expression in renal proximal tubule, NaPi-2 has been taken as a specific marker of BBM in some recent renal histological studies (Biemesderfer *et al.*, 1999; Schmitt *et al.*, 1999).

The mRNA of the flounder type II Na^+/Pi cotransporter NaPi-5 is expressed in the kidney, intestine and erythropoietic tissue. No NaPi-5 mRNA was detected in rat and shark kidneys (Werner *et al.*, 1994). The renal and the intestinal species of NaPi-5 mRNA encode the same type II Na^+/Pi cotransporter (NaPi-5). Interestingly, immunofluorescence revealed different subcellular expression of NaPi-5 protein. In the renal proximal tubule NaPi-5 protein is localized in the BLM, while in intestinal mucosa it is localized in the apical membrane (Kohl *et al.*, 1996). The mechanisms underlying the differential expression of NaPi-5 protein in different subcellular compartments of epithelial cells are unknown.

Although NaPi-6 has been detected in the rabbit renal BBM (Verri *et al.*, 1995), details of its histological distribution have not been investigated. We can only assume that NaPi-6 might be expressed only in the proximal tubules in the same way as NaPi-2, since their amino acid sequences are nearly 90% identical and both are regulated similarly by dietary Pi (Verri *et al.*, 1995). The mRNA of mouse type II Na^+/Pi cotransporter NaPi-7 is expressed in the renal proximal tubule as analyzed with *in situ* hybridization (Collins and Ghishan 1994). Although it hasn't been studied, NaPi-7 is most likely distributed in the same way as NaPi-2, as both proteins share 98% identity in their amino acid sequence (Collins and Ghishan 1994).

The recently identified murine intestinal type IIb Na^+/Pi cotransporter has a wide pattern of tissue distribution. It is expressed in the mucosa of the upper small intestine, colon, liver, lung, kidney and testis, as tested with RT-PCR. In contrast, the type IIa cotransporter NaPi-7 was only found in mRNA isolated from kidney (Hilfiker *et al.*, 1998). Immunofluorescence demonstrated that the type IIb cotransporter is expressed in the apical

membrane of enterocytes (Hilfiker *et al.*, 1998), indicating its role in intestinal Pi absorption. The newly reported human NaPi-3b is expressed most abundantly in the lung, and to a lesser degree, in several other tissues of epithelial origin including small intestine, pancreas, prostate, and kidney (Field *et al.*, 1999).

In summary, as the number of the type II Na^+/Pi cotransporters increased, more diversity was found in their structures and tissue distribution patterns. Unlike the "classical" type II cotransporters such as NaPi-2/3 that are distributed "exclusively" in renal proximal tubules, the newly identified members have been found in other tissues as well. However, regardless of their tissue distributions, all members of the type II cotransporter family are expressed in epithelial cells. This tissue expression profile implies that the type II cotransporters are the major transporters in trans-epithelial Pi transport and thus in overall Pi homeostasis. By contrast, the universal expression of the type I Na^+/Pi cotransporters in both epithelial as well as non-epithelial tissues suggests that they are meanly housekeeping Pi transporters.

I.6.3 Type III Na^+/Pi cotransporters

In contrast to type I and type II cotransporters, both Glvr-1 and Ram-1 exhibit a broad pattern of tissue distribution. The transcripts of both transporters were found in relatively high abundance in total mRNA prepared from kidney (Kavanaugh *et al.*, 1994). High levels of expression were also seen in tissues outside the kidney, including liver, lung, striated muscle, heart, and brain. The relative abundance of the two type III cotransporters varies in different tissues. The greatest difference is seen in heart, which expresses Ram-1 in higher abundance, and in bone marrow, where the expression of Glvr-1 is higher. Each transcript of Glvr-1 or Ram-1 is also abundantly expressed in rat fibroblasts (Kavanaugh *et al.*, 1994). In bone, Glvr-1 was found in osteoblast-like cells (Palmer *et al.*, 1997). Western blot analysis also showed a broad expression of Glvr-1 protein (Boyer *et al.*, 1998). The subcellular distribution of type III cotransporters in epithelial cells has not been elucidated.

I.7. Role of different types of Na^+/Pi cotransporters

I.7.1 Type I Na^+/Pi cotransporters

Expression of type I cotransporters in *Xenopus* oocytes as well as in MDCK cells resulted in elevated Na^+/Pi uptake in those cells (Quabius *et al.*, 1995; Werner *et al.*, 1990; Werner *et al.*, 1991), demonstrating that these transporters are indeed Na^+/Pi cotransporters. Recent electrophysiological studies revealed that type I transporters produced electrogenic transport only at high extracellular Pi concentrations (≥3 mM). On the other hand, expression of NaPi-1 induced a Cl^- conductance in *Xenopus laevis* oocytes, which was inhibited by Cl^- channel blockers and the organic anions phenol red, benzylpenicillin (penicillin G), and probenecid. These results indicate that the type I Na^+/Pi cotransporter could also serve an important role in the transport of Cl^- and the excretion of anionic xenobiotics in the kidney (Busch *et al.*, 1996; Busch *et al.*, 1996).

Since renal Na^+/Pi cotransport is under the control of dietary Pi content, effect of low phosphate diet (LPD) on the expression of type I Na^+/Pi cotransporter has been investigated in several studies. In rabbit adapted to LPD for 7 days, the abundance of NaPi-1 mRNA in the cortex and NaPi-1 protein in BBM was not changed (Biber *et al.*, 1993). In comparison, the type II Na^+/Pi cotransporter was upregulated by LPD (Verri *et al.*, 1995). In BBM of rats fed with LPD the NaPi-1 immunoreactive signal was not changed although the Na^+/Pi cotransport activity was increased (Delisle *et al.*, 1994).

Thus, the above studies clearly indicate that the type I Na^+/Pi cotransporters are not involved in Pi homeostasis. Currently these transporters are considered as the housekeeping Pi transporters in a number of tissues. Since they also function as anion channels for xenobiotics, they might be involved in excretion in the kidney proximal tubules.

Interestingly, recent reports indicated that the brain specific type I Na^+/Pi cotransporter BNPi is localized to the synaptic vesicles in the brain and to intracellular membranes in transfected cells, and also functions as glutamate transporter (Bellocchio et al., 1998, Bellocchio et al., 2000). This suggests that it is also involved in the process of neuroexcitation.

I.7.2 Type II Na^+/Pi cotransporters

The type II Na^+/Pi cotransporters are well qualified to be those responsible for Pi homeostasis. The first evidence came from kinetic analysis of Pi uptake activity of the oocytes transfected with type II cotransporter (Magagnin *et al.*, 1993). The Pi transport induced by NaPi-2 is strictly Na^+-dependent with a sigmoidal model (Hill coefficient approximates 2), the apparent *Km* for Pi ranges from 0.1 to 0.3 mM, and the transport is pH dependent. All these characteristics are in good agreement with the Pi transport properties of the renal proximal tubules (Biber and Murer 1994). The exclusive localization of the mRNA to proximal tubules and restricted BBM expression of the protein strongly support the role of type II Na^+/Pi cotransporters in renal Pi handling.

Crucial evidence supporting the importance of type II cotransporters came from studies on the regulation of these transporters by dietary Pi content, PTH, as well as many other factors that will be discussed later (see Sections 9 to 13). Briefly, the increased Na^+/Pi cotransport activity in animals or cells by Pi deprivation, PTH restriction (thyroid-parathyroidectomy), etc. is associated with upregulation of type II Na^+/Pi cotransporters in the BBM or cell surface. On the contrary, the decreased Na^+/Pi cotransport activity in animals or cells by high Pi diet (HPD) or administration of PTH is associated with downregulation of these cotransporters in the BBM and cell surface. In addition, intravenous injection of antisense oligonucleotides of type II Na^+/Pi cotransporter in mice resulted in reduced specific mRNA and protein content, accompanied by decreased Na^+/Pi cotransport activity in isolated renal BBM vesicles (Oberbauer *et al.*, 1996). In *Hyp* mouse with hereditary hyperphosphaturia, the level of type II cotransporters is significantly lower than that in normal controls (Tenenhouse *et al.*, 1994). Gene inactivation of type II cotransporters in mice leads to retarded skeletal development (Beck *et al.*, 1995). These studies support the conclusion that the type II Na^+/Pi cotransporters are the regulated renal Na^+/Pi cotransporters that are responsible for Pi homeostasis.

I.7.3 Type III Na^+/Pi cotransporters

Originally described as retrovirus receptors (Kavanaugh *et al.*, 1995), the type III Na^+/Pi cotransporters are widely expressed in different tissues (Boyer *et al,* 1998) and are not found in BBM of renal proximal tubule (unpublished data). Both type I and type III

cotransporters are not target transporters for Pi homeostasis. They are most likely as housekeeping Pi transporters in the cells where they reside.

I.8. Molecular characterization of type II Na^+/Pi cotransporters

I.8.1 Topology

Type II Na^+/Pi cotransporters share a common predicted structure based on hydropathy analysis but experimental evidence in support of the predicted structure has not been available until recently. In one study, a 33-amino acid fragment containing a tag epitope was inserted into seven loops connecting the TMs and into the NH_2- and COOH- ends of the protein (Lambert *et al.*, 1999). The protein was expressed in oocytes and was detected with antibodies against the tag sequence. Using this method, it was shown that the NH_2- and the COOH- termini are located intracellularly while the loops connecting the TM1 and TM2 as well as TM3 and TM4 are located extracellularly, in agreement with the predicted structure of NaPi-2 protein (Murer and Biber 1994; Murer and Biber 1996). In another study the flounder type II Na^+/Pi cotransporter NaPi-5 was expressed in oocytes, followed by immunohistochemical determination of sideness of the inserted tags. As a result, epitopes within the loop between the TM3 and TM4 enhanced fluorescnece while no fluorescence was detected for the N- and C-terminal tags (Kohl *et al.*, 1998), indicating that the N- and C-termini are located intracellularly and the loop between TM3 and TM4 is located extracellularly. These results suggest that the two terminal domains are located inside the cell, while the loop between TM3 and TM4 is located on the cell surface.

I.8.2 Cleavage

Western blotting analysis indicated that in the presence of disulfide bond reducing agents (ß-EtSH, for example), the N-terminal antibody detected a polypeptide of 50 kDa, while the C-terminal antibody detected one of 40 kDa (Biber *et al.*, 1996). This suggests that NaPi-2 might be cleaved into two segments in the BBM. Results from our lab showed that the limited proteolysis profiles of the NaPi-2 and the 40-kDa C-terminal segment are identical, confirming that this C-terminal segment is a part of NaPi-2, and might result from the cleavage of NaPi-2 (Boyer *et al.*, 1996). However, the exact site of the cleavage and its physiological implications are not known.

I.8.3 Posttranslational modification

The predicted secondary structure of NaPi-2 exhibits several N-glycosylation concensus sites (NXS/T) (Figure 6). However, only residues Asn 298 and Asn 329 are used for N-glycosylation as proved with site-directed mutagenesis (Hayes *et al.*, 1994). The unglycosylated NaPi-2 transports Pi as well as the glycosylated form in terms of transport kinetics (*Km* and *Vmax*), but the rate of membrane insertion is slightly decreased (Hayes *et al.*, 1994). Mutation of Y402, a non-concensus glycosylation site, resulted in the unglycosylation and reduced surface expression of NaPi-2 (Hernando *et al.*, 1999).

I.8.4 Phosphorylation

Involvement of protein kinase C (PKC) and protein kinase A (PKA) mediated phosphorylation in the regulation of renal Na^+/Pi cotransport by PTH as well as other related agents was demonstrated before Na^+/Pi cotransporters were discovered (Mizgala and Quamme 1985; Murer *et al.*, 1992). The predicted structure of NaPi-2 protein possesses several PKC but no PKA concensus sites (Magagnin *et al.*, 1993) (Figure 6). Studies using polymutant NaPi-2 proteins expressed in oocytes showed that the Na^+/Pi cotransport properties (*Km, Vmax* and pH dependency) were unaffected by the removal of the PKC phosphorylation sites. In addition, PKC-mediated inhibition of Na^+/Pi cotransport by phorbol 12,13-didecanoate (PDD) was not prevented by the removal of the concensus sites. Activation of PKA with dibutyryl cAMP or forskolin had no effect on oocyte Na^+/Pi cotransport induced by NaPi-2. It is believed that the PKC-mediated regulation of Na^+/Pi cotransport does not involve the predicted concensus sites, thus a "cryptic" phosphorylation site or a phosphorylated "regulatory" protein may be involved in this regulation (Hayes *et al.*, 1995).

I.8.5 Intracellular trafficking

The mechanisms involved in the intracellular trafficking of NaPi-2 protein have been studied recently using site directed mutagenesis (Hernando *et al.*, 1999). Mutation of the two intracellular trafficking concensus sites, GY402FAM (GYXXZ) and Y509RWF (YXXO), abolished NaPi-2 mediated Pi-uptake after injection of NaPi-2 cRNA into oocytes. Mutation of Y402 resulted in “unglycosylation” and reduced surface expression, while the mutation of Y509 lead to a functional inactivation of the protein expressed in the plasma membrane. Pharmacological activation of the PKC cascade by diacylglycerol (DAG) induced the

retrieval of both wild-type and Y509 mutant cotransporters from the oocyte plasma membrane. These data suggest that the Y402 is important for the surface expression whereas Y509 is important for the function of type II Na^+/Pi cotransporters. Y509 seems not to be involved in the membrane retrieval of the cotransporter (Hernando *et al.*, 1999).

I.8.6 Functional size

Functional size refers to the molecular weight of the functional unit of a protein. The functional size of Na^+/Pi cotransporters in rat renal BBM has been studied extensively by radiation inactivation. This approach provides a mean of estimating the *in situ* functional molecular size of proteins. This makes it possible to study their structure/function relationships without purification or even solubilization of the transporters from the membrane (Harman *et al.,* 1985; Beliveau and Potier 1989). The radiation inactivation size (RIS) of rat BBM Na^+/Pi cotransporters obtained from the radiation-induced loss of transport activity, measured in the presence of a sodium gradient, was 234 kDa (Beliveau *et al.*, 1988a). This size is very close to the RISs of rabbit and mouse Na^+/Pi cotransporters (223 and 242 kDa, respectively) (Delisle *et al.*, 1994, Tenenhouse *et al.*, 1990). In rat, rabbits and mice, the type II Na^+/Pi cotransporters are the major phosphate transporter with predicted molecular weights of 68 kDa (Magagnin *et al.*, 1993), the functional units of these cotransporters should be at least dimers. The monomeric forms of rat BBM Na^+/Pi cotransporters could also be active phosphate transporters, since a RIS of 62 kDa has been determined under sodium equilibrium conditions (Jette *et al.*, 1996).

I.8.7 Involvement of disulfide bonds

When detected in the absence of disulfide bond reduction, NaPi-2 protein appeared as two polypeptides: one with an apparent MW of 180 kDa (named p180), the other with an apparent MW of 70 kDa (p70) (Biber *et al.*, 1996; Boyer *et al.*, 1996). Since NaPi-2 is a glycoprotein with a predicted unglycosylated size of 68 kDa (Magagnin *et al.*, 1993), the p70 must be the monomeric form of NaPi-2, while the p180 is probably the dimeric form of NaPi-2. When detected in the presence of reducing agents, such as ß-mercaptoethanol (ß-EtSH), two smaller polypeptides were revealed in addition to p70, and p180 disappeared. Using the N-terminal antibody, NaPi-2 is detected as p70 and a polypeptide of 45 kDa (p45); whereas using the C-terminal antibody, it is detected as p70 and a polypeptide of 40 kDa (p40). The

disappearance of p180 under reducing conditions indicates that p180 must be composed of p70s linked together via disulfide bonds. In fact, a number of cysteine residues that are the bases of disulfide bond formation are found in the amino acid sequence of NaPi-2 (Magagnin *et al.*, 1993). The appearance of p45 and p40 in the presence of reducing agents suggests that disulfide bonds exist between these two segments. Thus, disulfide bonds are involved in the formation of NaPi-2 oligomers and may influence Na^+/Pi cotransport activity.

I.8.8 Isoforms of NaPi-2

Recently three isoforms of NaPi-2 have been identified from a rat kidney cDNA library: NaPi-2α, NaPi-2β, and NaPi-2γ (Tatsumi *et al.*, 1998). NaPi-2α consists of 337 amino acids and is highly homologous to the N-terminal half of NaPi-2 containing 3 transmembrane domains. NaPi-2β consists of 327 amino acids identical to the N-terminal region of NaPi-2 containing 4 transmembrane domains, whereas the 146 amino acids in the C-terminal region are completely different. In contrast, NaPi-2γ consists of 268 amino acids identical to the C-terminal half of NaPi-2. Functional analysis demonstrated that NaPi-2γ and NaPi-2α markedly inhibited NaPi-2 activity in *Xenopus* oocytes, suggesting that these short isoforms may function as a dominant negative inhibitor of the full-length transporter.

I.9 Regulation of type II cotransporters by dietary phosphate

I.9.1 LPD

Earlier *in vivo* functional studies demonstrated that proximal tubular BBM Na^+/Pi cotransport adapts to LPD or HPD with increased or decreased Pi transport rate (Mizgala and Quamme 1985; Berndt *et al.*, 1992; Dennis 1982; Murer *et al.*, 1992; Murer *et al.*, 1991). These responses occur rapidly (within a few hours) and protein synthesis has been shown to be involved in this process (Kempson *et al.*, 1980).

Recent molecular biological studies revealed that type II but not type I cotransporters are involved in this adaptive response. In rabbits adapted to LPD for two weeks, the levels of NaPi-1 mRNA (detected with Northern blotting) in the cortex and NaPi-1 protein (detected with Western blotting) in isolated BBM were not changed, although injection of total mRNA from kidney cortex of LPD rabbits into *Xenopus laevis* oocytes resulted in a higher stimulation of Na-dependent oocyte Pi uptake (Biber *et al.*, 1993). Studies from our

laboratory showed that, in rats under LPD for three days, the abundance of NaPi-1 immunoreactive protein remained unchanged, although the initial rates of Na^+/Pi cotransport in BBMV from outer cortex and juxtamedullary cortex are increased by 3.4- and 2.0-fold, respectively (Delisle *et al.*, 1994). Studies by others compared the effect of LPD on type I and type II mRNAs and proteins in rabbit on LPD for one week (Verri *et al.*, 1995). The result showed that Na^+/Pi cotransport activity was increased by 2.5 fold with a 1.5 fold increase in NaPi-6 mRNA and a 4-fold increase in NaPi-6 protein in BBM, while NaPi-1 mRNA and protein remained unchanged (Verri *et al.*, 1995). Thus, type I Na^+/Pi cotransporters are not responsible for the LPD induced adaptive response in BBM Na^+/Pi cotransport in the kidneys of rabbits and rats. In contrast, the type II cotransporters have been shown to play a major role in this process.

Involvement of type II cotransporters in LPD-induced upregulation of Na^+/Pi cotransport has also been demonstrated in a number of other studies in animals and cell lines. In a study of the time course of the effect of LPD on the expression of NaPi-2 proteins in rats, we demonstrated that the level of NaPi-2 proteins increase 2-fold as early as 2 hours after

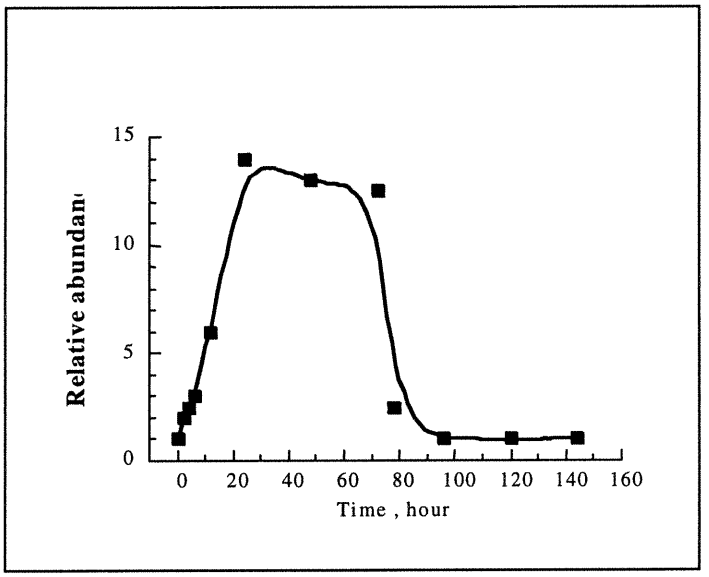

Figure 8. Effect of LPD and restoring normal Pi diet on the expression of NaPi-2 proteins in the renal proximal tubular BBM. Rats were subject to LPD for 72 hours and then were transferred to normal Pi diet from 72 hour to 144 hour. This figure is made with the data from our lab.

LPD feeding. This increase continued gradually, peaking in 24 hours, and remaing stable for 3 days. After switching from LPD to control diet, NaPi-2 protein expression decreased rapidly towards control levels in 6 hours and returned to control levels in 24 hours (Boyer *et al.*,

1996) (Figure 8). Immunohistochemistry experiments also showed that the level of NaPi-2 expression was significantly increased in the BBM of proximal tubules in rats switched to a LPD diet for 2 or 3 hours from a high Pi diet (Murer and Biber 1997; Murer *et al.*, 1996; Levi *et al.*, 1996). In rabbits, the type II Na^+/Pi cotransporter NaPi-6 was also found to be increased after one week of LPD (Verri *et al.*, 1995). In both normal and *Hyp* mice a 7-day LPD lead to increased BBMV Na^+/Pi cotransport accompanied by increased NaPi-2 protein expression and NaPi-2 mRNA abundance (Collins *et al.*, 1995; Tenenhouse *et al.*, 1995). In OK cells, expression of the type II Na^+/Pi cotransporter NaPi-4 was maximal 2-4 hours after removal of Pi from the culture media, in association with increased Na^+/Pi cotransport activity (Markovich *et al.*, 1995; Pfister *et al.*, 1998). These studies with different models indicate that (1) the type II Na^+/Pi cotransporters are responsible for Pi deprivation-induced increases in Na^+/Pi cotransport activity; (2) the response in the level of cotransporters occurs very rapidly and lasts as long as the Pi deprivation exists. Since *de novo* synthesis of the cotransporters takes a relatively long time, this is unlikely to mediate the early response of the animal or cell to Pi deprivation. Thus, the mechanism of the adaptive response might be different at different stages of LPD treatment.

Earlier studies in rats showed that short-term adaptation to Pi deprivation is independent of *de novo* protein synthesis or altered rate of transcription, since actinomycin D or cycloheximide did not prevent the increase in Na^+/Pi cotransport activity (Levine *et al.*, 1986). Adaptation to long-term (longer than 6 hours) LPD *in vivo* or Pi deprivation in cultured cells was abolished by inhibitors of transcription and protein synthesis (Caverzasio *et al.*, 1985, Biber *et al.*, 1988, Shah *et al.*, 1979).

Recent molecular biological studies demonstrated that short-term LPD and long-term LPD activate different adaptation mechanisms. In rats switched from high Pi diet to LPD, the abundance of BBM NaPi-2 proteins was markedly upregulated, while the level of NaPi-2 mRNA remained unchanged for the first two hours. Prolonged treatment with LPD increased both NaPi-2 protein and mRNA (Ritthaler *et al.*, 1999). Similar studies also showed that 2-h LPD increased BBM NaPi-2 protein by 1.8-fold without changes in mRNA abundance, while 7-day LPD increased NaPi-2 protein and mRNA by 4.9-fold and 2.2-fold, respectively (Levi *et al.*, 1994). In OK cells, Pi removal from the medium induced overexpression of NaPi-4 as well as NaPi-2 without changing the amount of mRNA (Pfister *et al.*, 1998). These studies suggest that the short-term response in Na^+/Pi cotransport does not involve transcriptional

changes of NaPi-2 cotransporters and translational or posttranslational mechanisms may play a role. In this regard, it has been shown that response in Na^+/Pi cotransport of OK cells to a low-Pi medium is significantly impaired in the presence of the microtubule disrupting agents nocodazole and colchicine. This suggests that insertion of Na^+/Pi cotransporters into the apical membrane is necessary for the adaptive response (Hansch *et al.*, 1993).

Immunohistochemical studies demonstrated that under normal Pi diet conditions, a large portion of NaPi-2 is distributed in subapical vesicles and other subcellular sites, while 2-h LPD rapidly recruited NaPi-2 to the BBM (Levi *et al.*, 1994). Enhanced insertion of proteins into the plasma membrane (exocytosis) in mediating acute regulation responses has been demonstrated for other transmembrane transport systems. For example, in adipocytes and muscle cells, the insulin-induced increase in glucose transport activity is closely correlated with enhanced translocation of the glucose transporters GLUT1 and GLUT4 to the plasma membrane (Gould and Holman 1993; Marette *et al.*, 1992; Marette *et al.*, 1992; Munoz *et al.*, 1995). Thus, one of the mechanisms involved in the acute response of proximal tubular cells to LPD could be the recruitment of pre-synthesized NaPi-2 proteins from intracellular vesicles to BBM.

As mentioned above, long-term adaptation to LPD in animals is mRNA-dependent. This means that long-term LPD may increase the amount of NaPi-2 by (1) increasing the rate of NaPi-2 gene transcription, (2) increasing NaPi-2 mRNA stability (decreasing NaPi-2 mRNA degradation) or (3) increasing the efficiency of mRNA translation (NaPi-2 protein synthesis). Studies in OK cells demonstrated that long-term (16h) Pi deprivation resulted in increased NaPi-4 mRNA abundance. This increase was attributed to increased NaPi-4 mRNA stability while the rate of gene transcription remained unchanged (Markovich *et al.*, 1995).

A role for type III Na^+/Pi cotransporters in the adaptive response to LPD can be ruled out. These transporters are not expressed in the BBM and their expression in the kidney cortex is unaltered after LPD treatment (unpublished data). In addition, studies from our laboratory showed that in OK cells exposed to Pi free medium, increased Pi transport occurs without changing Glvr-1 protein abundance (Boyer *et al.*, 1998). However, Glvr-1 and Ram-1 are regulated by extracellular Pi at both the mRNA and functional level in fibroblasts (Kavanaugh *et al.*, 1994). These studies suggest that in different tissues, the type III cotransporters may be under different control mechanisms.

Taken together, short-term LPD increases Na^+/Pi cotransport activity by enhancing the insertion (exocytosis) of presynthesized NaPi-2 proteins into the BBM without alteration in mRNA stability. Long-term LPD increases Na^+/Pi cotransport activity by increasing the *de novo* NaPi-2 synthesis via increased NaPi-2 mRNA stability without altered NaPi-2 gene transcription.

The effect of hypophosphatemia on the expression of NaPi-2 protein has recently been demonstrated. In rats with hypophosphatemia, some renal hypophosphatemic proteins increased the binding of cytosolic proteins to the 5'-untranslated region of NaPi-2 mRNA, leading to 6-fold increase in NaPi-2 mRNA stability, and thus an increase in the abundance of NaPi-2 protein and phosphate transport in the BBM (Moz *et al.*, 1999). These findings show directly that Pi regulates NaPi-2 expression by affecting protein-RNA interactions in vivo.

A summary on the mechanisms involved in the regulation of NaPi-2 by Pi and thyroid hormone (PTH) is shown in Figure 9.

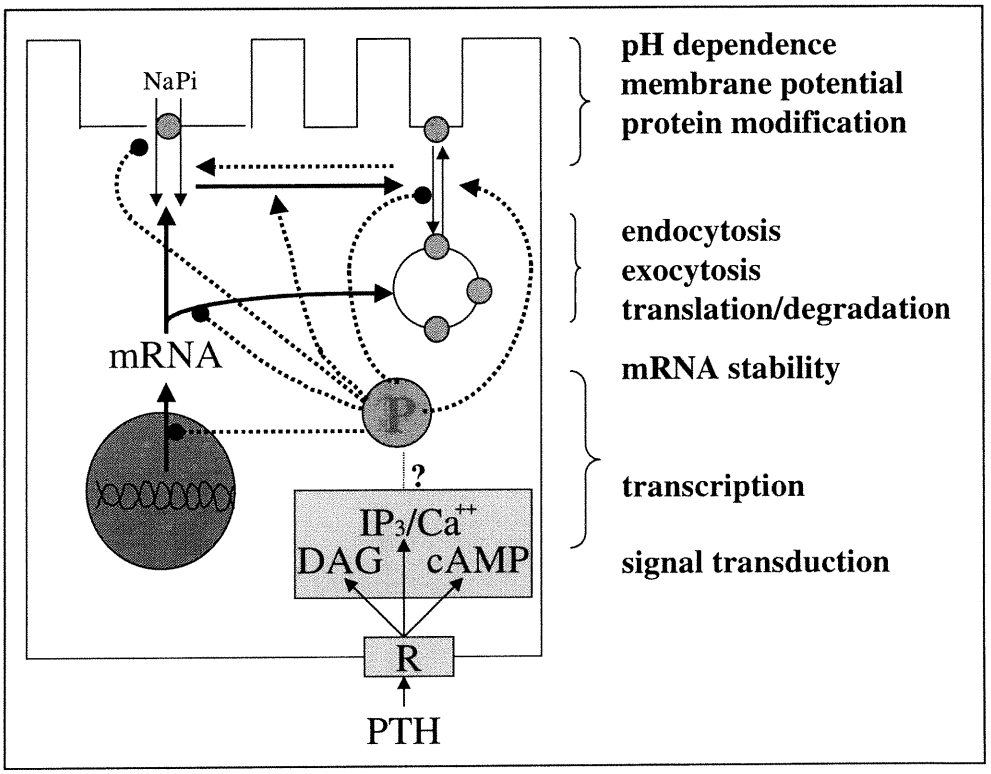

Figure 9. Summary on the mechanisms involved in the regulation of type II Na/Pi cotransporters, especially by parathyroid hormone (PTH) and dietary Pi.····· ● inhibitory effect;····· ▶ stimulatory effect. Adapted from Murer et al., 1996.

I.9.2 HPD

In contrast to LPD, HPD caused down-regulation of Na^+/Pi cotransport both *in vivo* and *in vitro*, accompanied by decreased expression of the type II Na^+/Pi cotransporters. In rats fed LPD for 7 days, switching to a HPD resulted in 1.9-fold decrease in BBM Na^+/Pi cotransport activity within 2h and a 2.3-fold decrease within 4h, without change in Na^+/glucose cotransport activity. Northern blotting analysis on cortex mRNA showed that NaPi-2 mRNA level was not changed, but Western blot analysis on the isolated cortical BBM showed that the level of NaPi-2 proteins was decreased by 4-fold. Immunohistochemical analysis further showed a marked downregulation in NaPi-2 expression in the apical membranes of the proximal tubule of rats acutely fed HPD, and the downregulation was much more pronounced in superficial and midcortical than in juxtamedullary nephrons (Levi *et al.*, 1994; Levi *et al.*, 1996). A similar study was conducted in which rats fed HPD for 2 hours showed decreased BBM Na^+/Pi cotransport activity and BBM NaPi-2 protein level without changes in mRNA level (Takai *et al.*, 1997). To avoid the effect of PTH on the type II Na^+/Pi cotransporters during changes in dietary Pi, thyroparathyroidectomized (TPTX) rats were used to study the effect of dietary Pi on Na^+/Pi cotransport and NaPi-2 protein and mRNA abundance. In TPTX rats adapted chronically to LPD, feeding a HPD for 4h resulted in a 50% decrease in Na^+/Pi cotransport activity and a 40% decrease in the amount of NaPi-2 protein; in contrast, the level of NaPi-2 mRNA remained unchanged (Takahashi *et al.* 1998). The large change caused by HPD in the expression of NaPi-2 is clearly demonstrated in Figure 8.

The cellular mechanisms involved in HPD-induced downregulation of Na^+/Pi cotransport have also been studied. It has been shown that short-term downregulation of BBM Na^+/Pi cotransport activity is independent of endogenous PTH activity (Takai *et al.*, 1997). Pretreatment with cycloheximide, actinomycin D or colchicine did not prevent the acute downregulation of the Na^+/Pi cotransporter in response to acute HPD (Loestcher *et al.*, 1996). HPD caused increased NaPi-2 immunoreactive signals in the late endosome/lysosome fractions of rat renal cortex. In accordance with this finding, the content of type II Na^+/Pi cotransporters was increased in lysosomes isolated from these tissues (Keusch *et al.*, 1998; Takai *et al.*, 1997). Thus, the short-term response to HPD is most likely mediated by rapid endocytotic internalization of Na^+/Pi cotransporters from the apical membrane of the proximal tubule by cellular mechanisms that are independent of endogenous PTH activity and the microtubular network.

Less data is available on the effect of long-term HPD on type II Na^+/Pi cotransporters. One study showed that in rats adapted to one-week high Pi intake, NaPi-2 mRNA and NaPi-2 protein in kidney cortical PTs were weakly detected (Ritthaler *et al.*, 1999). This data suggests that adaptation of rats to long-term HPD is mediated by decreased levels of NaPi-2 mRNA in the PTs. This decrease was possibly caused by decreased transcription of NaPi-2 genes and/or decreased stability of NaPi-2 mRNA, since regulation of NaPi-2 at these levels has been observed (Markovich *et al.*, 1995).

I.9.3 Sensing of altered Pi by the cell

Although the cellular mechanisms of regulation of Na^+/Pi cotransport activity and type II Na^+/Pi cotransporters by dietary Pi have been extensively studied, how the proximal tubular cell senses changes in the extracellular Pi concentration is still not understood. The sensing process must be an intrinsic property of the PT cells since it occurs in isolated tubules as well as proximal tubular cells in culture. Thus, as suggested, this process may be related to small changes in intracellular Pi availability (Mizgala and Quamme 1985; Caverzasio *et al.*, 1985; Biber *et al.*, 1988).

Recently the Pi responsive element (PRE) in the gene of mouse type II Na^+/Pi cotransporter has been identified. LPD induces the overexpression of the mouse transcription factor μE3 (TFE3), which binds to the PRE sequence of the mouse *NPT2* gene. The binding of TFE3 to PRE significantly increases the rate of NPT2 gene transcription, leading to increased expression of the cotransporter and Na^+/Pi cotransporter activity (Kido *et al.*, 1999). This indicates another mechanism of upregulation of Na^+/Pi cotransport by long-term LPD, in addition to increased mRNA stability.

I.10. Regulation of type II cotransporters by PTH

I.10.1 Effect of PTH on renal phosphate transport

The effect of PTH on Na^+/Pi cotransport is another heavily studied aspect of the regulation of phosphate transport. PTH is described as a phosphaturic hormone because administration of PTH in animals results in increased Pi excretion in the urine (Agus *et al.*, 1973, Aurbach and Heath, 1974). Initial micropuncture studies in dogs indicated that the proximal convoluted tubule was the critical site of PTH action on the kidney (Agus *et al.*,

1971). Later transport studies using BBMVs isolated from the renal cortex of animals treated with PTH showed a decreased Na^+/Pi cotransport activity, while the Na^+/glucose and other solutes remained unchanged. This indicates that the apical membrane of the PT is the subcellular site of PTH action (Hamerman *et al.*, 1980; Evers *et al.*, 1978; Murer *et al.*, 1977). Studies using OK cells demonstrated that addition of PTH inhibited, while removal of PTH restored, Na^+/Pi cotransport at the membrane level by decreasing the *Vmax* but not the *Km* of the transport activity (Malmstrom and Murer, 1986). The action of PTH on Na^+/Pi cotransport is due to the irreversible inactivation of an unknown membrane protein (Malmstrom and Murer 1987). The recent identification of the renal Na^+/Pi cotransporters greatly extended our understanding in the effect of PTH on phosphate transport.

In parathyroidectomized rats, administration of PTH caused decreased BBM Na^+/Pi cotransport activity accompanied by decreased expression of BBM NaPi-2 proteins within 2h (Kempson *et al.*, 1995). The action of PTH is mainly at the protein level since NaPi-2 mRNA is only slightly decreased (Kempson *et al.*, 1995). PTH may selectively affect type II Na^+/Pi cotransporters since the expression of NaSi-1, a Na^+-dependent sulfate (SO4) cotransporter in BBM, was not altered (Lotscher *et al.*, 1996). The decrease in BBM NaPi-2 expression is caused by the PTH-induced endocytosis of this protein from the BBM, since after PTH infusion, NaPi-2 redistributes to higher density intracellular fractions (Zhang *et al.*, 1999). In agreement with this, a strong overlap of internalized type II cotransporters with late endosomes/lysosomes was observed by immunohistochemistry. The content of type II Na^+/Pi cotransporters as detected by Western blotting was increased in lysosomes isolated from the corresponding tissues. This suggests that in proximal tubular cells, type II Na^+/Pi cotransporters internalized in response to administration of PTH and acute high Pi-diet are routed to the lysosomes and likely do not enter a recycling compartment (Keusch *et al.*, 1998). Using electrophysiological and immunodetection techniques it has been showed that in OK cells transfected with NaPi-2 cDNA, PTH caused retrieval of both NaPi-2 and the native NaPi-4 from the cell membrane followed by rapid degradation of these transporters. Recovery of these cotransporters and the transport function from PTH inhibition requires *de novo* protein synthesis (Pfister *et al.*, 1997).

Further morphological studies in OK cells showed that PTH removed NaPi-4 proteins into late endosomes/lysosomes where the transporters are degraded rapidly (Pfister *et al.*, 1998). It is unlikely that the internalized transporters are recycled for transport because

inhibition of internalization increased the protein abundance but did not increase the Na^+/Pi cotransport activity (Pfister *et al.*, 1998). The internalization process does not involve the wholesale nonspecific retrieval of BBM fragments since the overall rate of endocytotic activity is not changed by PTH as evidenced by horseradish peroxidase uptake experiments in OK cells (Paraiso *et al.*, 1995). The involvement of type III Na^+/Pi cotransporters in PTH-induced inhibition of Na^+/Pi cotransport has been excluded in our experiments in OK cells (Boyer *et al.*, 1998). Whether the type I cotransporters are involved in this process is not known.

I.10.2 Signaling pathways of PTH action on Na^+/Pi cotransport

Although a large number of studies have been focused on the PTH effect on Na^+/Pi cotransport, the signaling pathways involved in PTH-induced internalization of type II cotransporters has not been clear. PTH receptors have been localized in both the BBM and the BLM of proximal tubules (Kaufmann *et al.*, 1994). These receptors belong to the GTP-binding protein-linked membrane receptor family (Juppner *et al.*, 1991). PTH binding activates a dual signaling cascade, the AC/cAMP/PKA pathway and the phospholipase/IP3/ DAG/PKC pathway (Muff *et al.*, 1992). Direct activation of either kinase in renal cells is accompanied by inhibition of Na^+/Pi cotransport (Dunlay and Hruska 1990). However, the link between PKA or PKC and the type II Na^+/Pi cotransporters is not known. NaPi-2 possesses several PKC concensus sites (Ser 5, 91, 462, 625 and Thr 508), but none of these sites has been shown to be used for phosphorylation, as determined by site-directed mutagenesis experiments (Hayes *et al.*, 1995). Thus, an altered phosphorylation of the transporter *per se* as a triggering mechanism for membrane retrieval, as well as the possible interaction of the transporter with a regulatory protein, or a phosphorylation-dependent control of membrane retrieval/insertion has been suggested (Murer *et al.*, 1996).

Recent studies have been focused on the contribution of these two pathways in the regulation of Na^+/Pi cotransporters. One study found that in OK cells, only the PKA pathway regulated the internalization of NaPi-4, while the inhibitory effect of the PKC pathway on phosphate transport was independent of NaPi-4 expression (Lederer *et al.*, 1998). However, a later study demonstrated that PTH signal transduction *via* cAMP-dependent (PKA), as well as cAMP-independent (including PKC) pathways leads to membrane retrieval and degradation of type II Na^+/Pi cotransporters and, thereby, to the inhibition of Na^+/Pi cotransport activity

(Pfister *et al.*, 1999). Using electrophysiological and immunodetection techniques, it has been shown that the PKC activator DOG (1,2-dioctanoyl-sn-glycerol) or PMA (phorbol 12-myristate 13-acetate) reduced more than 50% the Na^+/Pi cotransport activity of *Xenopus laevis* oocytes transfected with type II Na^+/Pi cotransporter messengers (NaPi-2, NaPi-5 and NaPi-7). This reduction was accompanied by the redistribution of these transporters from the oolemma to the submembrane region (Forster *et al.*, 1999). Interestingly, it is suggested that 20-hydroxyeicosatetraenoic acid, the product of phospholipase A2, may pass the signal from PKC to the internalization of Na^+/Pi cotransporters in proximal tubule cells (Silverstein *et al.*, 1998). The involvement of PI 3-kinase in the synthesis (and/or routing) of the apical type II Na^+/Pi cotransporter in OK cells has also been demonstrated (Pfister *et al.*, 1999).

I.11 Effect of acid/base balance

Alterations in systemic acid/base balance affect renal Pi excretion. Metabolic acidosis results in impaired renal tubular Pi reabsorption and proximal tubular BBM Na^+/Pi cotransport activity (Mizgala and Quamme 1985). Studies in rats with metabolic acidosis induced by ingestion of NH_4Cl showed that in chronic ($\geq$12 hr) metabolic acidosis, the progressive decrease in BBM Na^+/Pi cotransport activity is most likely mediated by decreases in BBM NaPi-2 protein and cortical NaPi-2 mRNA abundance. In contrast, in acute ($\leq$6 hr) metabolic acidosis the decrease in BBM Na^+/Pi cotransport is likely mediated by enhanced internalization from and/or impaired delivery of the NaPi-2 protein to the apical BBM (Ambuhl *et al.*, 1998). However, LPD treatment can diminish the effect of acidosis on Na^+/Pi cotransport in rats (Ambuhl *et al.*, 1998).

Studies in OK cells, however, showed different results. Incubation of OK cells in acidic medium led to an increase in Na^+/Pi cotransport activity accompanied by increased expression of NaPi-4. The short-term effect is due to an increased rate of NaPi-4 protein insertion while the long-term effect involves *de novo* NaPi-4 synthesis without changes in the transcription rate (Jehle *et al.*, 1997). The concentration of HCO_3^- and the partial pressure of CO_2 (P_{CO2}) can influence the transport activity independent of hydrogen ion concentration. This is because isohydric decrease in HCO_3 concentration and P_{CO2} exert a significant, direct stimulatory effect on Na^+/Pi cotransport and NaPi-4 protein expression in OK cells by affecting NaPi-4 mRNA transcription (Jehle *et al.*, 1999).

In isolated renal BBM vesicles, it is well established that Na^+/Pi cotransport is highly pH dependent. The rate of Pi uptake increases 7-fold when the pH of the medium is increased from 6.0 to 8.0 (Amstutz *et al.*, 1985). Kinetic analysis of the Na and H^+ dependency of Na^+/Pi cotransport suggested that these two ions may interact competitively with the transporter at sites different from the Pi-binding site and thus control the affinity of the transporter for Pi. In this way, H^+ may inhibit the Na^+/Pi cotransport by the renal BBM (Murer and Biber 1992).

I.12. Regulation of type II cotransporters by other hormone/factors

I.12.1 Glucocorticoids

Glucocorticoids have been suggested to play an important role in the maturational decrease in proximal tubule phosphate transport (Arar *et al.*, 1994). Dexamethasone-treated rats had decreased *Vmax* without a change in *Km* for Na^+/Pi cotransport in renal BBM. This decrease was accompanied by a decrease in mRNA and protein abundance for NaPi-2 (Levi *et al.*, 1995). In neonatal rabbits, decreased Na^+/Pi cotransport was associated with decreased NaPi-6 protein expression but not with changes in NaPi-6 mRNA (Prabhu *et al.*, 1997). In rats, adrenalectomy increased NaPi-2 protein abundance in the proximal tubule, whereas dexamethasone at pharmacological doses dramatically suppressed NaPi-2 protein on the apical membrane in both adrenalectomized and sham-operated animals without significant reciprocal increases in subapical NaPi-2 staining in the dexamethasone-treated rats, compared to a significant increase in NHE-3 (Loffing *et al.*, 1998).

I.12.2 Vitamin D_3

Vitamin D_3 ($1,25(OH)_2D_3$) is a steroid hormone whose receptor is located in cytoplasm. A stimulatory effect of Vitamin D_3 on Na^+/Pi cotransport has been observed previously, and its action is independent of other endocrine factors such as PTH (Murer *et al.*, 1992). One mechanism by which vitamin D regulates Pi homeostasis is the modulation of the expression of type II Na^+/Pi cotransporter genes (Taketani *et al.*, 1998). The Vitamin D_3 responsive element in the promoter of the gene encoding the human type II Na^+/Pi cotransporter NaPi-3 has been identified (Taketani *et al.*, 1997). In OK cells, Vitamin D_3 *per se* does not affect the Pi transport activity, but it may antagonize the inhibitory effect of PTH-

induced activation of AC/cAMP system on Pi transport partly via the suppression of PTH/PTHrP receptor mRNA expression (Wald *et al.*, 1998).

I.12.3 Thyroid hormone

Thyroid hormone increases Na^+/Pi cotransport in the renal BBM (Yusufi *et al.*, 1985). Thyroid hormone leads to precocious maturation of renal Pi transport by increasing NaPi-2 protein expression in BBM in 14-day-old weaning rats, suggesting that thyroid hormone is normally involved in this maturation (Euzet *et al.*, 1995). Administration of T_3 restored the expression of NaPi-2 mRNA and proteins levels in rats treated with 6-n-propyl-2-thiouracil (to induce hypothyroidism) or prevented from weaning, these effects are independent of PTH, GH, or glucocorticoids (Euzet *et al.*, 1996). In a similar study in young hypothyroid rats the renal Na^+/Pi cotransport activity decreases markedly in association with decreases in type II Na^+/Pi cotransporter (NaPi-2) protein and mRNA abundance (Alcalde *et al.*, 1999). This decrease was reversed by the administration of long-term physiological and supraphysiological doses of T_3 (Alcalde *et al.*, 1999). Nuclear run-on experiments indicated that T_3 regulates NaPi-2 mRNA levels by a transcriptional mechanism (Alcalde *et al.*, 1999). In OK cells, T_3 stimulates Na^+/Pi cotransport most likely via increased rate of transcription, since actinomycin D completely prevented this stimulatory effect (Sorribas *et al.*, 1995).

I.12.4 Epidermal growth factor (EGF)

In contrast to the factors mentioned above, epidermal growth factor (EGF) exhibited an inhibitory effect on renal Na^+/Pi cotransport. Administration of EGF suppressed the Na^+/Pi cotransport activity in BBMV isolated from suckling and weaned rats via a decrease in NaPi-2 protein abundance without any change in NaPi-2 mRNA (Arar *et al.*, 1999). In OK cells, the EGF-induced decrease in Na^+/Pi cotransport is mediated through a decrease in NaPi-4 mRNA and activation of the phospholipase C signaling pathway (Arar *et al.*, 1995).

I.12.5 Insulin-like growth factor (IGF)

IGF-I has been shown to stimulate Na^+/Pi cotransport in OK cells (Caverzasio and Bonjour; 1989). The mechanisms of this stimulatory effect involve distinct protein tyrosine phosphorylation processes (Palmer *et al.*, 1996). Tyrosine phosphorylation led to increased expression of NaPi-4 in OK cell membranes without changes in transcription and protein synthesis since this process was not prevented by actinomycin D and cycloheximide.

Therefore phosphorylation increases Na^+/Pi cotransport in OK cells by increasing the stability of NaPi-4 (Jehle *et al.*, 1998). IGF-II was also shown to increase apical Pi transport in cultured proximal tubule cells (Hirschberg *et al.*, 1995). However, the effect of IGFs on phosphate transport has not been studied in relation to the known renal Na^+/Pi cotransporters.

I.12.6 Integrin

A NaPi-2 immunoreactive protein with a molecular weight of about 100 kDa has been found in the avian osteoclast (Gupta *et al.*, 1996). Co-incubation of the avian osteoclast with bone particles led to an immediate increase in Na^+/Pi cotransport. This effect was inhibited in the presence of Arg-Gly-Asp-Ser peptide (Gupta *et al.*, 1996), suggesting that integrins and cell-matrix interactions may be involved in the regulation of Na^+/Pi cotransport mediated presumably *via* type II Na^+/Pi cotransporters. However, this process seems to be involved only in bone metablism, and NaPi-2 immunoreactive protein itself needs to be identified.

I.12.7 IPAN

Interestingly, an anti-sense product of flounder NaPi-5 has been cloned recently and was designated IPAN to allude to the sense transcript of NaPi-5 (Huelseweh *et al.*, 1998). IPAN encodes a 68-amino acid peptide that is expressed transiently in a submembrane compartment during the flounder oocyte development, suggesting a role of IPAN during development and in the regulation of type II cotransporter (Huelseweh *et al.*, 1998). However, further study is needed to reveal its role in phosphate transport since no direct interaction of IPAN with other proteins has been established.

I.13. Ontogenesis of type II Na^+/Pi cotransporters

Ontogenesis of type II Na^+/Pi cotransporters has received special attention because it has been found previously that neonates have greater ability than adults to maintain a higher plasma Pi level. In humans, for example, the plasma Pi concentration at birth is in excess of 6 mg/dl, falling thereafter to 5 mg/dl by 1 year of age, and the subsequent decrease to approximately 1 mg/dl to adult values occurs very slowly over the entire period of growth (Aperia and Celsi, 1992). Studies in isolated perfused kidneys demonstrated that the kidney of the newborn guinea pig reabsorbed almost four times as much as Pi as that of the adult (Neiberger *et al.*, 1989). The higher Pi reabsorption rate in the newborn guinea pig kidney is

due to the higher Na^+/Pi cotransport activity of the renal proximal tubule BBM of the newborn animal (Neiberger *et al.*, 1989). This indicates that the higher plasma Pi in the immature animals is due to higher renal Pi reabsorption rate. Using *in situ* hybridization and immunohistochemical methods, it was shown that expression of NaPi-2 starts as early as the post-S-shape stages of tubulogenesis (Schmitt *et al.*, 1999).

In kidneys of newborn (13-day-old suckling and 22-day-old weaned) rats, the appearance of NaPi-2 protein and mRNA coincided with the development of the brush border (assessed by actin staining) on proximal tubular cells. NaPi-2 was not detectable in the nephrogenic zone or in the outgrowing straight sections of proximal tubules, which lack a brush border. In 13-day-old suckling rats, strong NaPi-2 staining was seen in the BBM of convoluted proximal tubules of all nephron generations. In contrast, in 22-day-old weaned rats, NaPi-2 staining in the BBM of superficial nephrons was weaker than that in the BBM of juxtamedullary nephrons. Western blotting demonstrated that the overall abundance of NaPi-2 protein in the BBM of 22-day-old rats was decreased to approximately 70% of that in 13-day-old rats. In kidneys of 6-week-old rats, the internephron gradient for NaPi-2 abundance in the BBM corresponded to that in adult rats. The data suggest that the NaPi-2 system in the kidney is fully functional and possesses the capacity for regulation as soon as nephrogenesis is completed. The manifestation of NaPi-2 internephron heterogeneity immediately after weaning might be related to the change in dietary inorganic phosphate content (Traebert *et al.*, 1999). In weaning rats, the expression of NaPi-2 in the BBM increases with age without changes in mRNA abundance (Euzet *et al.*, 1995; Euzet *et al.*, 1996; Arar *et al.*, 1999; Taufiq *et al.*, 1997).

Thyroid hormone may play an important role in renal phosphate transport maturation, especially during the weaning stage of rats in which the Na^+/Pi cotransport activity increases abruptly from a very low level to the adult level (Euzet *et al.*, 1995; Euzet *et al.*, 1996; Alcalde *et al.*, 1999). Interestingly, the development of type II Na^+/Pi cotransporter found in frog intestine is tightly regulated by T3 and its mRNA is restricted to the differentiated epithelial cells (Ishizuya-Oka *et al.*, 1997). From the weaning stage to 4 months of age, expression of NaPi-2 and BBM Na^+/Pi cotransport activity decreases, due to posttranscriptional mechanisms since the mRNA level remains unchanged (Taufiq *et al.*, 1997). The increased phosphaturia observed in rats older than 4 months is due to decreased expression of NaPi-2 mRNA and NaPi-2 proteins (Sorribas *et al.*, 1996). Other species of

Na^+/Pi cotransporters that are only partially homologous to NaPi-2 in their amino acid sequence have been reported in 3-week-old but not in 12-week-old rats. These cotransporters could be attributable to the higher Na^+/Pi cotransport activity of the BBM in the younger rats (Silverstein *et al.*, 1996; Silverstein *et al.*, 1997).

I.14. Type II Na^+/Pi cotransporters and XLH

Several Mendelian disorders (X-linked and autosomal forms) related to renal Pi wasting have been described (Rasmussen and Tenenhouse 1995). X-Linked Hypophosphatemia (XLH) is a dominantly inherited disorder of Pi homeostasis, characterized by rachitic bone disease, short stature, hypophosphatemia arising from a specific defect in renal Pi reabsorption, and abnormal regulation of renal vitamin D metabolism (Rasmussen and Tenenhouse 1995). The *Hyp* mice with an X-linked mutation homologous to human XLH display a specific defect in Na^+/Pi cotransport in renal BBM that accounts for the hypophosphatemia and bone disease (Tenenhouse *et al.*, 1978a; Tenenhouse *et al.*, 1978b). Morever, the deficit in Na^+/Pi cotransport is associated with a decrease in transport maximal velocity (*Vmax*) that is proportional to a decrease in the relative abundance of the type II Na^+/Pi cotransporter (NaPi-7) mRNA and immunoreactive protein (Tenenhouse *et al.*, 1994). However, the responsive mechanism in the *Hyp* mice to a LPD is as intact as that of normal control mice (Tenenhouse *et al.*, 1995). The *Hyp* mice fail to exhibit an increase in tubular maximum for phosphate reabsorption (TmP/GFR) following the administration of a LPD diet (Muhlbauer *et al.*, 1982). Thus it is suggested that the adaptive increase in Na^+/Pi cotransport in the BBM of the proximal tubule is not sufficient for the overall increase in Tmp/GFR in the whole kidney (Tenenhouse *et al.*, 1995). The type I Na^+/Pi cotransporter seems not to be affected in *Hyp* mice (Chong *et al.*, 1995).

Although the *Hyp* mice have low levels of renal Na^+/Pi cotransport activity due to the suppressed expression of the type II Na^+/Pi cotransporter, this cotransporter has been ruled out as a product of the XLH gene in human, as the type II cotransporter in humans is encoded by a gene located on chromosome 5 (Kos *et al.*, 1994). Thus the type II Na^+/Pi cotransporter may represent a final target in the regulatory cascades affected in XLH.

Recently the gene causing XLH has been identified by positional cloning and has been named PEX for "phosphate regulating gene with homology to endopeptidase on the X-chromosome" (Econs 1996; Francis *et al.*, 1995). Several mutations in the PEX gene have

been observed in patients with HYP, confirming that defects in PEX might be responsible for HYP (Holm *et al.*, 1997). How a mutation in PEX encoding a neutral endopeptidase could suppress the renal expression of the type II Na^+/Pi cotransporter is still unclear. It is possible that a defective PEX gene product (an endopeptidase) acts through its substrates (peptides, such as hormones) and results in either a lack of stimulation of the type II Na^+/Pi cotransporter expression/function and/or in inhibition of type II Na^+/Pi cotransporter expression/function. In this regard, it has been shown that some newly found peptide hormones (such as stanniocalcin and phosphatonin) exert their stimulatory or inhibitory effect on renal Na^+/Pi cotransport (Olsen *et al.*, 1996; Cai *et al.,* 1994; Econs and Drezner 1994).

I.15 Nephrotoxicity related to BBM Na^+/Pi cotransport

Some heavy metals such as cadmium (Cd), lead (Pb) and mercury (Hg) are well-known occupational and environmental hazards with potent nephrotoxic action. Nephropathy due to chronic Cd exposure in humans and experimental animals is characterized by defects in reabsorptive and secretory functions in the proximal tubule that clinically resemble acquired Fanconi's syndrome (Kim *et al.*, 1990). In rats with nephrotoxicity induced by chronic subcutaneous injection of Cd, the observed phosphaturia and decreased BBMV Na^+/Pi cotransport activity is accompanied by decreased expression of NaPi-2 in the BBM. The effect of Cd on NaPi-2 is selective since other transport systems such as Na^+/sulphate cotransporter and aquaporin 1 were not affected (Herak-Kramberger *et al.*, 1996). The decreased expression of NaPi-2 is due to the suppressed transcription of the 2.7 kb NaPi-2-related mRNA as revealed by Northern blot analysis (Herak-Kramberger *et al.*, 1996).

The inhibitory effect of heavy metals on Na^+/Pi cotransport also occurs through the direct contact of the heavy metals with the cotransporters: electrophysiological studies in *Xenopus* oocytes transfected with NaPi-3 showed that Hg^{2+}, Pb^{2+} and Cd^{2+} inhibited the phosphate-induced current (*Ip*) (Wagner *et al.*, 1996). But, the mechanisms of each ion's action on the *Ip* are different: the Hg^{2+}-induced inhibition was not reversible while the Pb^{2+}- and Cd^{2+}-induced inhibitions were rapidly reversible upon washout (Wagner *et al.*, 1996). Other substances, such as arsenate, induced inward currents through NaPi-3 and decreased the apparent *Km* in measurements of *Ip*, while the specific Na^+/Pi cotransport inhibitor phosphonoformic acid (PFA) itself induced no currents but inhibited Pi-induced currents (Busch *et al.*, 1995).

I.16 Effect of renal ischemia on renal Na^+/Pi cotransport

Tissue ischemia occurs when a reduction in regional blood flow decreases delivery of oxygen and substrates to levels inadequate to maintain cellular energy status. Renal ischemia has been extensively studied because of its importance in clinical issues such as kidney transplantation and acute renal failure. Due to their polarized structural orientation and high metabolic rate, the renal proximal tubular epithelial cells are particularly susceptible to ischemic injury (Weinberg 1991). The rapidly-occuring, duration-dependent disruption and dissociation of the actin cytoskeleton system plays an important role in the morphological changes induced by renal ischemia. During ischemia, the cytoskeletal structures such as zonula occludens, zonula adherens, terminal web, cortical web and focal adhesions separate from each other. F-actin aggregates redistribute throughout the cytoplasm and accumulate perinuclearly. Other actin cytoskeletal elements, such as spectrin and ankyrin, redistribute throughout the cytoplasm. Apical and basolateral integral membrane proteins (e.g., apical leucine aminopeptidase; basolateral Na^+/K^+-ATPase and integrins) distribute randomly over the surface membrane of the cell. Redistribution of the Na^+/K^+-ATPase from the BLM to the BBM may lead to apical sodium secretion and induce high urinal sodium excretion (Leiser and Molitoris 1993).

Details of the events occuring in the proximal tubule during renal ischemia gradually become clear at the molecular level. Of the many proteins involved in the initial steps in ischemia induced PT structural and functional alterations, the actin binding protein, actin depolymerizing factor (ADF), plays a central role (Ashworth *et al.*, 2001). ADF is a 19-kDa phosphoprotein activated by dephosphorylation (Agnew *et al.*, 1995), it acts as the major regulator of actin filament turnover in vivo (Lappalainen *et al.*, 1997). Due to the lack of ATP for kinase activity in the presence of continued phosphatase activity (Chen and Mandel, 1997), the level of phosphorylated ADF drops dramatically to 19% within 15 minutes of ischemia (Schwartz *et al.*, 1999). Because the unphosphorylated or activated form of ADF binds actin with a much higher affinity than does phosphorylated ADF (Lueck *et al.*, 1998), majority of the ADFs relocated to the microvilli and bind to the actin filament (Schwartz *et al.*, 2001). This results in the depolymerization of the actin filaments and consequently, the destruction of the microvilli. The decreased intracellular pH, which occurs early during ischemia, enhances the binding of ADF to actin (Hawkins *et al.*, 1993).

Cytoskeletal disruption would alter functions of the proximal tubule BBM transport. The effect of renal ischemia on BBM transport activities of several substrates has been investigated in previous studies. Rabbits with renal ischemia induced by occlusion of the left renal artery showed selective effect on proximal tubular transport activity. For example, ischemia of 30 or 60 min did not change the uptake of glucose, succinate and L-glutamate by BBM vesicles, while the H^+/tetraethylammonium antiport was significantly inhibited (Kim *et al.*, 1995). Prolonged ischemia of up to 120 min inhibited glucose and succinate transport by BBM vesicles while L-glutamate uptake remaind unchanged (Kim *et al.*, 1995). Ischemia of 30 or 60 min has stimulatory effect on the uptake of p-aminohippurate (PAH) and tetraethylammonium (TEA) in cortical slices. This stimulatory effect was diminished upon reperfusion for 30 min. During ischemia, the BBMV uptake of PAH was not changed while the uptake of TEA was significantly reduced, due to decreased *Vmax* (Kim *et al.*, 1996). In rats, TEA and Na/D-glucose reabsorption by renal BBM was also reduced following renal ischemia, and was further impaired by reperfusion (Maeda *et al.*, 1993).

In nephrons isolated from rat kidney subjected to 60 min of ischemia, the proximal convoluted tubule fluid reabsorption was greatly reduced (Hanley 1980). Microperfusion experiments showed that fluid reabsorption and total glucose efflux from the proximal convoluted tubule were decreased by 35 min of ischemia in rat kidney (Johnston *et al.*, 1984). Clearance experiments in rats also showed that fluid (Na) reabsorption was greatly impaired by ischemia (Herminghuysen *et al.*, 1985).

Although it has been observed that renal ischemia caused phosphaturia (Kim *et al.*, 1995), ischemic effect on the Na^+/Pi cotransport of proximal tubule BBM has not been studied in detail. Recent studies have been focused on the long-term effects of ischemia on renal transporters of Pi and other solutes. In rabbits, renal pedicle clamping for 60 minutes caused phosphaturia and glucosuria during reperfusion, accompanied by decreased Na^+/Pi and Na^+/glucose uptakes of BBM vesicles (Jung *et al.*, 2000). The mRNA levels of Na^+/Pi, Na^+/glucose and Na^+/succinate transporters analyzed by RT-PCR were not changed by 60 min of ischemia alone but were significantly reduced by 24 h of reperfusion (Jung *et al.*, 2000). In rats, 30 min of bilateral ischemia followed by 24 h of reperfusion decreased the protein levels of the type 3 Na^+/ H^+ exchanger (NHE-3) and NaPi-2 to 28% and 14 %, respectively (Kwon *et al.*, 2000). The Na^+-K^+-ATPase levels were also significantly decreased (51%), whereas there was no significant decrease in type 1 bumetanide-sensitive contransporter and thiazide-

sensitive cotransporter levels (Kwon *et al.*, 2000). Thirty minutes of ischemia also decreased the abundance of renal aquaporin1 and aquaporin2 to 30% and 40%, respectively, which can explain the impairment in urinary concentration in the postischemic period (Kwon *et al.*, 1999). The suppressed expression of Na-K-2Cl cotransporter during ischemia/reperfusion is also responsible for the natriuretic and diuretic effects of ischemia (Wang *et al.*, 1998). Results of these studies indicate that renal ischemia/reperfusion causes global destruction and suppression on the important renal transmembrane transporters, including NaPi-2. However, the early changes of these transporters during ischemia have not been reported. Since phosphate is involved in energy metablism and NaPi-2 is the key factor in Pi absorption, it should be interesting to examine the early alteration of NaPi-2 in BBM by ischemic injury.

Membrane fluidity has been shown to influence the transport activity of glucose, Pi, and sulfate. In contrast to glucose and sulfate transport, Na^+/Pi cotransport is positively related to membrane fluidity. It was shown that fluidization of the proximal tubule cell membrane with benzyl alcohol increased Na^+/Pi cotransport (Freidlander *et al.*, 1988; Levine *et al.*, 1991; Lee *et al.*, 1999). On the other hand, decreasing membrane fluidity by incorporating cholesterol into the renal BBM decreased Na^+/Pi cotransport dose-dependently (Levi *et al.*, 1990). Ischemia not only has profound influence on cell structures, but also affects the lipid fluidity of cell membranes due to changes occurred in membrane lipid composition and redox status. First, reversible renal ischemia causes decreases in renal BBM sphingomylin-to-phosphatidylcholine (SPH/PC) and cholesterol-to-phospholipid ratios (Molitoris and Kinne 1987). A decreased cholesterol-to-phospholipid ratio would be expected to increase the membrane fluidity (Molitoris *et al.*, 1985; Levi *et al.*, 1990). Secondly, ischemia-induced renal hypoxia produces a number of free radicals that impair the cellular redox status. As a result, the membrane lipids are peroxidized (Montagna *et al.*, 1998). Lipid peroxidation has been suggested to increase membrane fluidity (Montagna *et al.*, 1998; Sen and Mukherjea 1998).

II. STRATEGY OF RESEARCH

II. STRATEGY OF RESEARCH

The aim of the present research project was to investigate the molecular mechanisms of renal Na^+/Pi cotransport. By comparing biochemical observation (using immunodetection) with physiological analysis (using vesicle transport), the structure-function relationship of the recently identified rat renal type II cotransporter NaPi-2 was explored. Information from these studies will help understand the physiology of renal Pi handling and the mechanism of Pi homeostasis, a topic that has evoked extensive investigation for many decades (Mizgala and Quamme 1985; Murer and Biber 1992).

As its amino acid sequence has been known (Magagnin *et al.*, 1993), we were able to make antibodies to characterize and to investigate the distribution, regulation, as well as structure-function relationship of NaPi-2. In fact, two antibodies, namely anti-N and anti-C, were raised against synthesized peptides corresponding to the N-terminal (NH_2-MMSYSERLGGPAVS-COOH) and C-terminal (NH_2-PRLALPAHHNATRL-COOH) residues, respectively. Pilot studies of our group have found that LPD led to the overexpression of p70 and p40 as detected with anti-C (Boyer *et al.*, 1996). The p70 ought to be the monomeric form of NaPi-2 according to its predicted primary structure (Magagnin *et al.*, 1993), but the identity and the origin of the p40 remained unclear. These have been the outstanding questions at the very beginning of this thesis project. Later, our preliminary experiments with anti-N showed the novel p45 form of NaPi-2 that was also upregulated by LPD. These pieces of information suggest that the p45 and p40 might be products of p70 cleavage. To clarify the relationships between these NaPi-2 forms, we have planned the following studies: 1. to prove that p45 and p40 are not experimental artifacts, i.e., they are not the result of protein degradation that might occur during sample preparation; 2. to obtain the amino acid sequence of p45 and p40 and prove directly that they are NaPi-2 cleavage products. The first study was carried out under extreme conditions (prolonged incubation of the BBM at different temperatures for different periods of time). In the second study the p40 were purified by electroelution and was subjected to N-terminal sequencing. Results of these studies should reveal convincingly that p45 and p40 are the products of p70 cleavage. Details of them have been described in MANUSCRIPT II and III.

A number of important questions regarding the different forms of NaPi-2 remain unanswered. To elucidate the extent of glycosylation, a deglycosylation experiment is

necessary. Only N-deglycosylation was performed in this thesis project, since several N-glycosylation but no O-glycosylation concensus site has been predicted (Magagnin *et al.*, 1993). These studies might also help to elucidate the cleavage site of p70 as the only two sites of N-glycosylation are at Asn 298 and Asn 328 (Hayes *et al.*, 1994). Thus, if both p45 and p40 are glycosylated, the cleavage site must lie between Asn 298 and Asn 328; if only p45 is glycosylated, the cleavage site must lie in the C-terminal of Asn 328; if only p40 is glycosylated, the cleavage site must be in the N-terminal of Asn 298. Results from this experiment should also support the results from sequence analysis. These studies have been incorporated into MANUSCRIPT II.

Our pilot experiments unveiled another form of NaPi-2, i.e., the p180. Thus, together with the p45, p40, and p70, there are four forms of NaPi-2 and disulfide bonds play an important role in their association. We performed two sets of experiments to examine this issue. First, BBM was subjected to SDS-PAGE analysis under reducing and non-reducing conditions with ß-EtSH or DTT, and the electrophoretic profiles were revealed by Western blotting with anti-N or anti-C. Second, the gel slice corresponding to the p70 obtained under non-reducing condition was treated with ß-EtSH and was loaded to another gel for Western analysis to see if p45 and p40 were generated. This would directly prove that p45 and p40 were from p70. These studies are presented in MANUSCRIPT I.

Of the different forms of NaPi-2, which are the active ones that transport Pi? This was answered by comparing the amounts of different forms of NaPi-2 with Na/Pi transport activity in BBM vesicles treated with different concentrations of reducing agents (i.e., ß-EtSH, DTT, and TCEP). As complementary evidence, radiation inactivation was used to measure the target size of NaPi-2 in BBM treated with or without the reducing agents. These studies are presented in MANUSCRIPT I.

After elucidating the properties of NaPi-2 proteins, it is necessary to understand their roles in Na/Pi cotransport under physiological and pathophysiological conditions. It has been known that renal BBM Na/Pi cotransport activity changes during development and dietary Pi deprivation. It also changes in different parts of the renal cortex, such as in the juxtamedullary cortex vs the superficial cortex. Thus, by comparing transport activity with the expression of different forms of NaPi-2 in each physiological situation, the role of each NaPi-2 form might be revealed. These studies are described in MANUSCRIPT III.

Renal ischemia is a well-known renal pathophysiological condition and has been paid extensive attention, but its effect on renal Na/Pi cotransport and NaPi-2 expression has not been clear. In one of my projects rats were subjected to reversible (30 minute) renal ischemia followed or not followed by reperfusion. Then renal BBM was isolated and used for solute transport assays and NaPi-2 immunodetection. Two-dimensional (IEF/SDS-PAGE) gel electrophoresis was also used to further clarify our observations. These studies were included in MANUSCRIPT IV.

III. MANUSCRIPTS

III. MANUSCRIPTS

III.1 Involvement of disulphide bonds in the renal sodium/phosphate cotransporter NaPi-2 (Published)

III.2 Membrane topography of the renal phosphate carrier NaPi-2: limited proteolysis studies (Published)

III.3 Different expression patterns of NaPi-2 transporter modulate sodium-phosphate cotransport by rat kidney (Under revision)

III.4 Effect of ischemia/reperfusion on renal brush-border membrane sodium-dependent phosphate cotransporter NaPi-2 (Published)

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Involvement of disulphide bonds in the renal sodium/phosphate co-transporter NaPi-2

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The rat renal brush border membrane sodium/phosphate co-transporter NaPi-2 was analysed in Western blots with polyclonal antibodies raised against its N-terminal and C-terminal segments. Under reducing conditions, proteins of 45–49 and 70–90 kDa (p45 and p70) were detected with N-terminal antibodies, and proteins of 40 and 70–90 kDa (p40 and p70) were detected with C-terminal antibodies. p40 and p45 apparently result from a post-translational cleavage of NaPi-2 but remain linked through one or more disulphide bonds. Glycosidase digestion showed that both polypeptides are glycosylated; the cleavage site could thus be located between Asn-298 and Asn-328, which have been shown to constitute the only two N-glycosylated residues in NaPi-2. In the absence of reducing agents, both N-terminal and C-terminal antibodies detected p70 and a protein of 180 kDa (p180), suggesting the presence of p70 dimers. Much higher concentrations of β-mercaptoethanol were required to produce a given effect in intact membrane vesicles than in solubilized proteins, indicating that the affected disulphide bonds are not exposed at the surface of the co-transporter. Phosphate transport activity decreased with increasing concentrations of reducing agents [β-mercaptoethanol, dithiothreitol and tris-(2-carboxyethyl)phosphine] and was linearly correlated with the amount of p180 detected. The target sizes estimated from the radiation-induced loss of intensity of p40, p70 and p180 were all approx. 190 kDa, suggesting that NaPi-2 exists as an oligomeric protein in which the subunits are sufficiently close to one another to allow substantial energy transfer between the monomers. When protein samples were pretreated with β-mercaptoethanol [2.5% and 5% (v/v) to optimize the detection of p40 and p70] before irradiation, target sizes estimated from the radiation-induced loss of intensity of p40 and p70 were 74 and 92 kDa respectively, showing the presence of disulphide bridges in the molecular structure of NaPi-2.

INTRODUCTION

Renal reabsorption of inorganic phosphate from the glomerular filtrate takes place predominantly across the brush border membrane (BBM) of the proximal tubule [1]. This process, under hormonal and cellular control [2,3], involves specific sodium gradient-dependent transport systems [4–6]. Renal phosphate transport has been functionally well characterized and the recent molecular identification of proximal tubular Na^+/P_i co-transporters from several animal species [7–14] and renal epithelial cell lines [15,16] has broadened our understanding of their molecular structure, function and regulation. Na^+/P_i co-transporters have been classified into two families, type I and type II, on the basis of their amino acid sequences [6]. Both types differ in molecular mass, possible transmembrane domains, functional motifs and modes of regulation [6]. From their predicted molecular masses, type I co-transporters [7–9] are smaller proteins (51–52 kDa) than type II co-transporters [10–16] (69–75 kDa). Translation experiments *in vitro* have demonstrated that co-transporters of both types are N-glycosylated [7,10,17]. Both type I and type II co-transporters are found mainly in kidney cortex, but type I co-transporters are more abundant in the juxtamedullary portion of the cortex than in its superficial portion [18,19].

Type II co-transporters have been shown to be important in the adaptive response to a low-P_i diet [14,20], but type I co-transporters do not seem to be involved [18,21]. Recently we demonstrated that phosphate deprivation not only results in higher levels of a 70 kDa protein, corresponding in size to that calculated from the amino acid sequence of the rat type II co-transporter NaPi-2 [10], but also to the overproduction of an immunologically related 40 kDa protein [22]. The present study was undertaken to further examine the possible relationship between these two proteins, which share similar immunological, chemical and physical characteristics. Earlier studies have shown that thiol groups are essential for the function of renal Na^+/P_i co-transporters, although these groups are not located at the substrate-binding sites [23–25]. The electrophoretic mobility of renal Na^+/P_i co-transporters is also known to be greatly influenced by the presence or absence of reducing agents [18,22]. Biochemical and immunochemical approaches were therefore used to study the involvement of disulphide bridges in the oligomeric structure and function of NaPi-2. Using the Western blot technique and polyclonal antibodies raised against its N-terminal and C-terminal portions, we demonstrate that this rat Na^+/P_i co-transporter is composed of two protein subunits linked together by disulphide bonds. In addition, dimers of this protein complex, also linked by disulphide bonds, were found to be present in the renal BBM. Finally, target size analysis [26–29] of the radiation-induced disappearance of NaPi-2 polypeptides revealed that this Na^+/P_i co-transporter forms a larger oligomeric

Abbreviations used: BBM, brush border membrane; DTT, dithiothreitol; TCEP, tris-(2-carboxyethyl)phosphine; TS, target size.

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structure, with considerable transfer of the absorbed energy between different subunits, as was previously shown for the type I co-transporters of rat and rabbit [30].

MATERIALS AND METHODS

Preparation of BBM vesicles

Male Sprague–Dawley rats (300–350 g; 2 months old) were purchased from the Charles River Co. (St-Constant, Que., Canada). To examine the effect of phosphate deprivation, rats were fed with either a low-phosphate (no. 86128) or a standard (no. 86129) diet from Teklad (Madison, WI, U.S.A.) containing 0.03% (w/w) or 1% (w/w) phosphate for 3 days. BBM vesicles were prepared from kidney cortex with a $MgCl_2$ precipitation method [31]. The final pellet was resuspended in 300 mM mannitol and 20 mM Hepes/Tris, pH 7.5 (buffer A) and the following protease inhibitors: chymostatin A (10 μg/ml), bacitracin (10 μg/ml), pepstatin (10 μg/ml) and aprotinin (1.85 μg/ml), or, for radiation inactivation experiments, in a cryoprotective medium composed of 150 mM KCl, 14% (w/v) glycerol, 1.4% (w/v) sorbitol and 5 mM Hepes/Tris, pH 7.5, and stored in liquid nitrogen until use. Protein concentration was determined by the method of Bradford [32] with BSA as standard protein. Alkaline phosphatase, a BBM marker enzyme assayed as described by Kelly and Hamilton [33], was enriched 10–15-fold in the final vesicle preparations relative to the cortex homogenate.

Immunization and antibody purification

The 15-residue peptide CPRLALPAHHNATRL, corresponding to the C-terminal portion of NaPi-2, has previously been synthesized and used for antibody production [22]. Here the 14-residue peptide MMSYSERLGGPAVS, corresponding to the N-terminal portion of NaPi-2 and synthesized with the multiple antigen peptide (MAP) system [34], was obtained from the Service de Séquence de Peptides de l'Est du Québec (Centre Hospitalier de l'Université Laval, Ste-Foy, Que., Canada) at a purity of more than 85%. This peptide was used for antibody production with previously described immunization and antibody purification protocols [22] except that the N-terminal peptide did not require coupling to keyhole limpet haemocyanin [34].

Western blot analysis

SDS/PAGE was performed with a Mini-Protean II electrophoresis apparatus (Bio-Rad, Mississauga, Ont., Canada) and the Laemmli buffer system [35]. The separating gel contained 7.5% (w/v) acrylamide and 0.23% bisacrylamide before polymerization. The samples were mixed with sample buffer containing a final concentration of 20% (w/v) glycerol, 1% (w/v) SDS, 0.05% Bromophenol Blue and 0.625 M Tris/HCl, pH 6.8, with the indicated concentrations of reducing agent [β-mercaptoethanol, dithiothreitol (DTT) or tris-(2-carboxyethyl)phosphine (TCEP)]. Broad-range protein standards (Bio-Rad) were used for the calibration of molecular masses. Electrophoresis was performed at 10 mA per 0.75 mm gel for approx. 1.5 h. Each well contained 12 μg of protein. After electrophoresis the proteins were transferred electrophoretically to poly(vinylidene difluoride) microporous (0.45 μm pore diameter) membranes (Millipore, Mississauga, Ont., Canada) in a Milliblot Graphite Electroblotter I apparatus (Millipore) at 1 mA/cm^2 for 1 h at room temperature. Non-specific binding sites on poly(vinylidene difluoride) membranes were blocked by incubation overnight in 150 mM NaCl/0.2% (w/v) Tween-20/50 mM Tris/HCl (pH 7.5) (buffer B), containing 5% (w/v) powdered milk, at 4 °C. The membranes were washed three times by gentle agitation in buffer B for 10 min and incubated for 1 h with a 1:10000 dilution of either anti-peptide (N-terminal or C-terminal) antibody in buffer B. After three more washes in buffer B, the membranes were incubated for 1 h with a 1:1000 dilution of anti-rabbit IgG horseradish peroxidase-linked whole antibody from donkey (Amersham, Oakville, Ont., Canada) in buffer B. The membranes were washed a final time as described above; labelled antigens were revealed with enhanced chemiluminescence (ECL) Western blotting solutions (Amersham). The membranes were exposed to Fuji films that had been preflashed with a Sensitize flashgun unit (Amersham). The X-ray films were developed with a Mini-Med/90 X-ray film processor (AFP Imaging, Elmsford, NY, U.S.A.), scanned with a Personal Densitometer SI scanner (Molecular Dynamics) and analysed with the IPLab Gel H program (Signal Analytics Corporation).

Deglycosylation

BBM proteins were solubilized in 1% (w/v) SDS for 10 min at room temperature and diluted to a final concentration of 1 mg/ml in a buffer composed of 0.3% (v/v) Brij-58 and 40 mM Tris/HCl, pH 7.2. Endoglycosidase F/N-glycosidase F mixture (Boehringer-Mannheim, Laval, Que., Canada) was added at 1 unit/mg of protein and the reaction continued for 17 h at 37 °C with gentle shaking. Sample buffer containing 5% (v/v) β-mercaptoethanol was added and the samples were analysed in Western blots.

Pretreatment of BBM vesicles with reducing agents

BBM vesicles (approx. 1 μg/μl protein) in buffer A were incubated at 25 °C with gentle stirring for 30 min in the presence of various concentrations of reducing agents (β-mercaptoethanol, DTT or TCEP). Buffer A was added and samples were centrifuged for 20 min at 50000 *g* at 4 °C to wash out the unreacted reagents. Two more washes were done before the pellets were finally resuspended at approx. 6 μg/μl protein in buffer A. These samples were used for parallel analysis of phosphate transport activity (described below) and detection of NaPi-2-related proteins in Western blots. For Western blot analyses, samples were mixed with sample buffer without any reducing agent and loaded on an SDS/PAGE gel.

Transport measurements

Uptake of radiolabelled phosphate into BBM vesicles was measured at 25 °C with a rapid filtration method [36]. For transport assays, 5 μl of the vesicles treated with reducing agent were added to 25 μl of a solution containing 200 μM [^{32}P]KH_2PO_4/K_2HPO_4, 5 mM Hepes/Tris, pH 7.5, and 150 mM NaCl or KCl. After incubation for 9 s, the reaction was stopped with 1 ml of ice-cold stop solution containing 150 mM KCl and 5 mM Hepes/Tris, pH 7.5. The suspension was filtered immediately under vacuum through a nitrocellulose (Millipore) filter (0.45 μm pore diameter). The filter was rinsed with an additional 7 ml of stop solution and the radioactivity from the filter was measured. For the estimation of the intravesicular volume we used the method of D-glucose at equilibrium [30].

Irradiation procedure

BBM vesicles were exposed to γ-rays in a Model 220 Gammacell ^{60}Co irradiator (Atomic Energy of Canada) at a dose rate of approx. 0.75 Mrad/h [37]. During this procedure the samples

were kept at −78 °C with crushed dry ice. The target size (*TS*) was calculated from the loss of intensity of the immunodetected protein bands corresponding to the phosphate co-transporter by using the following empirical equation [38], which is valid for samples irradiated at −78 °C:

$$TS = 1.29 \times 10^6 / D_{37}$$

where D_{37} is the radiation dose (in Mrad) at which the intensity of the immunodetected bands has been decreased to 37 % of its initial value. D_{37} values were obtained from semilogarithmic plots of the absorbance against irradiation dose by using a least-squares fit. As an internal control for the irradiation procedure we used alkaline phosphatase and 5′-nucleotidase. Both were assayed by standard procedures [39].

RESULTS

Polyclonal antibodies directed against the N-terminal and C-terminal portions of the rat renal Na^+/P_i co-transporter NaPi-2 [10] were raised in rabbits, affinity-purified and used to identify related proteins in BBM from rats. Adaptation to chronic dietary phosphate deprivation is well known to involve an increase in Na^+/P_i co-transport in BBM from the proximal tubule [40,41]. Under such conditions of low dietary phosphate, the initial rate of Na^+-dependent phosphate transport into BBM vesicles is increased 3–4-fold [18]. The low dietary phosphate intake led to a 15–25-fold increase in the expression of NaPi-2-related proteins as assessed by Western blot analysis (Figure 1). Such an induction has previously been shown for p70 [22,42]. When 1 μM antigenic peptide was added during incubation with the antibodies, no proteins were detected (results not shown), demonstrating the specificity of the antibodies. Under non-reducing conditions, probing with N-terminal antibodies revealed proteins of 70–90 and 180 kDa, the first being designated the p70 group, and the latter p180. C-terminal antibodies also detected a 180 kDa protein along with a large band migrating as proteins of 70–90 kDa (also designated the p70 group). In the presence of 1 % (v/v) β-mercaptoethanol or 5 mM DTT, p180 was not detected by either antibody. However, new lower-molecular-mass polypeptides became apparent. N-terminal antibodies revealed proteins of 45 and 49 kDa (together designated the p45 group), while C-terminal antibodies recognized a protein migrating slightly faster, with a molecular mass of approx. 40 kDa (p40). Not only is the expression of p40 induced by a low-phosphate diet as previously shown [22], but so is the expression of p45. The amount of detected proteins was a linear function of the amount of protein from rat BBMs loaded on the gel (Figure 2).

To investigate the relationship between these NaPi-2-related proteins, slices corresponding to the regions of 70–90 and 180 kDa were excised from a first gel made without any reducing agent. Proteins from the excised gel slices were resolved on a second SDS/PAGE gel in the presence or absence of β-mercaptoethanol and analysed by Western blotting with C-terminal antibodies (Figure 3). In the presence of β-mercaptoethanol, the p70 band became much fainter and a strong band corresponding to p40 became apparent. Under the same conditions, p180 was resolved into p40 and p70, with very little p180 remaining. Reprobing of the blot with N-terminal antibodies yielded the same results, except that p45 was detected instead of p40 (results not shown).

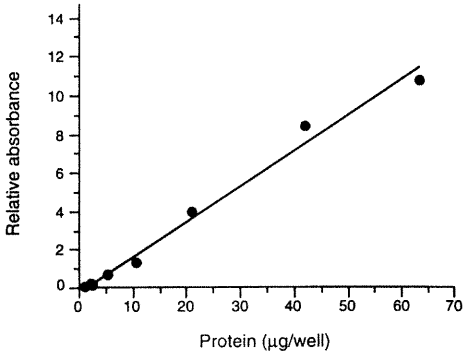

Figure 2 Calibration of immunoblots

BBM proteins were resolved by SDS/PAGE and transferred to poly(vinylidene difluoride) membranes as described in the Materials and methods section. The Western blots were incubated with affinity-purified antibodies, followed by donkey anti-rabbit IgG horseradish peroxidase-linked antibodies. The labelled proteins were revealed with enhanced chemiluminescence Western blotting solutions. The intensities of the specifically labelled protein bands corresponding to p70 were measured by densitometric scanning of the Western blots and analysed with IPLab Gel H program.

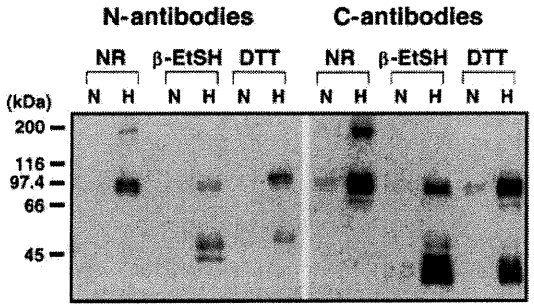

Figure 1 Immunodetection of rat renal BBM proteins with polyclonal antibodies directed against the sodium/phosphate co-transporter NaPi-2

BBM were prepared from rats fed with a control (N) or a low-phosphate (H) diet. Proteins were solubilized in sample buffer without any reducing agent (NR) or containing 1 % β-mercaptoethanol (β-EtSH) or 5 mM DTT. These protein samples were resolved by SDS/PAGE [7.5 % (w/v) gel]. Immunoblots were performed as described in the Materials and methods section with antibodies directed against the N-terminal and C-terminal portions of NaPi-2.

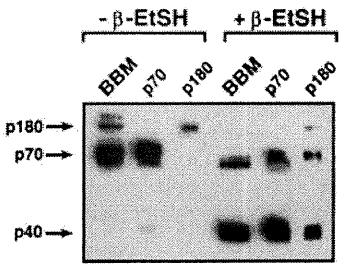

Figure 3 Relationship between NaPi-2-related proteins

BBM proteins were resolved on a first gel under non-reducing conditions. The regions of the gel containing proteins of 70–90 kDa (p70) and 180 kDa (p180) were obtained by slicing the gel. Excised gel slices were incubated in sample buffer containing either 5% (+) or no (−) β-mercaptoethanol (β-EtSH) for 30 min at 25 °C. Samples were loaded on a second gel (12.5% polyacrylamide) for Western blot analysis. BBM protein preparations (BBM) were also loaded on the second gel as controls. The blots were probed with antibodies directed against the C-terminal portions of NaPi-2 and analysed as described in the Materials and methods section.

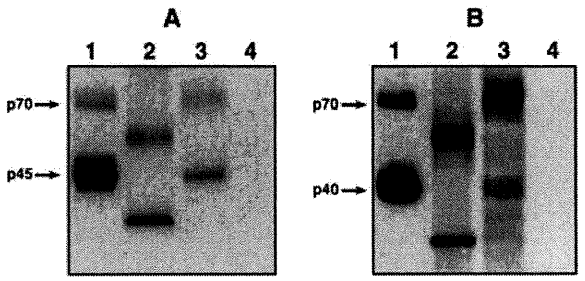

Figure 4 Deglycosylation of renal BBM NaPi-2-related proteins

BBM proteins were solubilized in 1% SDS, followed by incubation overnight at 37 °C with endoglycosidase F/N–glycosidase F, and resolved by SDS/PAGE [7.5% (w/v) gel] under reducing conditions (lanes 2). Both N-terminal (**A**) and C-terminal antibodies (**B**) were used for the detection of NaPi-2-related proteins. Three controls were included: BBM proteins loaded directly on the gel without incubation (lanes 1), BBM proteins incubated overnight without glycosidase (lanes 3) and the enzyme mixture alone loaded on the gel without incubation (lanes 4).

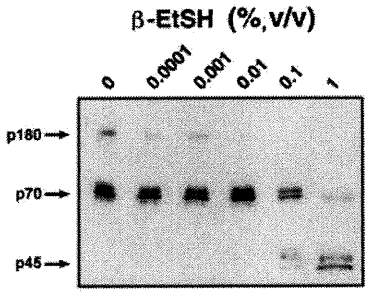

Figure 5 Effect of β-mercaptoethanol concentration on the electrophoretic mobility of solubilized NaPi-2-related proteins

BBM proteins were first solubilized in sample buffer containing 1% SDS without reducing agent. β-mercaptoethanol (β-EtSH) was then added at the indicated concentrations. Membrane proteins were resolved and Western blots were probed with N-terminal anti-NaPi-2 antibodies as described in the Materials and methods section.

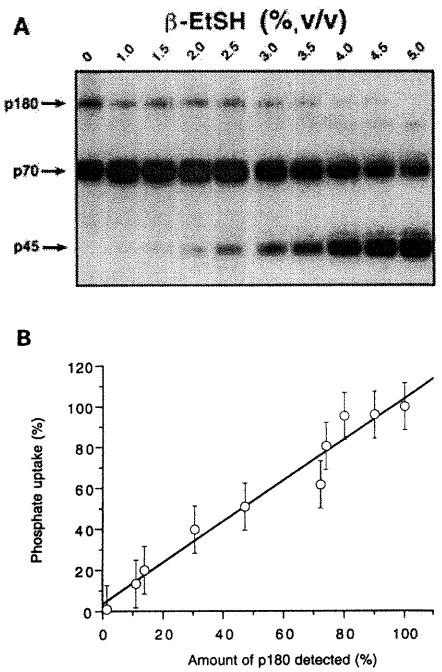

Figure 6 Effect of β-mercaptoethanol on the electrophoretic mobility of NaPi-2-related proteins and phosphate uptake into BBM vesicles

BBM vesicles were incubated in buffer A containing the indicated concentrations of β-mercaptoethanol (β-EtSH) as described in the Materials and methods section. To remove any unreacted β-mercaptoethanol, samples were centrifuged and pellets were washed twice with buffer A. Proteins were resolved under non-reducing conditions and analysed in Western blots with N-terminal antibodies (**A**). Phosphate uptake into vesicles and the amount of p180 detected were plotted, with each point corresponding to a different concentration of β-mercaptoethanol (**B**): 100% phosphate uptake represents the phosphate transported in the absence of any reducing agent, which corresponds to 3.6×10^5 pmol of P_i/s per μg of protein; 100% p180 detection represents the amount of p180 detected under non-reducing conditions as obtained by densitometric scanning of the band obtained on the X-ray film after Western blotting.

Glycosidase digestion of BBM proteins resulted in a significant shift in the migration of NaPi-2-related proteins (Figure 4). The apparent molecular mass of p70 was decreased to 57 kDa (Figures 4A and 4B, lanes 2) and that of p40 was decreased to 30 kDa (Figure 4B, lane 2). Reprobing the same blot with N-terminal antibodies showed that p45 was deglycosylated to a 35 kDa polypeptide (Figure 4A, lane 2). The control samples incubated overnight without endoglycosidase F/N–glycosidase F (Figures 4A and 4B, lanes 3) were unchanged. No protein band was detected when the glycosidase was loaded alone (Figures 4A and 4B, lanes 4).

To examine further the effect of reducing agents on the electrophoretic mobility of NaPi-2-related proteins, BBM vesicles from rats fed with a low-phosphate diet, which express a greater amount of NaPi-2, were either solubilized before (Figure 5) or after (Figures 6A, 7A and 8A) treatment with reducing agents. First, BBM vesicles were previously solubilized with sample buffer containing SDS, incubated with increasing concentrations of β-mercaptoethanol and analysed with N-terminal antibodies (Figure 5). The amount of p180 detected in the absence of β-mercaptoethanol was much smaller than that of p70. Between 0.01 and 1% β-mercaptoethanol the detection of p180 was abolished. The amount of p70 detected increased as the concentration of β-mercaptoethanol was raised from 0.001 to 0.01%, and decreased rapidly as the reducing agent was brought to higher concentrations. p45 became apparent when at least 0.1% β-mercaptoethanol was added to the BBM proteins, and the amount detected increased further when the concentration of β-mercaptoethanol was raised to 1%.

Secondly, when intact membrane vesicles were pretreated with various concentrations of reducing agents and proteins were then solubilized under non-reducing conditions before analysis in Western blots, p180 was still detected along with p70 with up to 3.5–4% β-mercaptoethanol (Figure 6A), 100 mM DTT (Figure 7A) or 5 mM TCEP (Figure 8A). This result also shows that all reducing agents do not possess the same reducing power on NaPi-2 (DTT reacts at higher concentrations than β-mercaptoethanol or TCEP). At higher concentrations p180 was no longer detected, whereas the p70 signal decreased and that of p45 increased. This pattern was similar to that observed with previously SDS-solubilized BBM proteins (Figure 5), with the notable difference that much higher concentrations of β-mercaptoethanol were required to obtain the same immunodetection band profile.

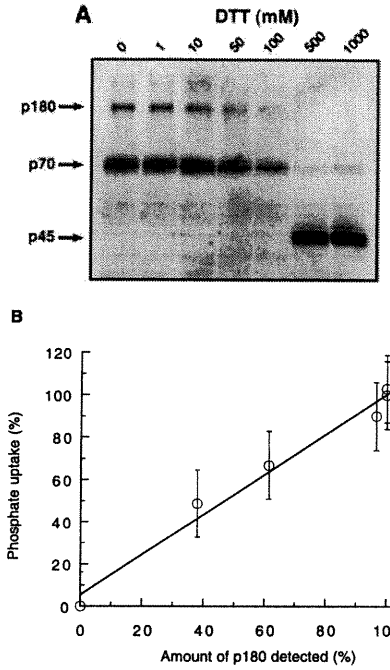

Figure 7 Effect of DTT on the electrophoretic mobility of NaPi-2-related proteins and phosphate uptake into BBM vesicles

BBM vesicles were incubated in buffer A containing the indicated amount of DTT as described in the Materials and methods section, and washed as described in the legend to Figure 6 to remove any unreacted DTT. Proteins were resolved under non-reducing conditions and analysed in Western blots with N-terminal antibodies (**A**). Phosphate uptake into vesicles and the amount of p180 detected were plotted, with each point corresponding to a different concentration of DTT (**B**); 100% phosphate uptake represents the phosphate transported in the absence of any reducing agent, which corresponds to 3.6×10^5 pmol of P_i/s per μg of protein; 100% p180 detection represents the amount of p180 detected under non-reducing conditions as obtained by densitometric scanning of the band obtained on the X-ray film after Western blotting.

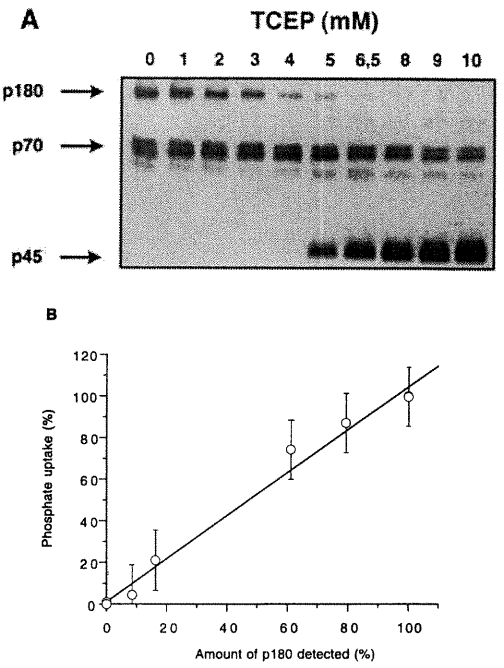

Figure 8 Effect of TCEP on the electrophoretic mobility of NaPi-2-related proteins and phosphate uptake into BBM vesicles

BBM vesicles were incubated in buffer A containing the indicated amount of TCEP as described in the Materials and methods section, and washed as described in the legend to Figure 6 to remove any unreacted TCEP. Proteins were resolved under non-reducing conditions and analysed in Western blots with N-terminal antibodies (**A**). Phosphate uptake into vesicles and the amount of p180 detected were plotted, with each point corresponding to a different concentration of TCEP (**B**); 100% phosphate uptake represents the phosphate transported in the absence of any reducing agent, which corresponds to 3.6×10^5 pmol of P_i/s per μg of protein; 100% p180 detection represents the amount of p180 detected under non-reducing conditions as obtained by densitometric scanning of the band obtained on the X-ray film after Western blotting.

A linear correlation was observed between phosphate transport activity and the amount of p180 detected in BBM vesicles pretreated with different concentrations of β-mercaptoethanol (Figure 6B), DTT (Figure 7B) or TCEP (Figure 8B). Such a correlation was observed only for p180; in fact, as the concentration of β-mercaptoethanol was brought from 0 to 3%, the intensity of p45 and p70 increased, whereas the transport activity decreased markedly (Figure 6A). When vesicles were pretreated with any of these three reducing agents and then washed (i.e. uptake and detection were determined under non-reducing conditions), their intravesicular volume remained unchanged, showing that the vesicles kept their integrity (results not shown). The uptake was linear for at least 10 s of incubation; the transport measurements used, which were done at 9 s, were thus within the linear portion of the curve (Figure 9).

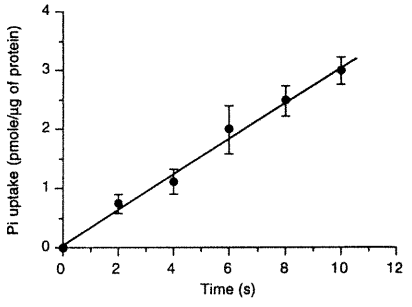

Figure 9 Initial rate of phosphate uptake by BBM vesicles

BBM vesicles were isolated as described in the Materials and methods section. Phosphate uptake was measured in the presence of 150 mM NaCl as described in the text. Each value represents the mean ± S.D. for three experiments in which each assay was performed in quadruplicate.

Exposure of BBM vesicles to ionizing radiation resulted in a dose-dependent decrease in the intensity of the NaPi-2-related proteins (Figure 10A). The radiation-induced disappearance of p40, p70 and p180, each detected with C-terminal antibodies, was a simple exponential function of the radiation dose (Figure 10B). The target sizes calculated from the data shown in Figure 10(B) were 178 ± 20 kDa (mean ± S.D.) for p40, 179 ± 18 kDa for p70 and 199 ± 22 kDa for p180. When the vesicles were pretreated with 2.5% or 5% β-mercaptoethanol [to obtain an optimal amount of p70 or p40 respectively (Figure 11A)] before irradiation, the *TS* values obtained from the radiation-induced disappearance of p40 and p70 (Figure 11B) were approx. 74 and 92 kDa respectively (from Figure 11C). As previously shown, the intravesicular volume was not affected by the radiation dose [30].

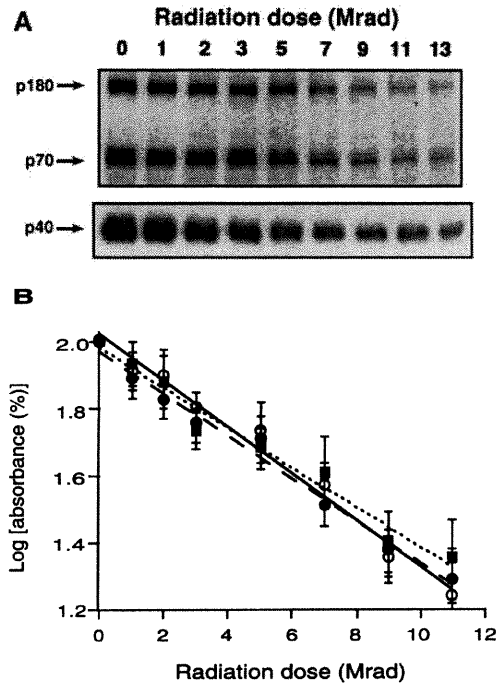

Figure 10 Determination of the target size of the rat renal sodium/phosphate co-transporter NaPi-2

BBM vesicles were irradiated and analysed in Western blots with C-terminal antibodies as described in the Materials and methods section. (**A**) Effect of radiation dose on the structural integrity of NaPi-2-related proteins. For the analysis of p180 and p70, electrophoresis was performed in the absence of reducing agents. Samples used for the analysis of p40 were solubilized in sample buffer containing 5% β-mercaptoethanol. (**B**) Disappearance of the immunodetected proteins was measured by laser densitometry. The results are expressed as the logarithm of the percentage area remaining under each peak relative to the unirradiated control for p180 (○), p70 (●) and p40 (■). Each value represents the mean ± S.D. for four independent experiments.

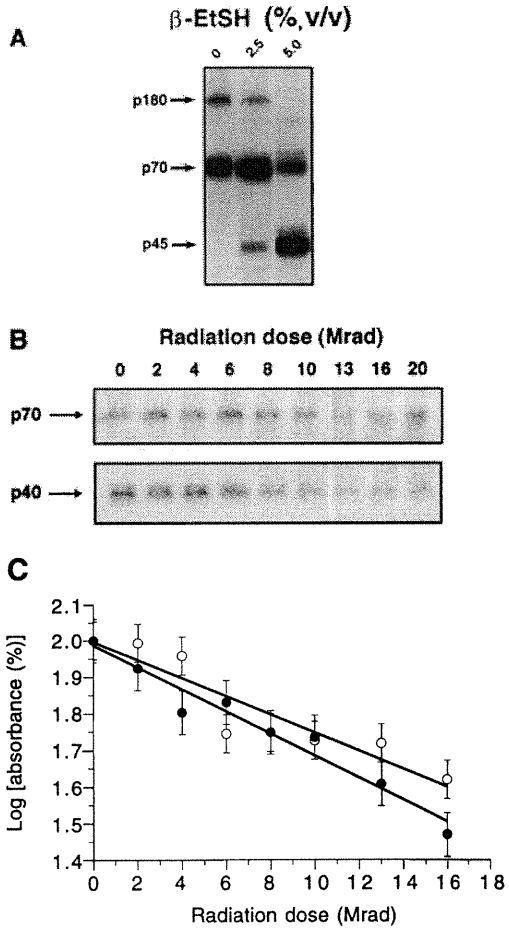

Figure 11 Determination of the target size of the rat renal sodium/phosphate co-transporter NaPi-2 after pretreatment with β-mercaptoethanol

BBM vesicles were pretreated with 0%, 2.5% and 5% β-mercaptoethanol (β-EtSH), then irradiated and analysed in Western blots with C-terminal antibodies as described in the Materials and methods section. (**A**) Immunodetection of NaPi-2-related proteins pretreated with 0%, 2.5% and 5% β-mercaptoethanol. (**B**) Effect of radiation dose on the structural integrity of NaPi-2-related proteins pretreated with 0%, 2.5% (for optimal p70 detection) or 5% β-mercaptoethanol (for optimal p40 detection). ECL detection was done with Bio-Rad's Molecular Imager System. (**C**) Disappearance of the immunodetected proteins was measured with Bio-Rad's Molecular Imager System and the IPLab Gel software from Molecular Dynamics. The results are expressed as the logarithm of the percentage area remaining under each peak relative to the unirradiated control for p70 (●) and p40 (○). Each value represents the mean ± S.D. for two independent experiments.

To establish the validity of the irradiation method in our system, the inactivation of two intrinsic enzyme markers was investigated. For alkaline phosphatase the inactivation curve gave a molecular mass of 104800 ± 3500 Da (Figure 12A). This value is similar to that reported for calf kidney alkaline phosphatase (115 kDa) estimated by the same method [43] and for isolated alkaline phosphatase [44] purified by SDS/PAGE (120 kDa). The estimated molecular mass of 5′-nucleotidase was 89400 ± 1800 Da (Figure 12B). This is similar to the value estimated in fibroblasts (80 kDa) [45], but somewhat higher than that found in rabbit kidney (58 kDa) [46].

DISCUSSION

The results of the present study clearly demonstrate a strong effect of reducing agents on the structure and function of the rat renal Na^+/P_i co-transporter NaPi-2. Under non-reducing conditions the major proteins detected with antibodies directed against the N-terminal or C-terminal portions of NaPi-2 had a molecular mass of 70–90 kDa (p70). Because NaPi-2 is glycosylated [10,17], this size is in good agreement with that calculated from its amino acid sequence (68.7 kDa) [10]. Previous studies with polyclonal antibodies have demonstrated that NaPi-2 is present as proteins of 80–90 kDa in the rat renal BBM [17,47] and 70–110 kDa in cRNA-injected *Xenopus laevis* oocytes [17]. Under reducing conditions, however, additional proteins of 45–49 kDa (p45) and 40 kDa (p40) were detected with N-terminal and C-terminal antibodies respectively. Recent studies have shown the presence of a 51 kDa protein in OK cells by using antibodies against NaPi-4 [48,49], and a NaPi-2-related 37 kDa protein in mouse BBM [50]. The authors assume these lower bands are degradation products. In contrast, our results clearly show that p45 and p40 are not degradation proteins (Figure 3). Furthermore the addition of protease inhibitors during BBM purification or the incubation of purified BBM with protease inhibitors were without any effect on the detection of p70, p45 and p40, indicating that p45 and p40 are not degradation artifacts created during membrane isolation. Using C-terminal

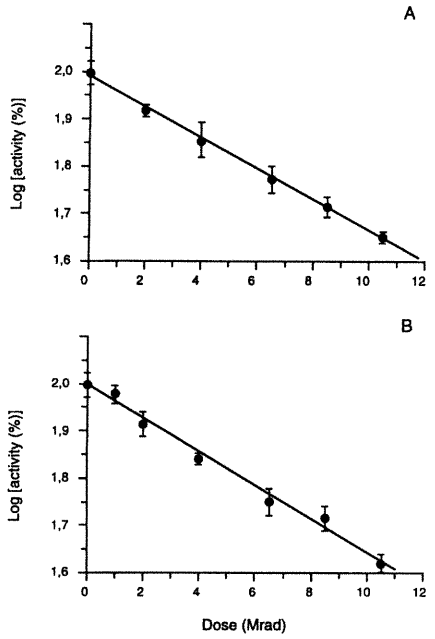

Figure 12 Radiation-inactivation curves for alkaline phosphatase and 5′-nucleotidase in membrane vesicles

BBM vesicles were irradiated at the indicated doses, and phosphatase (**A**) and 5′-nucleotidase (**B**) activities were measured with *p*-nitrophenyl phosphate or AMP as substrate respectively [37] ($n = 4$).

antibodies, we have recently shown by tryptic proteolysis experiments that p40 is closely related to p70 [22]. In the present study we demonstrate that an increase in the amount of p40 or p45 detected, owing to the presence of β-mercaptoethanol, DTT or TCEP, is accompanied by a loss of p70. Each of these three reducing agents possesses a different strength of reduction on NaPi-2 (e.g. DTT reacts at much higher concentrations than β-mercaptoethanol or TCEP). These results strongly suggest that p70 is composed of two protein fragments held together by one or more disulphide bonds and resulting from a specific post-translational proteolytic cleavage of the NaPi-2 polypeptide. Both of these fragments, p40 and p45, were shown to be glycosylated. Because NaPi-2 has previously been shown, with site-directed mutagenesis experiments, to be glycosylated at only two of its four potential N-glycosylation sites [17], the proteolytic cleavage site could be located between Asn-298 and Asn-328. Our proposed model is reinforced by a recent report suggesting that NaPi-II is a co-transporter that could originate from two ancient independent genes [51]. The first would represent a protein with three transmembrane regions; the second would represent a protein with four transmembrane regions. These proteins could represent p45 and p40 respectively.

Glycosidase digestion significantly decreased the apparent molecular masses of p40, p45 and p70. After deglycosylation, p70 migrated as a protein of 57 kDa, a value somewhat smaller than that calculated from the amino acid sequence of NaPi-2 (68.7 kDa) [10]. This difference, however, might be due to the presence of disulphide bonds in p70, which could affect its electrophoretic mobility. In contrast, once deglycosylated, p40 and p45 had apparent molecular masses of 30 and 35 kDa respectively. The sum of these values is in remarkably good agreement with the predicted size of NaPi-2, suggesting that little or no further proteolysis occurs after the initial cleavage of the native polypeptide.

Both antibodies also detected a protein of 180 kDa (p180) that disappeared gradually as the concentration of reducing agent was increased, with a concomitant increase in the detection of the lower-molecular-mass NaPi-2-related proteins. p180 thus seems to correspond to an oligomeric form of NaPi-2 composed of p70 subunits linked through disulphide bonds. These disulphide bridges could be involved in stabilizing the functional oligomeric structure of the Na^+/P_i co-transporter molecule, as was demonstrated for the type-C natriuretic peptide receptor [52] and the GLUT1 glucose transporter [53]. A striking correlation was demonstrated between the transport activity and amount of p180 detected. When p180 was reduced by the addition of any of the three reducing agents, detection of p70 was increased but p180 disappeared in correlation with a diminution of phosphate uptake into the vesicles. This, as well as the target sizes obtained for NaPi-2-related proteins under non-reducing conditions, strongly suggests that the protein responsible for the transport of phosphate into vesicles is an oligomer rather than a monomer.

At a given concentration the reducing agents had a much weaker effect on the electrophoretic mobility of NaPi-2-related proteins when added to intact BBM vesicles than when added to solubilized proteins. The inter-polypeptide disulphide bonds thus seem to be protected in the intact membrane and become readily exposed only once the membrane proteins have been solubilized and unfolded by SDS.

Our results are at variance with those of Suzuki et al. [54], who reported a slight but significant stimulation of phosphate transport in rabbit renal BBM vesicles preincubated with 5 mM DTT. Phosphate transport was also increased in vesicles reconstituted from bovine renal BBM when 1 mM DTT was included in the buffer used to solubilize the proteins [55]. The different effects of reducing agents observed in these studies could, however, be due to the use of different animal species and experimental conditions. For example, in the present study, the vesicles were incubated at least twice as long and, for most experiments, with higher concentrations of the reducing agents.

Similar target sizes, ranging from 178 to 199 kDa, were estimated from the radiation-induced disappearance of p40, p70 and p180 protein bands in Western blots probed with C-terminal antibodies. These values, which are not significantly different from one another, strongly suggest that all three polypeptide species are part of the same oligomeric structure. Because the oligosaccharide moiety of glycoproteins does not contribute to the molecular sizes determined with the radiation inactivation method [26–29], these TS values correspond to almost three times the size of a p70 subunit. Target sizes corresponding to three times the molecular mass of the constituent monomer have also been measured for the type I Na^+/P_i co-transporter [30]. In both types of phosphate co-transporter, the different polypeptides thus seem to be closely associated with one another because, after a single radiation hit, the absorbed energy causes the fragmentation of three subunits. Disulphide bonds between p70 subunits might contribute to this energy transfer. The results obtained from vesicles pretreated with β-mercaptoethanol before irradiation demonstrate the effect of reducing agents on the state of oligomerization of NaPi-2. Under these reducing conditions, NaPi-2 exists as a monomer, not an oligomer.

The size of the functional rat renal Na^+/P_i co-transporter complex, estimated from the radiation-induced loss of phosphate transport activity in BBM vesicles, has been previously shown to be 234 kDa [56]. Similarly, large radiation inactivation sizes have also been reported for the Na^+/P_i co-transporters of rabbit [30],

mouse [57] and cow [58]. Phosphate transport across the rat renal BBM is, however, probably not performed only by NaPi-2 but also by a type I phosphate co-transporter. Although no type I co-transporter has yet been cloned from rat, both types of phosphate co-transporter have been cloned from rabbit [7,14], human [8,10] and mouse [9,12,13] kidney cortex. In addition, a protein of 65 kDa is detected specifically in Western blots of rat BBM proteins with antibodies raised against the C-terminal portion of the rabbit type I co-transporter NaPi-1, a region in which NaPi-1 and NaPi-2 share little similarity [18,30]. Because type I co-transporters are smaller than type II co-transporters, the size of the functional NaPi-2 oligomer is thus probably larger than 234 kDa. NaPi-2 would consist of a tetramer composed of four p70 subunits each composed of a p40 and a p45 polypeptide. Disulphide bonds would play an important role in the structure and function of this Na^+/P_i co-transporter by holding p40 and p45 polypeptides together within each subunit and forming bridges between some of the p70 subunits.

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Membrane topography of the renal phosphate carrier NaPi-2: limited proteolysis studies[1]

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Abstract

The rat sodium/phosphate cotransporter NaPi-2 is a 70 kDa polypeptide (p70) for which eight transmembrane segments have been predicted. We have shown that p70 exists predominantly as p45 and p40 fragments which are linked by disulfide bonds. In this work, the p40 fragment, corresponding to the C-terminus of NaPi-2, was purified from renal brush-border membranes using non-reducing and then reducing column electrophoresis followed by enzymatic deglycosylation and SDS-PAGE. The N-terminal sequence obtained for this fragment, VEAIG, indicates that the formation of p45 and p40 arises from the cleavage of p70 between arginine-319 and valine-320. In order to determine the membrane topography of NaPi-2, brush-border membrane vesicles were digested with various proteases and the transporter-derived proteolytic peptides were subsequently identified by Western blotting using N- and C-terminal-directed antibodies. Our results lead us to propose an alternative topographical model in which p45 and p40 possess three transmembrane domains each and indicate that the processing site of p70 for the generation of p45 and p40 is localized in a large protein core facing the extracellular milieu. This localization of the cleavage site indicated that NaPi-2 could either be processed intracellularly by vesicular proteases or extracellularly by secretory proteases or by brush-border membrane ectoenzymes.

Keywords: Phosphate transport; Rat kidney; Immunoblotting; Microsequence; Protein structure; Membrane vesicle

1. Introduction

Renal re-absorption of inorganic phosphate (Pi) from the glomerular filtrate takes place essentially across the brush-border membrane (BBM) of the proximal tubule. This process, under hormonal and cellular control [1–3], involves specific sodium gradient-dependent transport systems [4,5]. These systems have been well-characterized at the functional level and the cloning of proximal tubular sodium/phosphate (Na^+/Pi) cotransporters from several animal species has expanded our knowledge at the molecular level [6–10].

Several studies have reported the primary structure

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of two types of renal Na^+/Pi cotransporter, types I and II [3]. Furthermore, the membrane receptor for the gibbon ape leukemia virus, or GLVR-1, has been identified as a third type of phosphate carrier [11,12]. In contrast to types I and II which are restrictively located in the kidney, type III shows a broad tissue distribution [11–13]. Among the three types, only type II was clearly shown to be implicated in the adaptive response to a low phosphate diet [10,14]. Type I transporters have a predicted M_r which is lower than that of type II molecules and show very little homology to them [3,6]. NaPi-2, a type II Na^+/Pi cotransporter cloned from a rat kidney cortex cDNA library, is encoded as an unique 637 residues polypeptide which behaves as a *N*-glycosylated 70 kDa protein (p70) when expressed in *Xenopus laevis* oocytes [6,7]. *N*-glycosylation occurs on the extracellular asparagine residues 298 and 328 [15] and, although not essential for the transport function, is thought perhaps to be important for the targeting of NaPi-2 to the plasma membrane [16]. NaPi-2 has been predicted to possess several phosphorylation sites for protein kinase C, a leucine zipper motif and eight transmembrane domains. The N- and C-termini of the polypeptide were shown to face the cytoplasm [3,7]. The leucine zipper structure could be used by NaPi-2 for dimerization since the cotransporter was demonstrated to form functional oligomers of 180 kDa (p180) [17]. We have shown that p70 also exists as a cleaved species formed by the disulfide linkage of the N-terminal (p45) and the C-terminal (p40) moieties of the polypeptide [18]. Cleavage of NaPi-2 into two fragments was previously reported by Biber et al. [19]. However, the site of proteolytic processing in the p70 sequence has not been identified.

Limited proteolysis is a powerful tool for determining the topography of membrane proteins since membrane-embedded regions are known to be protected from digestion [20–22]. This approach was used to study the membrane arrangement of troponin C [23], of the band 3 erythrocyte anion transport protein [24] and of the red cell membrane calcium pump [25] and should be applicable for studying the topography of NaPi-2 in BBM and hence determining if p70-specific cleavage could be an intra- or extracellular phenomenon.

In the present study, we first purified p40 and subjected it to microsequencing in order to determine the site used in vivo for cleavage of the p70 species. The purification protocol combined two different steps of continuous elution electrophoresis, one in the absence and one in the presence of reducing agents, followed by enzymatic deglycosylation and SDS-PAGE. We also combined limited proteolysis and Western blotting, using polyclonal antibodies recognizing the N- and C-termini of NaPi-2, to experimentally determine the topographical arrangement of the transporter in BBM and the relation of the p70-specific cleavage site with this arrangement. We propose a model in which the cleaved bond of NaPi-2, Arg_{319}-Val_{320}, is located in a large protein core of the polypeptide exposed to the extracellular environment.

2. Materials and methods

2.1. Preparation of BBM vesicles

Two-months-old male Sprague-Dawley rats (300–350 g) were purchased from Charles River (St-Constant, Que., Canada) and fed with a low phosphate diet (number 86128 from Teklad, Madison, WI, USA) containing 0.03% (w/w) phosphate, for 3 days, as described [18]. This diet was shown to increase the NaPi-2 expression [26]. BBM vesicles were prepared from kidney cortices of these rats using a $MgCl_2$ precipitation method [27]. The final pellet was resuspended in buffer A (20 mM HEPES/Tris, pH 7.5, 300 mM mannitol) containing the following protease inhibitors at the indicated concentrations: chymostatin A 10 μg/ml, bacitracin 10 μg/ml, pepstatin 10 μg/ml and aprotinin 1.85 μg/ml and was stored in liquid nitrogen until use. The protein concentration was determined by the method of Bradford [28] using bovine serum albumin as a standard. Alkaline phosphatase, a BBM marker enzyme, was assayed as described by Kelly and Hamilton [29] and was found to be enriched 10–15-fold in the final vesicle preparations relative to the cortex homogenates. It has already been shown that more than 85% of BBM vesicles prepared according to the above protocol have a right side out orientation [30].

2.2. Antibodies

The synthetic 15-mer CPRLALPAHHNATRL and 14-mer MMSYSERLGGPAVS peptides, corresponding to the C- and N- termini of NaPi-2, respectively, have previously been used for the production of polyclonal antibodies in rabbits [18,26]. The resulting antibodies were purified by affinity chromatography as described previously [18,26].

2.3. Western Blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as by Laemmli [31] using a Mini-Protean II apparatus (Bio-Rad, Mississauga, Ont., Canada). Unless indicated otherwise, the samples were mixed prior to electrophoresis with sample buffer to a final concentration of 20% (w/v) glycerol, 2% (w/v) SDS, 0.05% (w/v) bromophenol blue and 62.5 mM Tris-HCl, pH 6.8, with or without β-mercaptoethanol (β-EtSH). Electrophoresis was carried out at 10 mA/0.75 mm gel for about 1.5 h. Following electrophoresis, the proteins were electro-transferred onto polyvinylidene difluoride (PVDF) microporous (0.45 μm pore diameter) membranes (Millipore, Mississauga, Ont., Canada) using a Milliblot Graphite Electroblotter I apparatus (Millipore) at 1 mA/cm^2 for 1 h at room temperature. Non-specific binding sites on the PVDF membranes were blocked by overnight incubation in 150 mM NaCl, 0.2% (w/v) Tween-20 and 50 mM Tris-HCl, pH 7.5 (buffer B), containing 5% (w/v) powdered milk, at 4°C. The membranes were washed three times by gentle agitation in buffer B for 10 min and incubated for 1 h with anti-N- or anti-C-terminal antibodies diluted 10 000-fold in buffer B. After three more washes in buffer B, the membranes were incubated for 1 h with a donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham, Oakville, Ont., Canada) and diluted 1000-fold in buffer B. The immune complexes were revealed by enhanced chemiluminescence using an ECL kit (Amersham). The membranes were exposed to X-ray films which were pre-flashed with a Sensitize flashgun unit (Amersham). The films were developed with a Mini-Med/90 X-ray film processor (AFP Imaging, Elmsford, NY, USA), scanned with a Personal Densitometer SI scanner (Molecular Dynamics) and analyzed with the IPLab Gel H program (Signal Analytics Corporation).

2.4. Purification of p40

BBM proteins were resolved by continuous elution electrophoresis on a model 491 Prep-Cell column (Bio-Rad, Mississauga, Ont., Canada) as follows. BBM proteins (60 mg) were mixed with sample buffer containing 20% (w/v) glycerol, 1% (w/v) SDS, 0.05% (w/v) bromophenol blue and 0.625 M Tris-HCl, pH 6.8, in the absence of a reducing agent. The mix was applied onto an electrophoresis column formed by a 6 cm long separating gel containing 4.5% (w/v) acrylamide/bisacrylamide (30% T, 2.67% C). Electrophoresis was carried out at 45 mA for about 7 h at a flow rate of 0.75 ml/min. Fractions of the effluent were collected and those containing NaPi-2 p180, as assayed by Western blotting, were pooled and concentrated at 2000×g for 10 min with a Centricon-30 concentrator (Amicon, Beverley, MA, USA), following which β-EtSH was added to a final concentration of 5% (v/v). This concentration of the reducing agent generates maximal levels of p40 and p45 from p180 [18]. The reduced sample was electrophoresed on a second continuous elution column under the conditions described above except that the separating gel contained 11% (w/v) acrylamide/bisacrylamide (30% T, 2.67% C). Fractions containing p40, as judged by a Western blot analysis, were pooled and concentrated to 300 μl with a Centricon-30 concentrator. An aliquot (65 μl) of the concentrated p40 pool was mixed with an equal volume of a 20 mM, pH 7.2, potassium phosphate buffer containing 0.6% (v/v) Brij-58, 0.2% (v/v) SDS and 50 mM EDTA. The resulting solution (130 μl) was subjected to deglycosylation by incubation with 6.5 μl of an endoglycosidase F/*N*-glycosidase F mixture (Boehringer-Mannheim, Laval, Que., Canada) for 17 h at 37°C with gentle shaking. The deglycosylated polypeptide solution was subjected to SDS-PAGE and to electrotransfer onto a PVDF (0.2 μm pore diameter) membrane (Bio-Rad) using 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid/NaOH, pH 8.0, as the transfer buffer. The membrane was stained with Coomassie blue and the protein band migrating with the size of deglycosylated p40 was analyzed by microsequencing.

2.5. Microsequencing

Microsequencing by automated Edman degradation was done for us by the Biotechnology Research Institute (Montreal, Que., Canada). Automated sequencing was performed with the 03RBLOT program on a model 470A gas phase sequencer from Applied Biosystems (ABI). The phenylthiohydantoin amino acid derivatives released at each cycle were determined by comparison with standard amino acid derivatives (ABI) during on-line analysis with a 120A PTH Analyser HPLC system (ABI).

2.6. Limited proteolysis of BBM vesicles

BBM vesicle samples containing to 100 μg of protein were incubated with trypsin (Sigma, St. Louis, MO, USA), chymotrypsin or proteinase K (both from Boehringer Mannheim, Que., Canada) for various times at 37°C in buffer A. Reactions were stopped by a 10-fold dilution of the reaction media with buffer A containing 1 mg/ml of the serine protease inhibitor PEFABLOC (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, from Boehringer Mannheim). Dilution in the presence of PEFABLOC completely inhibited further proteolysis. The resulting samples were then centrifuged at 50 000 × *g* for 15 min at 4°C. The pellets were resuspended in sample buffer at a final protein concentration of 3 μg/μl and analyzed by SDS-PAGE in the presence of 5% β-EtSH, followed by Western blotting as described above. Identification of the protease cleavage sites within the NaPi-2 polypeptide was done by comparing the sizes of the immunoreactive fragments obtained experimentally with the fragment sizes predicted from the amino acid sequence of the transporter using the MacVector software. Localization of potential transmembrane domains in the NaPi-2 structure was performed with the amino acid sequence of the protein according to Kyte and Doolittle's algorithm [32].

3. Results

3.1. Purification and microsequencing of p40

Analysis of BBM proteins by SDS-PAGE and Western blot techniques using antibodies raised against the N- and the C-terminal portions of NaPi-2 has previously shown that the transporter exists as a p180 (180 kDa) and a p70 (70–90 kDa) protein species in the absence of a reducing agent [18]. However, under reducing conditions, the p180 band disappeared whereas that of p70 remained, although at a lower level, and was detected along with a 45–49 kDa (p45) protein by the N-terminal antibody and a 40 kDa (p40) protein by the C-terminal antibody [18], two immunoreactive polypeptides that were previously detected by Biber et al. [19]. Both p45 and p40 were found to be glycosylated [18]. According to these results, we have proposed that, in the rat kidney, p70 exists not only as a single polypeptide chain but also as a cleaved species formed by the disulfide linkage of the two glycosylated protein fragments, p45 and p40 [18]. In addition, both the full-length and the cleaved p70 molecules are linked together to form the p180 species [18]. Cleavage of p70 is likely not an artefact of preparation. Indeed, as stated by Xiao et al. [18], addition of protease inhibitors during BBM vesicles preparation did not change the relative recovery of each protein species. Moreover, incubation of BBM vesicles for up to 120 min at 37°C in the absence or presence of protease inhibitors did not change immunoreactive profiles on SDS-PAGE (A. Dugré, unpublished results). In order to identify the site of cleavage for formation of p45 and p40, we purified p40, the C-terminal portion of NaPi-2 and determined its partial N-terminal sequence.

To purify p40, we designed a purification strategy that exploited the glycosylated character of the transporter [18] and its capacity to form oligomers [17]. We reasoned that, among the 180 kDa proteins that exist in the BBM, only NaPi-2 was likely to elute as a 40 kDa peptide in reducing SDS-PAGE and subsequently to be deglycosylated to a 30 kDa species. The purification protocol therefore comprized the following steps: (i) preparation of BBM vesicles from rats submitted to a low phosphate diet since this diet increases the expression of NaPi-2 in the kidney by about 10-fold [26], (ii) isolation of p180 by SDS-PAGE on a continuous elution column under non-reducing conditions to obtain maximal amounts of this form of the transporter, (iii) isolation of maximal amounts of p40 by column electro-

phoresis of p180 under reducing conditions and finally (iv) deglycosylation of p40 and purification of the 30 kDa deglycosylated polypeptide by SDS-PAGE.

The proteins obtained after each of the two electrophoresis procedures and after the deglycosylation reaction have been analyzed by SDS-PAGE and Western blotting using the NaPi-2 N- and C-terminal antibodies. Fig. 1A shows that the p180 forms of NaPi-2 eluted in fractions 22–37 from the first electrophoresis. These fractions were pooled, concentrated, mixed with 5% β-EtSH and subjected to the second electrophoresis column. Immunodetection indicated that fractions 60–77 from the second electro-

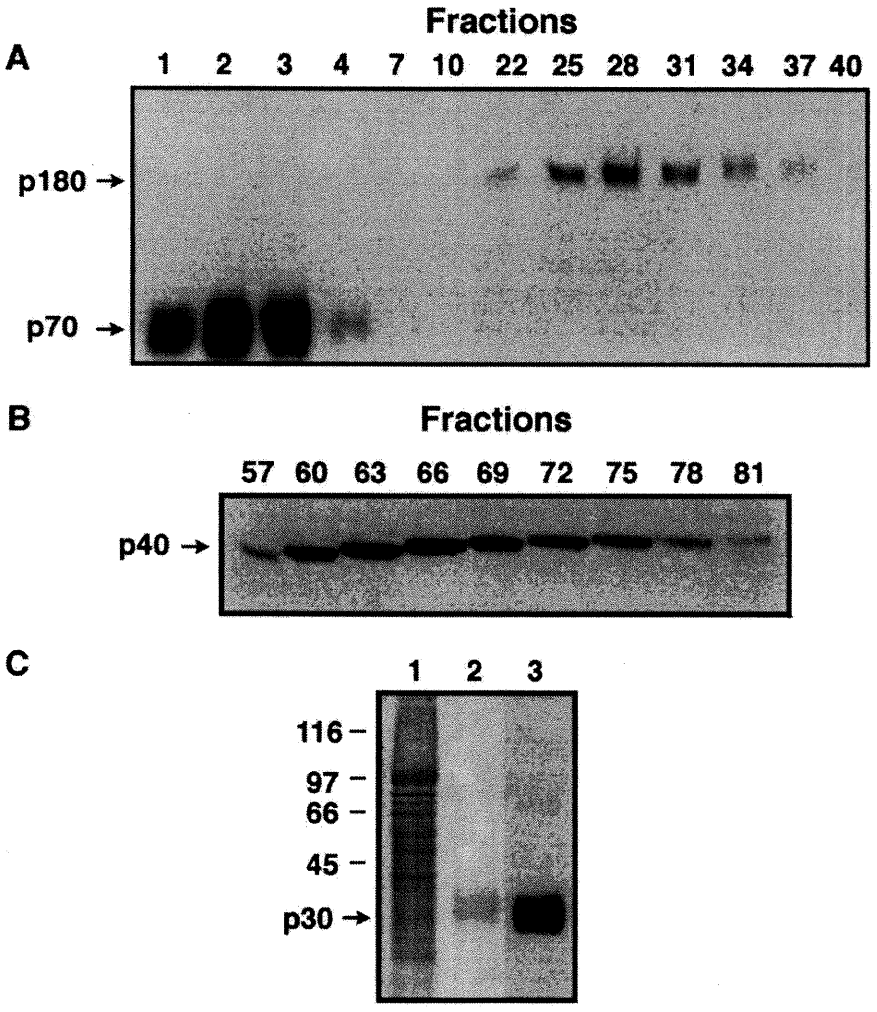


Fig. 1. Purification of the NaPi-2 p40 fragment by continuous elution electrophoresis and deglycosylation. (A) Aliquots of fractions (10 μl) obtained from the first electrophoresis (in the absence of a reducing agent) were subjected to SDS-PAGE followed by immunodetection with the antibody directed against the C-terminus of NaPi-2. Fractions 22–37 containing the p180 form of the protein were pooled, concentrated and submitted to a second continuous elution electrophoresis in the presence of 5% β-mercaptoethanol. (B) Aliquots of fractions (10 μl) obtained from the second electrophoresis were subjected to SDS-PAGE followed by immunodetection with the same antibody as in A. Fractions 60–77, containing p40, were pooled and concentrated and an aliquot of this pool was submitted to enzymatic deglycosylation. (C) A 5 μl aliquot of the deglycosylation mix containing p40 and endoglycosidases was subjected to SDS-PAGE and electrotransfer (lanes 2 and 3), together with 5 μg of BBM proteins (lane 1). The blot was stained with Coomassie blue (lanes 1 and 2) or immunodetected with the same antibody as in A (lane 3). The immunoreactive p30 band seen in lane 3 corresponds to the deglycosylated form of p40.

phoresis contained the p40 fragment resulting from the reduction of NaPi-2 (Fig. 1B). The apparent increase in the molecular size of the fragment seen from fractions 60–75 was likely due to the existence of p40 polypeptide subpopulations differing by their level of glycosylation [18]. Fractions 60–77 were pooled, concentrated and treated with endoglycosidase F/*N*-glycosidase F to generate the p30 deglycosylated derivative. The electrophoretic profile of the deglycosylation mix is compared with that of the corresponding BBM vesicle preparation in Fig. 1C. In contrast to the BBM preparation (Fig. 1C, lane 1), the deglycosylation mix almost exclusively contained proteins of about 30 kDa (lane 2). The 30 kDa band was immunoreactive when probed with the NaPi-2 C-terminal antibody (lane 3), indicating that it contained the transporter sequence. No other immunoreactive bands were detectable (lane 3), indicating that the deglycosylation was complete.

The deglycosylation mix was subjected to reducing SDS-PAGE and electro-transferred to a PVDF membrane, then the piece of membrane containing the deglycosylated p40 was excized for protein microsequencing. Two amino acids were released at each cycle of the sequencer but the predominant signal (about 60% of the total signal) identified the sequence VEAIG which corresponds to residues 320–325 of intact NaPi-2. The other signal, which accounted for approximately 30% or less of the total signal, gave a sequence (GLDRP) that neither exists in the structure of NaPi-2 p70 or in that of recently identified isoforms: NaPi-2α and NaPi-2β (both beginning with MMSYS) and NaPi-2γ (beginning with MLNSL) [33]. These isoforms, which have a M_r around 30 kDa once deglycosylated [33], could potentially participate in the assemblage of the p180 species. Furthermore, no alternate combination beginning with VL or GE can be matched with specific sites in the sequence of NaPi-2 forms indicating that p40 was the major species present in the deglycosylated mix and its formation was dependent on the processing of a unique site on the p70 structure. There are several explanations to the presence of the much weaker GLDRP signal. (i) It could arise from the endoglycosidases themselves since a control deglycosylation mix, containing these enzymes but not p40, produced a band at 35 kDa when analyzed by SDS-PAGE (not shown), (ii) it could arise from a BBM protein(s) which behaved like NaPi-2 during the electrophoretic purification steps or (iii) it could correspond not to a peptide sequence but to amino acids released by random chemical cleavages of endoglycosidase, NaPi-2 and/or other polypeptides during sequencing. This last explanation is plausible since GLDRP amino acids were identified from peaks extending only modestly beyond the baseline on the sequencer. Overall, the sequencing results indicate that the cleavage site in p70 which yields p40 and p45 is the peptide bond between Arg-319 and Val-320. If we subtract the contribution of the GLDRP signal to the yield of recovered amino acids on the sequencer, the sequencing results also indicate that the deglycosylated p40 obtained from BBM preparations was more than 87% pure. In addition, this sequencing analysis provided a direct proof that p180, p40 and p45 immunoreactive polypeptides are all directly related to NaPi-2.

3.2. *Limited proteolysis of BBM vesicles*

In the second part of this work, we studied the topography of NaPi-2 by subjecting BBM vesicles to limited proteolysis with enzymes of different specificities and by using Western blotting with the N- (Fig. 2) or C-terminal (Fig. 3) antibodies to identify the immunoreactive proteolytic fragments derived from the transporter. BBM vesicles were incubated with trypsin (Figs. 2A and 3A), chymotrypsin (Figs. 2B and 3B) or proteinase K (Figs. 2C and 3C) for various times. At time zero, the two bands seen with the N-terminal antibody correspond to NaPi-2 p45 (45 kDa) and to uncleaved p70 polypeptide (about 70 kDa) (Fig. 2), while those seen with the C-terminal antibody correspond to p40 (40 kDa) and to uncleaved p70 (Fig. 3). The intensity of these bands decreased or disappeared as the proteins they contained were digested by the proteases. Tryptic digestion generated two fragments from the N-terminus of the transporter (Fig. 2A) and two fragments from its C-terminus (Fig. 3A), chymotryptic proteolysis also generated two fragments from the N-terminus (Fig. 2B) but four fragments from the C-terminus (Fig. 3B) and digestion with proteinase K generated four fragments from both termini (Figs. 2C and 3C). Production of the fragments seen in Figs. 2 and 3 was due to proteolysis since no degradation of the p70,

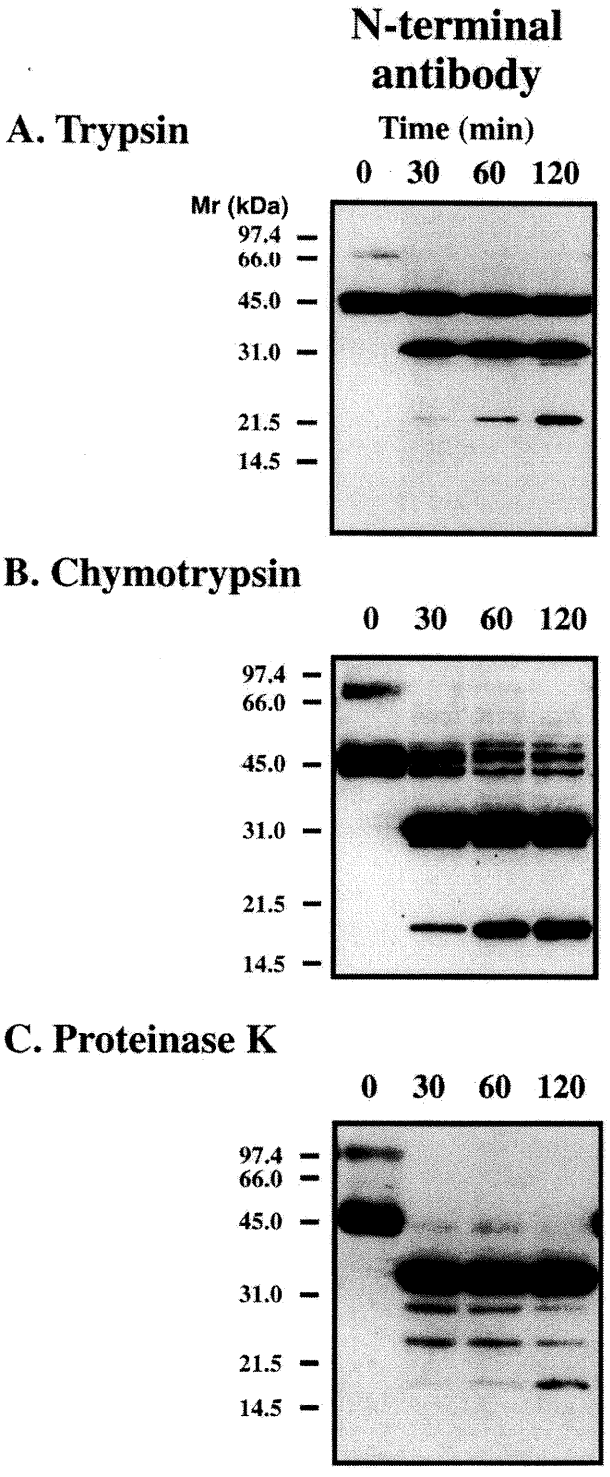
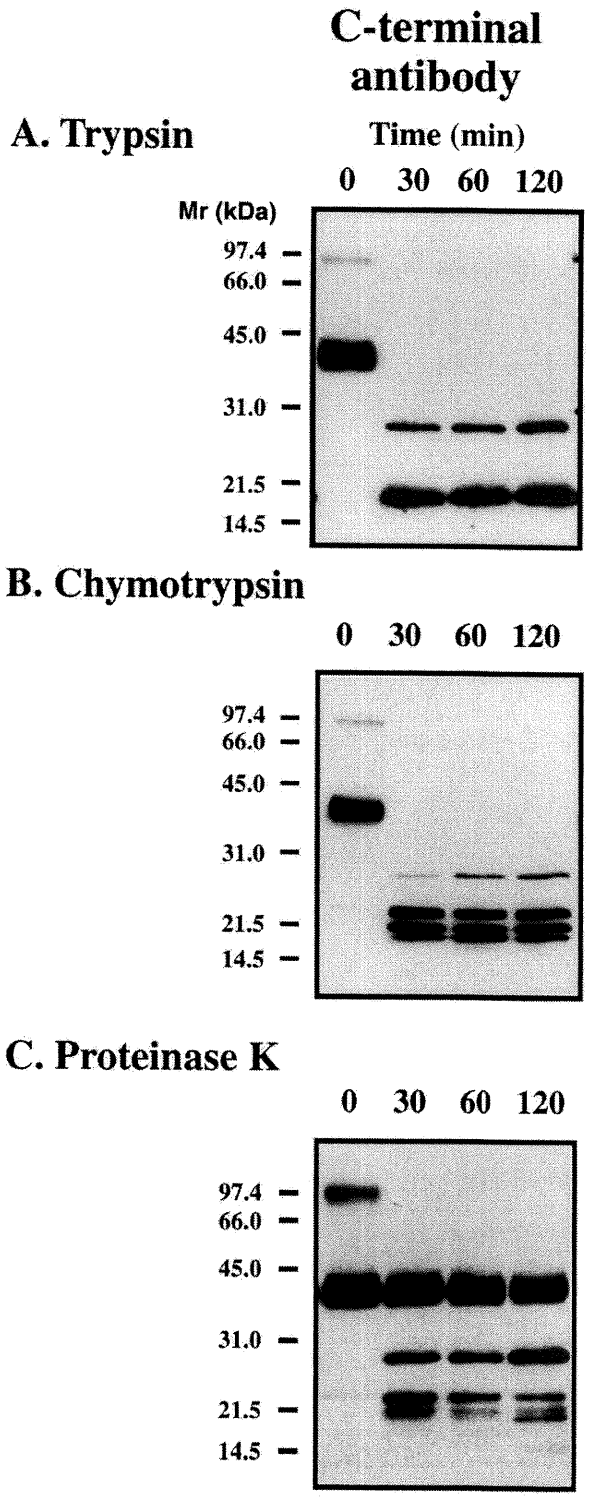


Fig. 2. Effects of limited proteolysis on the immunodetection profile of NaPi-2-related proteins using the N-terminal antibody. BBM vesicles (100 μg protein) were incubated with trypsin (A, 127 mU/μg), chymotrypsin (B, 900 μU/μg) or proteinase K (C, 17.7 μU/μg) for intervals varying from 0 to 120 min, at 37°C, in a pH 7.5 buffer containing 300 mM mannitol and 20 mM HEPES/Tris. Proteolysis was stopped by dilution of the samples with the same buffer containing 1 mg/ml PEFABLOC. Samples were then centrifuged and the resulting pellets resuspended in sample buffer at a protein concentration of 3 μg/μl. BBM proteins were resolved by SDS-PAGE, done under reducing conditions on 12.5% polyacrylamide gels and NaPi-2-related peptide fragments were detected by immunoblotting as described under section Section 2 using the N-terminal antibody. Under the reducing conditions used for electrophoresis, NaPi-2 p180 is completely reduced to p45 and uncleaved p70 and cleaved p70 is completely reduced to p45 (see the zero min lanes). P45 is detected as a triplet (particularly visible at lower concentrations of the polypeptide), most probably due to differences in the level of glycosylation.
←

p45 and p40 bands was observed after incubation of control BBM vesicles in the absence of protease. Table 1 summarizes the apparent molecular weight values and relative yields of the fragments obtained in Figs. 2 and 3. In all cases, the number of immunoreactive proteolytic fragments generated from NaPi-2 in vesicles is small compared to the number of potential proteolytic sites (which are presented in Fig. 4), indicating that many of these sites in the protein structure were inaccessible to the proteases.

Candidate cleavage sites used by trypsin and chymotrypsin on NaPi-2 were assigned according to the best match that can be achieved between the apparent M_r of peptide fragments (which were used to derive corresponding numbers of amino acids on the known sequence of the protein [3]) and existence of a suitable residue for recognition by a given protease in the proximity of derived amino acid positions. Sometimes, more than one cleavage site could be assigned to each fragment if considered individually. However, the number of possibilities was narrowed down by having to match the position and M_r of not only one but several tryptic and chymotryptic sites along p45 and p40 polypeptides. On the other hand, the assignment of cleaved tryptic and chymotryptic sites was not complicated by variation in the glycosylation level since all immunoreactive fragments obtained from these digestions were likely not glycosylated. Indeed, by calculating a mean elec-

Fig. 3. Effects of limited proteolysis on the immunodetection profile of NaPi-2-related proteins using the C-terminal antibody. Proteolysis of BBM vesicles and analysis of proteolytic fragments derived from NaPi-2 were performed as described in the legend to Fig. 2, with the exception that immunodetection used the C-terminal antibody. Under the reducing conditions used for electrophoresis, NaPi-2 p180 is completely reduced to p40 and uncleaved p70 and cleaved p70 is completely reduced to p40 (see the zero min lanes).



trophoretic size per residue for the deglycosylated form of each polypeptide, we determined that NaPi-2 1–297 and 329–637 (respectively corresponding to p45 and p40, truncated at their glycosylation site, see Fig. 4) would migrate respectively as a 34 and a 30 kDa fragment. We can have confidence in such an estimation because on the one hand, the calculated and experimentally determined size of deglycosylated p45 and p40 are in close agreement (respectively 36.6 and 36 kDa for p45 and 32.1 and 30 kDa for p40 [18]) and on the other hand, the truncation is modest and would not result in a significant change in the amino acid composition of the truncated versus the non-truncated forms and hence in their relative mobilities on gels. All tryptic and chymotryptic fragments were of a smaller size than reference values calculated above (Table 1), indicating that they did not contain a glycosylation site and were therefore not glycosylated. Although there are many potential tryptic and chymotryptic sites distributed along the p70 structure, including sites within the cytoplasmic N- and C-tails and within the predicted transmembrane domains M1 and M4–M8 (see Fig. 4A which presents Biber and Murer's proposed model), almost all immunoreactive fragments generated by both proteases could only be formed by cleavages that occurred between the M3 and M6 domains (see sites identified by T and C in Fig. 4A). These results suggest that amino acid residues located between the p70 N-terminus and position 130 and between the p70 C-terminus and position 468 were protected from digestion because they are indeed embedded in the membrane or located in the cytoplasm as was predicted by Biber and Murer [3]. However, this line of reasoning does not apply to the M2 and M3 domains since they do not contain tryptic or chymotryptic sites (Fig. 4A). We therefore exposed BBM vesicles to proteinase K, a protease dis-

playing a very broad target specificity. Sizes of the main fragments produced by this enzyme indicated that the most accessible sites were between the M3 and M6 domains. M2 and M3 remained resistant to proteolysis, suggesting their insertion in the lipid bilayer. Proteinase K generated a 17 kDa fragment which was only slightly detectable after 120 min of digestion (Fig. 2C), suggesting a cleavage near the M2 domain at a site not easily accessible (Fig. 4A). The N- and C-termini of p70 were not digested by proteases (Fig. 4A) in accordance with other results showing their cytoplasmic location [3,7,34]. Unexpectedly, when considering Biber and Murer's model, important cleavages occurred at sites located in the proposed M4–M5 intracellular loop (Fig. 4A), suggesting that this loop is exposed to the extracellular environment. To account for these results, we suggest an alternative topographic model of the transporter whereby the M4 and M5 domains faced the extracellular milieu (Fig. 4B).

4. Discussion

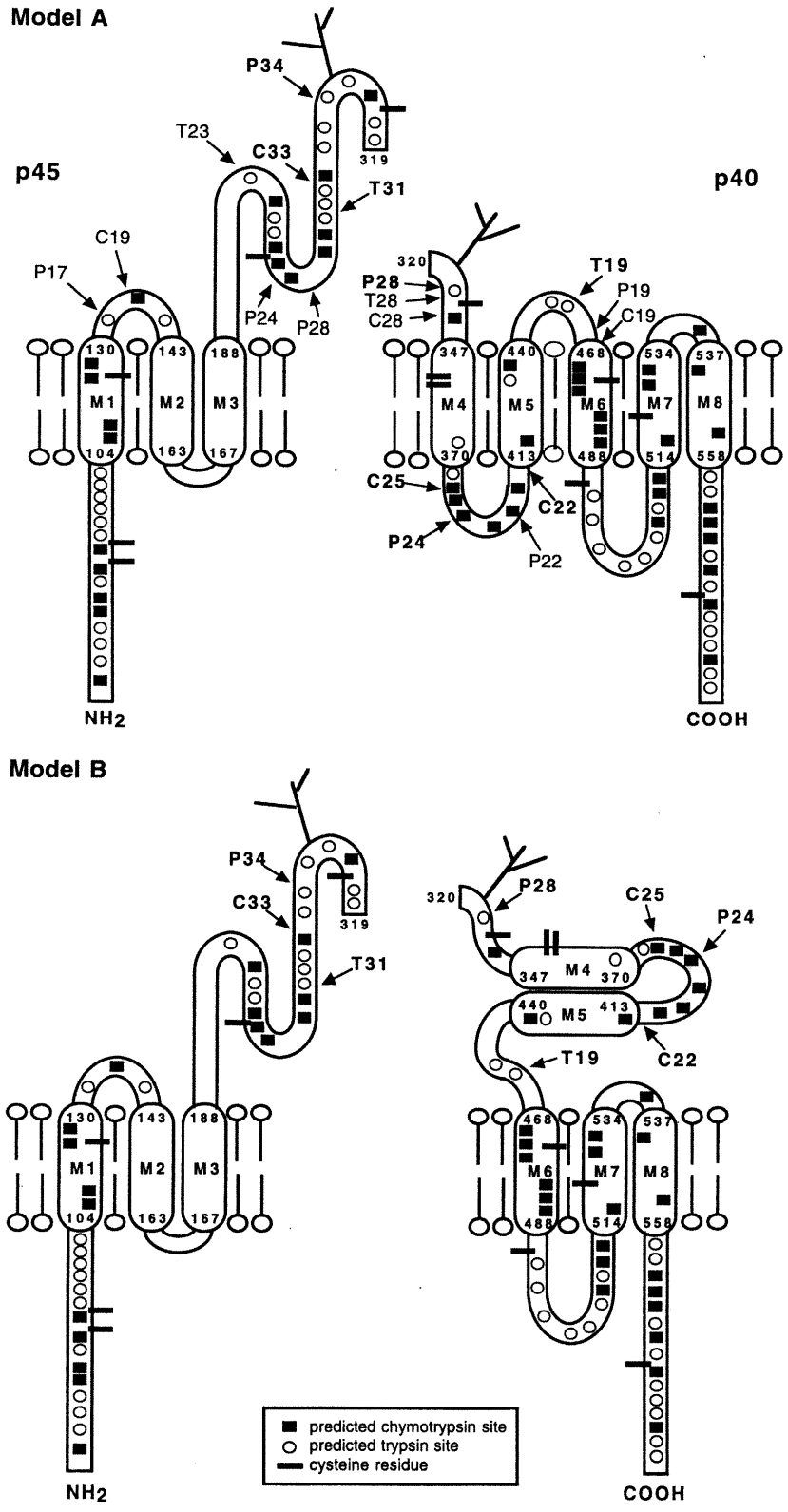
The rat kidney BBM NaPi-2 transporter is encoded as a single 637 amino acid polypeptide which undergoes glycosylation in vivo to yield a full-length 70 kDa polypeptide (p70). However, p70 also exists as a cleaved species formed by the disulfide linkage of the N-terminal (p45) and the C-terminal (p40) moieties of the original polypeptide [18,19]. The results of p40 microsequencing and of partial mapping of the transporter in BBM vesicles by limited proteolysis lead us to propose a topographic model of NaPi-2 (Fig. 4B), whereby the site for the cleavage which generates p45 and p40 is located after Arg-319 in a large protein core of p70 facing the extracellular environment. As discussed in the next two paragraphs, our study provides experimental evidence which overall confirms and modifies the model hypothesized by Biber and Murer for NaPi-2 on the basis of the transporter amino acid sequence [3].

Several characteristics of model A (Fig. 4), which is that originally proposed by Biber and Murer [3] except that the Arg-319-Val-320 peptide bond is not left, agree with the results of limited proteolysis on BBM vesicles. For instance, N- and C-termini of p70 as well as M1, M2, M3, M6, M7 and M8-predicted transmembrane domains were not cleaved by trypsin, chymotrypsin or proteinase K, in accordance with their proposed localization inside the cell or within the membrane whereas the loop extending from residues 188–347 (the M3–M4 loop) was digested, in accordance with its proposed extracellular localization (Fig. 4A). In addition, the proposed intracellular M2–M3 and M6–M7 loops were not sensitive to proteolysis (Fig. 4A). On the other hand, significant cleavage occurred within the proposed M4–M5 cytoplasmic loop, indicating that this region of the NaPi-2 transporter is in an environment more accessible to proteases that can be appreciated from Biber and Murer's model. To account for these results, a modi-

Table 1
Molecular weights and relative intensities of NaPi-2 immunoreactive peptides obtained following limited proteolysis of BBM vesicles

Protease	N-terminal peptides		C-terminal peptides	
	M_r (kDa)	Relative intensity	M_r (kDa)	Relative intensity
Trypsin	31	++++	28	+
	23	±	19	+++
Chymotrypsin	33	++++	28	±
	19	+	25	++
			22	++
			19	+
Proteinase K	34	++++	28	++
	28	+	24	++
	24	+	22	+
	17	±	19	±

Apparent molecular weights were determined by densitometric analysis of the immunoblots shown in Figs. 2 and 3. Relative intensities are reported for the 30 min digestion profiles: trace (±), low (+), moderate (++) and high (+++ or ++++) levels.


Model A
p45
p40
P34
T23
C33
319
T31
C19
P17
P24
P28
320
P28
T28
C28
T19
P19
C19
130
143
188
347
440
468
534
537
M1
M2
M3
M4
M5
M6
M7
M8
104
163
167
370
413
488
514
558
C25
C22
P24
P22
NH2
COOH
Model B
P34
C33
319
T31
320
P28
C25
P24
347
M4
370
440
M5
413
T19
C22
130
143
188
468
534
537
M1
M2
M3
M6
M7
M8
104
163
167
488
514
558
NH2
COOH
predicted chymotrypsin site
predicted trypsin site
cysteine residue

fied model can be proposed such that segments encompassing residues 188–319 and residues 320–468, approximately, form an important protein core that faces the extracellular milieu and can be immersed to a variable degree in the plasma membrane (Fig. 4, model B). This model suggests that a larger part of the transporter than previously thought is in contact with the exterior of the kidney cell, an arrangement which could provide room not only for sites involved in the Pi transport but maybe also for sites that could 'sense' the extracellular Pi concentration.

As a criticism of our results, presence of membrane leaflets and/or vesicles with a wrong orientation in our BBM vesicle preparations could have conceivably facilitated access of proteases to the M4–M5 loop. However, several lines of evidence indicate that the immunoreactive fragments seen in the present study are mostly derived from NaPi-2 in intact, right side out vesicles. First, at least 85% of the BBM vesicles prepared according to the protocol used were shown to be right side out orientated [30]. Secondly, no immunoreactive peptide could be detected when p70 isolated from denaturing SDS-polyacrylamide gels was incubated with chymotrypsin concentrations similar to those used in this work, indicating that the transporter terminal epitopes are easily digested by chymotrypsin when they are exposed (Y. Xiao, unpublished results). In addition, digestion of SDS-denatured p70, p45 or p40 with trypsin produced peptide profiles that were not seen in the present study. As an example, tryptic digestion of denatured p40 generated equivalent amounts of 5–6 fragments with M_r size values ranging from 21 to 30 kDa, in addition to several other fragments [26]. Similar studies on denatured polypeptides were not done for proteinase K, but with BBM vesicles, this enzyme generated NaPi-2 fragments of a similar size to those found with trypsin and chymotrypsin (Table 1), indicating that it cleaved within the same accessible regions of the transporter. Therefore, if NaPi-2 fragments were released from leaflets or inside out vesicles by cleavages at sites normally hidden, they did not interfere in our analysis because they lost epitopes recognized by our antibodies or were present at much lower concentrations than fragments released from right side out vesicles. One limitation of the topographic analysis combining proteolysis and M_r determination by SDS-PAGE is the accuracy of the determination of the cleavage sites. In our study, the accuracy was increased by having to localize fragments of similar sizes produced by two different proteases of strict cleavage specificity, namely trypsin and chymotrypsin. Any reasonable upstream or downstream displacement of cleavage sites that we proposed leads invariably to the same conclusion: the C-terminal moiety of p45 (residues 188 approximately to 319) and the N-terminal moiety of p40 (residues 320–468 approximately) are accessible to proteases present outside vesicles while remaining portions of these polypeptides are resistant to enzymatic digestion.

We were able to purify and microsequence the p40

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Fig. 4. Proposed topographic models of NaPi-2 p70 in the BBM of the rat kidney cortex. Model A (original proposal of Biber and Murer [3] except that the 319-320 peptide bond is cleaved) and model B (a modified model that meets the results of our proteolysis studies) are shown looking across the plane of a lipid bilayer. In both models, the N- and C-termini of p70 are on the cytoplasmic side of the membrane. Numbers refer to amino acid positions on the polypeptide and M1–M8 identify the eight predicted transmembrane domains. Since the structure of p70 is stabilized by at least one disulfide bond between p45 and p40, all cysteine residues are also indicated (black bars). The cleavage site for the generation of p45 and p40 polypeptides is after Arg-319 and between the two *N*-glycosylation sites (Asn-298 and Asn-328), which are identified by branched structures. Potential cleavage sites for trypsin and chymotrypsin are indicated by empty circles and filled squares, respectively. Candidate sites that were cleaved in the present work are indicated, in model A, by an arrow which is accompanied by a letter identifying the active protease (T = trypsin, C = chymotrypsin and P = proteinase K) and by a number indicating the size (in kDa) of the resulting immunoreactive fragment. Molecular weight values were taken from Table 1. The sites where cleavage was most prominent are shown in bold characters, only these cleavages are indicated in model B for the purpose of clarity. Trypsin (T) and chymotrypsin (C) sites between residues 130 and 488 (proposed cleavage sites are underlined): T131-C137-T138-T210-C213-T214-T215-C217-C226-C228-C243-C255-T258-T261-T268-C274-T276-T283-T297-T303-C305-T311-T319...T333-C338-T369-T385-C391-C395-C397-C401-C402-C412-C420-T437-C439-T462-T464-C469-C476-C477-C478-C486-C487. Although the polypeptide region encompassing residues 188 to 468 is entirely drawn outside the plasma membrane in model B, it could also form a large protein core connecting the interior and exterior of the kidney cell.

species derived from the cleavage of NaPi-2 p70. Calculation of the recovery yield on the sequencer and comparison of the intensities of the NaPi-2 C-terminal immunoreactive signals in BBM extracts with that of known amounts of purified p40, allow us to estimate that NaPi-2-related proteins represent approximately 0.005% of the total BBM proteins for rats fed with a normal diet and 0.03% for rats fed with a low phosphate diet (results not shown), indicating a low level of the transporter in BBM of the kidney. In addition, the percentage of cleaved versus full-length p70 changed with variations in dietary phosphate since about 80% of the p70 molecules exist as the cleaved form in animals on a low phosphate diet but only 50% of the p70 molecules are cleaved in animals on a normal phosphate diet (this work and that of Boyer et al. [26]). These numbers indicate that a higher level of cleavage is associated with a higher rate of phosphate transport and suggest that p70 cleavage could be necessary for activity. In support of this reasoning, recombinant expression studies clearly indicated that the cleaved form is active although they did not provide information on the transport capacity of the full-length polypeptide [34]. In fact, transport activity was observed when NaPi-II mRNA was injected in *Xenopus* oocytes as two pieces, one encoding the N-terminal moiety and the other encoding the C-terminal moiety of the transporter polypeptide [34]. Both regions of the transporter molecule are necessary for activity since the injection of either truncated mRNA alone did not induce phosphate transport [34]. The injection of the full-length mRNA also induced transport [34] although it was not determined whether the transporter existed as a full-length and active polypeptide or whether the transport capacity was acquired as a consequence of a post-translational activating cleavage. Therefore, the relationship between the NaPi-2 transport activity and p70 cleavage still remains to be determined. Convertases of the furin family are candidate proteases for the cleavage of NaPi-2 since they cleave at arginine and lysine residues and they are involved in the bioactivation of a variety of secretory and membrane proteins [35,36].

Cleavage of p70 could serve a purpose other than transporter bioactivation in vivo. It is possible that this cleavage is a means of controlling the cycling of NaPi-2 molecules to and from the kidney cell surface. In fact, studies have shown that the kidney cell promptly changes the number of transporter molecules at its apical membrane in response to acute alterations in dietary phosphate [26,37]. Since this response is not accompanied by changes in the protein synthesis or mRNA abundance, it has been suggested that the response depends on modifications in the rate of apical membrane insertion of transporter molecules from intracellular pools or of their internalization from the apical membrane [37]. It is therefore possible that cleavage of NaPi-2 at Arg-319 can target the transporter for rapid retrieval from the BBM, in a similar way as proteolysis at specific sequences has been shown to target cytoplasmic proteins for destruction [38]. The proportion of cleaved p70 transporters could conceivably be greater in low phosphate than in normal phosphate diets in preparation for the return to basal expression levels at the renal apical membrane.

In conclusion, p45 and p40 are generated by cleavage of the arginine-319 and valine-320 peptide bond in NaPi-2 p70. This cleavage site is localized in an important, extracellularly exposed protein core of the transporter and is thus accessible to a variety of proteases, including vesicular, secretory and ecto-enzymes. Since a large proportion of all P70 molecules exists as the cleaved p70 species, the cleavage between p45 and p40 likely has an important physiological role. We propose that this cleavage could serve to regulate the number of active transporter molecules in the BBM, either by activating the precursor form of the transporter or by affecting the transit rate of the transporter molecules at the surface of the kidney cells.

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Different expression patterns of NaPi-2 transporter modulate sodium-phosphate cotransport by rat kidney[1]

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Abbreviations: BBM, brush border membrane; ß-EtSH, ß-mercaptoethanol; LPD, low phosphate diet; Na^+/Pi, sodium-dependent phosphate (transport); NPD, normal phosphate diet; PAGE, polyacrylamide gel electrophoresis; Pi, inorganic phosphate; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

Summary

The rat type II sodium/phosphate (Na^+/Pi) cotransporter NaPi-2 exists as monomers (p70) and dimers (p180) in preparations of renal brush border membranes (BBM). Each species is composed of intact and/or cleaved p70 polypeptides. Previous studies showed that Na^+/Pi cotransport activity as well as expression of all forms of NaPi-2 is regulated by dietary Pi. However, it was not determined how NaPi-2 forms relate to each other in modulating activity. To investigate this aspect, the present work analyzed the expression profile of NaPi-2 proteins in three situations where Na^+/Pi transport is affected, including dietary Pi, developmental age and subtissular localization in the kidney cortex. Higher transport activity was observed in BBM of adult rats exposed to low compared to normal phosphate diet, in BBM of adult compared to suckling rats, and in BBM of juxtamedullary compared to superficial kidney cortex of adult animals. Higher activity was always associated with a higher expression of monomers and dimers by Western blotting, indicating that both species are involved in modulating transport. Changes in expression were however disproportionately more pronounced than those in activity, being 3 to 4-fold higher than activity for age and localization, and 13-fold higher for diet. The contribution of intact and cleaved p70 polypeptides to the level of monomers and dimers varied from one situation to another, and variations can even be in opposite direction without any apparent influence on activity. These findings indicate that there is not a unique expression pattern controlling the transport of Pi by NaPi-2.

Keywords: NaPi-2 expression; Brush border membrane; Phosphate transport; Dietary phosphate; Renal development; Renal cortex,

Introduction

The sodium-dependent reabsorption of phosphate (Pi) in the kidney plays an important role in the maintenance of Pi homeostasis. The rate-limiting step of this process occurs at the brush border membrane (BBM) of proximal tubules [1]. Recently, a number of Na^+/Pi cotransport systems have been identified and classified into three types [2, 3]. Type I cotransporters are expressed in renal BBM and may be involved in renal secretion since they also function as anion channels [4, 5]. Originally described as retrovirus receptors [6], type III cotransporters are widely expressed in different tissues [3] although they are not found in BBM of renal proximal tubules (unpublished data), and could be housekeeping Pi transporters. Types I and III cotransporters are not considered as actors in the maintenance of Pi homeostasis, since their expression is not regulated by dietary Pi [7, 8, 9].

In contrast to types I and III, the expression of type II Na^+/Pi cotransporters which is restricted to renal proximal tubule BBM [10], responds drastically to dietary phosphate changes [9, 11, 12, 13] as well as to parathyroid hormone treatment [14, 15]. This behavior clearly demonstrates that type II cotransporters play an important role in Pi homeostasis. The mechanisms responsible for the regulation of these cotransporters have been investigated in a couple of studies. It has been shown that short-term regulation (within a few hours) involves a rapid insertion of cotransporter molecules into or their internalization from the apical membrane of proximal tubular cells [16, 17]. In contrast, long-term regulation (more than several hours) involves modulation at mRNA and/or protein levels [16, 17].

Rat NaPi-2 is the most extensively studied type II Na^+/Pi cotransporter. This cotransporter was predicted to be a polypeptide of 70 kDa (p70) possessing eight transmembrane domains [18]. Recent topological studies confirmed the intracellular localization of both the NH_2- and the COOH-termini as well as the existence of at least six transmembrane domains and one important extracellular loop in the middle of the polypeptide [19, 20]. In rat renal BBM, significant amounts of p70 are cleaved into two moieties, corresponding to the N-terminal (p45) and the C-terminal (p40) fragments, each one possessing a glycosylated site [21, 22, 23]. Amino acid sequence analysis indicated that cleavage occurs between residues R319 and V320 [20].

Different forms of NaPi-2 proteins are observed in preparations of renal BBM. When these preparations are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) under non-reducing conditions, dimers (p180) and monomers (p70) are detected, while under conditions that reduce all disulfide bonds, both p180 and p70 are converted to p45, p40 as well as intact p70 [13, 21, 22]. There could thus exist as much as five different organizations of NaPi-2 at the quaternary structural level, as illustrated in Fig. 1. However, little is known about the contribution of each form to the transport activity of the protein. Radiation inactivation studies indicated that transport is linked to a large protein species with a molecular weight of □ 200 kDa [22], suggesting that association of p70 units may be necessary for activity. On the other hand, coexpression of p45 and p40 entities in *Xenopus* oocytes by gene transfer techniques induced a high Na^+/Pi cotransport activity [24], indicating that combination of p70 moieties is also functional. One approach that can help assessing the contribution of the different forms of NaPi-2 in modulating transport activity is to determine if changes in activity are accompanied by a preferential change in the expression level of one form of the protein. The present study applied this strategy to three different *in vivo* situations, including developmental age, subtissular localization in the kidney cortex and dietary Pi. The results showed that there is not a unique pattern of response linking changes in expression and activity. This observation indicates the existence of a complex mechanism(s) for the modulation of renal Na^+/Pi cotransport.

2. Materials and Methods

2.1. Animals and BBM preparations

For developmental studies, twenty-one 5-day old and sixteen 15-day old male suckling rats (each group from three litters), and three 2-month old male rats, all of the Sprague-Dawley strain (Charles River, St-Constant, Quebec, Canada), were used after the nursing and the adult rats were accommodated for 48 h to a regular diet. For diet studies, 3 adult male rats were exposed to normal (NPD) or low (LPD) phosphate diet (Teklad, Madison, WI, U.S.A.) for 12 h. Whole kidney cortex was taken from all of these animals for BBM preparation. For subtissular localization studies, the superficial kidney cortex (SC) and the juxtamedullary cortex (JC) were isolated from two groups of 3-month old male adult rats. SC was defined as the tissue found in the outer 1.5 mm thickness of the cortex, while JC corresponded to that of the outer 2 mm thickness of the rest of the kidney. BBM vesicles were purified from JC, SC, and whole cortex with an $MgCl_2$ precipitation method as described [25]. Briefly, cortex were carefully dissected on ice and then homogenized in 50 mM mannitol, 20 mM Tris/HCl, pH 7.4. After a series of centrifugations, the final pellet, which contained BBM, was resuspended in 300 mM mannitol, 20 mM Tris/HCl, pH 7.4, to induce vesicularization. The resulting BBM vesicle suspensions were stored at -80□C until their use in both transport and Western blotting analyses. Protein concentration was determined with the Bradford method using BSA as the standard [26]. BBM proteins were enriched 9.0 ± 0.5 folds (n=3) in those suspensions versus cortex homogenates based on the content of alkaline phosphatase activity.

2.2. Na^+/Pi and Na/glucose cotransport analyses

Na^+/Pi and Na^+/glucose cotransport activities of isolated BBM vesicles were analyzed using a rapid filtration method as described [27]. For the Na^+/Pi assay, 5 µL of BBM vesicles containing 70-80 µg of total proteins was pre-warmed to 25□C for 5 min and then incubated with a reaction medium containing 150 mM NaCl or KCl, 200 µM (2 µCi/assay) [^{32}P]-KH_2PO_4 and 25 mM Tris/HCl pH 7.4 for 5 s, followed by the addition of 1 mL ice cold stop solution containing 150 mM KCl and 5 mM Tris/HCl, pH 7.4. A similar medium was used for the Na/D-glucose assay, except that 50 µM (0.5 µCi/assay) [^{3}H]-D-glucose was used instead of KH_2PHO_4. Samples from each group of animals were assayed in triplicate.

2.3. Western blotting analysis

Two antibodies raised in rabbit were used: an anti-N antibody produced against the N-terminus of NaPi-2 p70 and recognizing p45, p70 and p180, and an anti-C antibody produced against the C-terminus of the transporter and recognizing p40, p70 and p180 [13, 22]. Immunodetection of NaPi-2 proteins by Western blotting was as described [13, 22]. Briefly, SDS-PAGE was done on mini gels using the Laemmli buffer system [28]. Running gels contained 8.0% (w/v) acrylamide and 0.23% bisacrylamide. BBM samples (12 μg protein) were solubilized in sample loading buffer containing 20% (w/v) glycerol, 1% (w/v) SDS, and 62.5 mM Tris/HCl, pH 6.8, in the presence or absence of 4% ß-mercaptoethanol (ß-EtSH), electrophoresed and electrotransfered on PVDF membranes. Membranes were blocked overnight at 4□C in a buffer containing 150 mM NaCl, 0.3% (w/v) Tween-20, 50 mM Tris-HCl, pH 7.5. Afterwards, membranes were incubated with anti-C (1:20,000) or anti-N (1:10,000) antibody in the blocking buffer for 1 h at room temperature and then, with a horseradish peroxidase-linked secondary antibody from donkey (Amersham Pharmacia Biotech, Montreal, Canada) at a dilution of 1:1000, for 1 h, at room temperature. Finally, membranes were incubated with enhanced chemiluminescence solutions (Amersham Pharmacia Biotech, Montreal, Canada) and exposed to X-ray films. Densitometric analysis of the autoluminograms was done with a Personal Densitometer SI scanner (Molecular Dynamics) and an IPLab Gel H program (Sigma Analytics Corporation).

2.4. Statistical analysis

Statistical analyses were done using Student's t test. Unless otherwise stated, the significance level chosen was $p < 0.05$.

3. Results

3.1. Transport activity of isolated BBM vesicles

To eventually identify which form(s) of NaPi-2 transporter is associated with variations in Pi uptake by the kidney, we first measured the Na^+/Pi cotransport activity of BBM vesicles obtained from three *in vivo* situations known to affect this activity: age of the animals [29], subcortical localization of the transporter [30, 31], and dietary Pi [32]. Fig. 2A shows that Na^+/Pi cotransport activity of BBM vesicles increased with age, the values being 1.7-fold higher for 15-day than for 5-day old suckling animals, and 2.1-fold higher for adult rats than for 15-day old suckling rats. Interestingly, the Na/D-glucose cotransport activity reached the adult value at an earlier age (Fig. 2B). Fig. 2C compares the transport activity of BBM vesicles isolated from SC and JC, and shows a 25% higher activity in the latter. Finally, Fig. 2D indicates that transport significantly increased (30%) after 12 h of a LPD. Thus, an increased Pi transport activity is associated with maturation of the kidney, with a deeper localization of cells in the kidney cortex, and with Pi deprivation. In the next sections, we used Western blotting techniques to determine whether these changes in transport activity can be correlated with a preferential modification in the expression of one form of NaPi-2 protein.

3.2. Detection and stability of NaPi-2 proteins

Disulfide bonds are involved in the association of p45 and p40 to form cleaved p70, as well as in the association of p70 (cleaved or intact) to form p180 (Fig. 1). Disulfide linkages between p70 subunits are more sensitive to reduction than those between p45 and p40 moieties [22]. To determine the conditions that will allow the respective quantification of p180, intact p70 and cleaved p70 (represented by either p40 or p45), we examined the effect of various concentrations of ß-EtSH in the sample buffer on the conversion of NaPi-2 to its reduced forms. As shown in Fig. 3, the proportions of the different NaPi-2 proteins depended on the concentration of ß-EtSH. For concentrations lower than 0.01%, only p180 and p70 were present and nearly no p40 was detected. For concentrations varying from 0.01 to 1 %, there was a gradual disappearance of p180 and p70, along with a concomitant appearance of p40, indicating that both p180 and p70 bands contained the cleaved p70 species. From 1 to 4 % ß-EtSH, the relative amounts of p70 and p40 remained stable, indicating that all cleaved p70 species contained in the original p180 and p70 bands were reduced to p40 and p45, and

that only the intact form remained in the p70 band. Higher concentrations of ß-EtSH affected the quality of the immunodetection of both intact p70 and p40. We therefore used no ß-EtSH to detect total p180 and p70 proteins in BBM samples, and 4 % ß-EtSH to separate cleaved from intact p70 species throughout this work.

We next performed two control studies to verify that NaPi-2 cleavage into p40 and p45 did not occur during the preparation of BBM samples. First, freshly isolated kidneys were incubated on ice for up to 3 h. BBM were then isolated and subjected to NaPi-2 immunodetection. Alternately, BBM prepared immediately after kidney dissection were incubated at 37□C for up to 2 h, before immunoblotting analysis. In both cases, the ratio of p40/intact p70 or p45/intact p70 did not change significantly during incubation (Fig. 4), indicating that NaPi-2 proteins are stable under the conditions used to prepare BBM samples. Thus the specific cleavage of intact p70 has already occurred *in vivo*, and might have some implication in the Pi transport activity.

3.3. Expression of NaPi-2 proteins as a function of age

BBM isolated from 5- and 15-day old suckling rats as well as from 2-month old rats were analyzed for their content of NaPi-2 proteins by Western blotting, using the anti-N and anti-C antibodies. Electrophoresis was done in the absence (for the separation of total p180 and p70, Fig. 5A) or presence (for the separation of intact and cleaved p70, Fig. 5B) of ß-EtSH. Results show that each form of the cotransporter was present at all ages (Figs 5A and B), and underwent upregulation with growth (Figs 5C, D and E). The ratios of total p70/p180 had similar values, being 1.9 ± 0.5 for 5-day old rats, 2.6 ± 0.3 for 15-day old rats, and 2.5 ± 0.3 for adult animals (Fig. 5). This indicates that the levels of p180 and total p70 increased with age at a coordinate rate. Cleaved p70 (represented by either p40 or p45) generally predominated over intact p70 (Figs 5D, E). While the levels of both cleaved and intact p70 increased with age, the change was modest (about 1.6-fold) for the cleaved form but more pronounced (□ 8-fold) for the intact form (Figs. 5D, E), suggesting that intact p70 contributed importantly to upregulation of Pi transport with growth.

3.4. Expression of NaPi-2 proteins in SC and JC

Western blot analysis was also done on BBM vesicles derived from SC and JC (Fig. 6). Fig. 6A shows that the levels of p180 and total p70 were significantly higher in JC than in SC,

although densitometric analysis (Fig. 6C) reveals similar ratio values for total p70/p180, respectively 2.0 ± 0.3 and 2.4 ± 0.4. Interestingly, the levels of intact p70 in JC BBM was only 75 % that of the SC BBM, while the levels of p40 and p45 in JC were respectively 140% and 390% higher than in SC (Figs. 6D and E). This differential expression of intact and cleaved p70 between JC and SC BBM suggests that cleaved p70 contributed to the higher transport activity exhibited by JC.

3.5. Expression of NaPi-2 proteins after exposure to LPD

Finally, Western blotting was applied to BBM proteins obtained from rats fed a NPD or a LPD for 12 h. As shown in Fig. 7A, the levels of p180 and total p70 were much higher in LPD than in NPD, in agreement with previous report [22]. Quantitative analysis (Fig. 7C) however indicates that the ratio value of total p70/p180 in NPD (1.2 ± 0.3) was similar to that seen in LPD (1.1 ± 0.2). Under reducing conditions, the levels of intact p70, p45 and p40 were also much higher in LPD than in NPD (Fig. 7B), but the ratio values of p45/intact p70 and p40/intact p70 were more than 2-fold higher in LPD than in NPD (Figs. 7D and E), indicating upregulation of transport induced by LPD preferentially affected cleaved p70 rather than intact p70. As LPD leads to an increase in Na^+/Pi cotransport, the overexpression of the cleaved relative to the intact form of NaPi-2 suggests the possible involvement of the former in modulating Na^+/Pi cotransport.

4. Discussion

The present study compared variations in renal Na^+/Pi cotransport activity with changes in the levels of different protein forms of rat kidney NaPi-2 in three different situations. This strategy was used to determine if a particular form(s) of the transporter could be involved in the modulation of activity. As summarized in Table 1 and discussed thereafter, the results show that increased activity does not translate into a unique protein expression pattern.

Na^+/Pi cotransport activity was higher in BBM obtained from adult compared to newborn rats, from juxtamedullary compared to superficial rat renal cortex, and from LPD compared to NPD fed rats. The stimulating effect of LPD on renal Na^+/Pi cotransport had already been extensively documented [1] and our results are not at variance regarding this well-established physiological effect. Results from our developmental study showed that levels of activity increased with growth, in the following ascending order : 5-day old suckling rats, 15-day old suckling rats and adult animals. This indicates that kidney must mature to exhibit full Na^+/Pi cotransport capacity. These findings agree with those of others demonstrating higher transport activity in rats at end-weaning stage and in adulthood than in rats at early-weaning stage [33]. Together with the present study, the data thus reveal a developmental pattern of regulation in rat renal Na^+/Pi cotransport whereby the transport activity increases quickly after birth, reaches its peak value at end-weaning stage, and then decreases to adult level. In adult rats, Na^+/Pi cotransport activity was reported to decline slowly with age [34]. The cotransport activities of SC and JC kidney subcompartments have been compared by others previously [30, 31]. Contrary to what observed in those studies, the present work indicated a higher transport activity in JC than in SC. We believe that this discrepancy is due to the difference in the criteria used to dissect JC and SC. In the present comparatively to other studies, much thinner sections were taken for SC while thicker sections were taken for JC. The difference had however no consequence in our work since protein expression and activity profiles were determined correspondingly on the same BBM fractions.

Examination of the BBM protein profiles by immunoblotting showed that monomeric and dimeric associations of NaPi-2 polypeptides as well as intact and cleaved p70 constituents (Fig. 1) are expressed in every situation, suggesting that all of these forms are somehow involved in Pi transport. Table 1 shows that higher amounts of dimers (p180) and monomers

(total p70) were always associated with higher Pi transport activity, indicating that the overall level of NaPi-2 proteins determines that of transport activity. However, there was a striking disproportionate relationship between expression and activity levels. For instance, a 3.6-fold change in activity was accompanied by a 11.8 (i.e. 5.1 + 6.7)-fold change in expression during development (adult / 5-day old), or by a 9.8 (i.e. 5.2 + 4.6)-fold change in expression for JC / SC (Table 1). Thus, change in expression was in average 3 to 4-fold more pronounced than that in activity. This indicates that NaPi-2 must function as multimers, supporting the results of radiation inactivation studies which suggested that tetramers could be the active assemblage of the cotransporter on the basis that target sizes of the protein ranged from 190 to 220 kDa [22, 35, 36].

Dietary Pi introduced even more important changes in expression than age and subcompartmental localization since the expression/activity ratio reached a value of 13 (i.e. [25.5 + 22.2] / 3.6) in that case (Table 1). One possible explanation is that some inhibitory components were added to the multimeric assemblages of NaPi-2 to inactivate transport activity. This is supported by the results of radiation inactivation studies showing an increase in the size of BBM Na^+/Pi cotransporter from 255 to 335 kDa upon lowering dietary Pi [38]. Interestingly, there are isoforms of the transporter that can fulfill this role. Indeed, NaPi-2 α and γ isoforms, respectively resembling the p45 and p40 moieties of p70 polypeptide, were detected in the kidney and found to decrease Pi transport when coexpressed with NaPi-2 itself [37]. Our antibodies would not distinguish p45 and NaPi-2 α isoform, or p40 and NaPi-2 γ isoform, since both members of each pair bears the recognized epitope [13, 22, 38]. It is not known whether the NaPi-2 isoforms exist in newly born rats or in adult rats exposed to NPD, but it seems that they have the potential to help the animals adapt to variations in dietary Pi. They could be part of a dynamic mechanism that has evolved to rapidly regulate the transport function in response to dietary, and perhaps also hormonal, fluctuations, since a tight regulation of phosphate retention is important to prevent acidosis. The binding of inhibitory components would exquisitely complement the already demonstrated processes of mobilization / demobilization of transporter molecules at the BBM [16-18]. In any case, it will be interesting to determine the stoichiometry of the inhibitory isoforms versus the multimers when appropriate antibodies will become available.

We also analyzed levels of the reduced forms (intact p70, p45 and p40) of the transporter to reveal any effect on transport activity. So far, variations in the relative amounts

of intact versus cleaved p70 appear to be not influential. Indeed, growth and subcompartmental localization exhibited almost exactly opposite results regarding the variation in the level of reduced species. In fact, for a similar increase in activity there was a 9.5- and 1.9-fold raise in the level of intact and cleaved p70, respectively, in the first situation, while the inverse was true for the latter (Table 1). These results clearly demonstrate that all reduced NaPi-2 proteins could be responsible for Pi transport. The intermediate values obtained with dietary phosphate (Table 1) do not contradict this conclusion. Our deduction that cleaved p70 must be active agrees with the study showing that coexpression of p45 and p40 corresponding segments of the flounder type II Na^+/Pi cotransporter resulted in full expression of Pi transport function in *Xenopus* oocytes [24].

Although there exist types of Na^+/Pi cotransporters other than type II, they could not be responsible for changes in transport activity in renal BBM since the type I cotransporter (NaPi-1) is expressed constitutively and distributed uniformly throughout the kidney cortex [4], while the type III (Glvr-1) cotransporter was found not to be expressed in the rat renal BBM (unpublished data).

In summary, the present study demonstrates that different protein expression patterns of NaPi-2 can lead to an increased Na^+/Pi cotransport activity in the rat kidney. The absence of a unique protein profile suggests that a complex mechanism modulates the cotransport function.

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Figure legends

Fig. 1. Proposed structures of NaPi-2 monomers and dimers. Based on previous studies [13, 20, 21, 22], native NaPi-2 transporter could exist as (A) monomeric intact p70 polypeptides, (B) monomeric cleaved p70 species comprising disulfide linked p40 and p45 moieties, (C) two intact p70s associated via disulfide bond(s), (D) one intact p70 disulfide linked to one cleaved p70, and/or (E) two cleaved p70s associated via disulfide bond(s).

Fig. 2. Transport activity of BBM vesicles obtained in different physiological situations. (A) Na^+/Pi and (B) Na^+/glucose cotransport activities for rats of different ages. (C) Na^+/Pi cotransport activity of JC and SC kidney subsites. (D) Na^+/Pi cotransport activity of rats exposed to NPD or LPD for 12 h. Results represent the means ± SEM of 3 independent assays.

Fig. 3. Effect of ß-EtSH concentration on the electrophoretic profile of NaPi-2 proteins. (A) Immunoblots. BBM proteins were pretreated for 20 min at 25□C, with sample buffer containing ß-EtSH at the concentrations (% v/v) indicated above lanes, resolved on an 8% acrylamide SDS-PAGE gel, electrotransferred on PVDF membranes and immunoblotted with anti-C antibody. (B) Relative density of p70 or p40 bands shown in A. The density ratio of p40/p70 is also shown. In this and the following figures, the multiplet signal associated with each protein species was considered as one signal for densitometric analysis, since it gives a single band after deglycosylation [22].

Fig. 4. Stability of NaPi-2 proteins *in vitro*. The upper curve (□) represents the ratio of p40/intact p70 in BBM isolated from kidneys that were left on ice for the indicated time (although not shown, there was no further change in the ratio value when the incubation was pursued until 3 h). The lower curve (□) represents the ratio of p45/intact p70 in BBM prepared immediately after kidney dissection and incubated at 37□C for the indicated time. Results were obtained from densitometric analysis of Western blots obtained under reducing conditions.

Fig. 5. Relative levels of NaPi-2 proteins in renal BBM of rats of different ages. BBM proteins (12 μg per lane) from rats of different ages were solubilized in electrophoresis sample buffer in absence (A) or presence (B) of 4% ß-EtSH before Western blotting. The probing of p180, p70, p45 and p40 species was done with anti-N or anti-C antibodies. (C), (D) and (E) summarize the densitometric results (mean ± SEM) of 3 independent assays. The levels of p180 and total p70 (C) were determined from the results of non-reducing SDS-PAGE (A), and are presented as the averaged values obtained from both anti-N and anti-C detection. The p45 doublet detected in adult rats (B) was considered as one species for densitometric analysis, since it migrates as a single band after deglycosylation [22]. 5d, 5-day old rats; 15d, 15-day old rats; A, adult rats. Significant differences ($p < 0.05$) in protein levels are indicated by * for 15d versus 5d, by # for A vs 15d, and by † for A vs 5d.

Fig. 6. Relative levels of NaPi-2 proteins in JC and SC BBM of rat kidneys. BBM were prepared from SC and JC of adult animals as described in Materials and Methods, and analyzed by Western blotting as indicated in the legend to Fig. 5. Immunoblots obtained under non-reducing (A) or reducing (B) electrophoresis conditions are shown. (C), (D) and (E) summarized the densitometric results (mean ± SEM) of 3 independent assays. * indicates a significant difference ($p < 0.05$) in protein level between JC and SC BBM.

Fig. 7. Relative levels of NaPi-2 proteins in renal BBM obtained from rats fed with a NPD or a LPD for 12 h. BBM were prepared and analyzed by Western blotting as indicated in the legend to Fig. 5. Immunoblots obtained under non-reducing (A) or reducing (B) electrophoresis conditions are shown. (C), (D) and (E) summarize the densitometric results (mean ± SEM) of 3 independent assays. * indicates a significant difference in protein level ($p< 0.05$) between LPD and NPD.

Table 1. Relative changes in Na^+/Pi transport and NaPi-2 protein level in different situations.

	Fold increase		
	Adult / 5-Day old	JC / SC	LPD / NPD
Na^+/Pi transport	3.6	1.25 (3.6)	1.3 (3.6)
NaPi-2 proteins			
Non-reducing conditions			
Total p180	5.1	1.8 (5.2)	9.2 (25.5)
Total p70	6.7	1.6 (4.6)	8.0 (22.2)
Reducing conditions			
Intact p70	9.5	0.8 (2.2)	4.6 (12.7)
Cleaved p70	1.9	3.4 (9.8)	12.7 (28.6)

Results summarizing the relative increases in transport activity and protein level in the adult versus the 5-day old rats, in JC versus SC, or in rats fed with a LPD versus NPD. The relative increase in cleaved p70 was averaged from the values obtained for p40 and p45. Numbers between parentheses were standardized to a 3.6-fold increase in Na^+/Pi cotransport, for comparison purpose.

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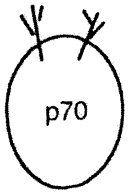
Manuscript III, Fig. 1

A. Intact p70



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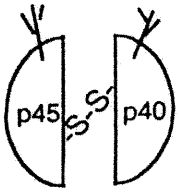
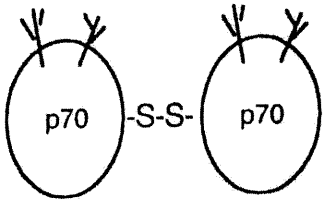


C. P180 composed of two intact p70s



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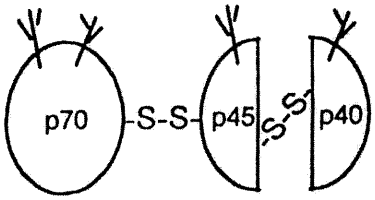

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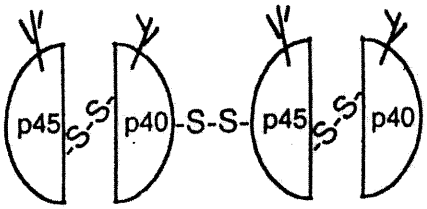

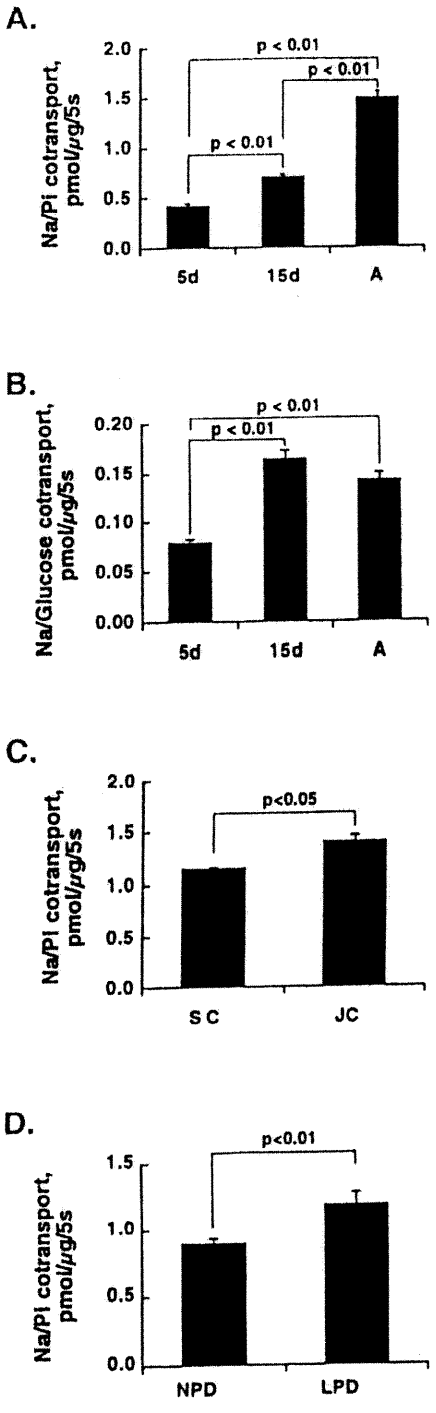
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Na/Pi cotransport, pmol/μg/5s
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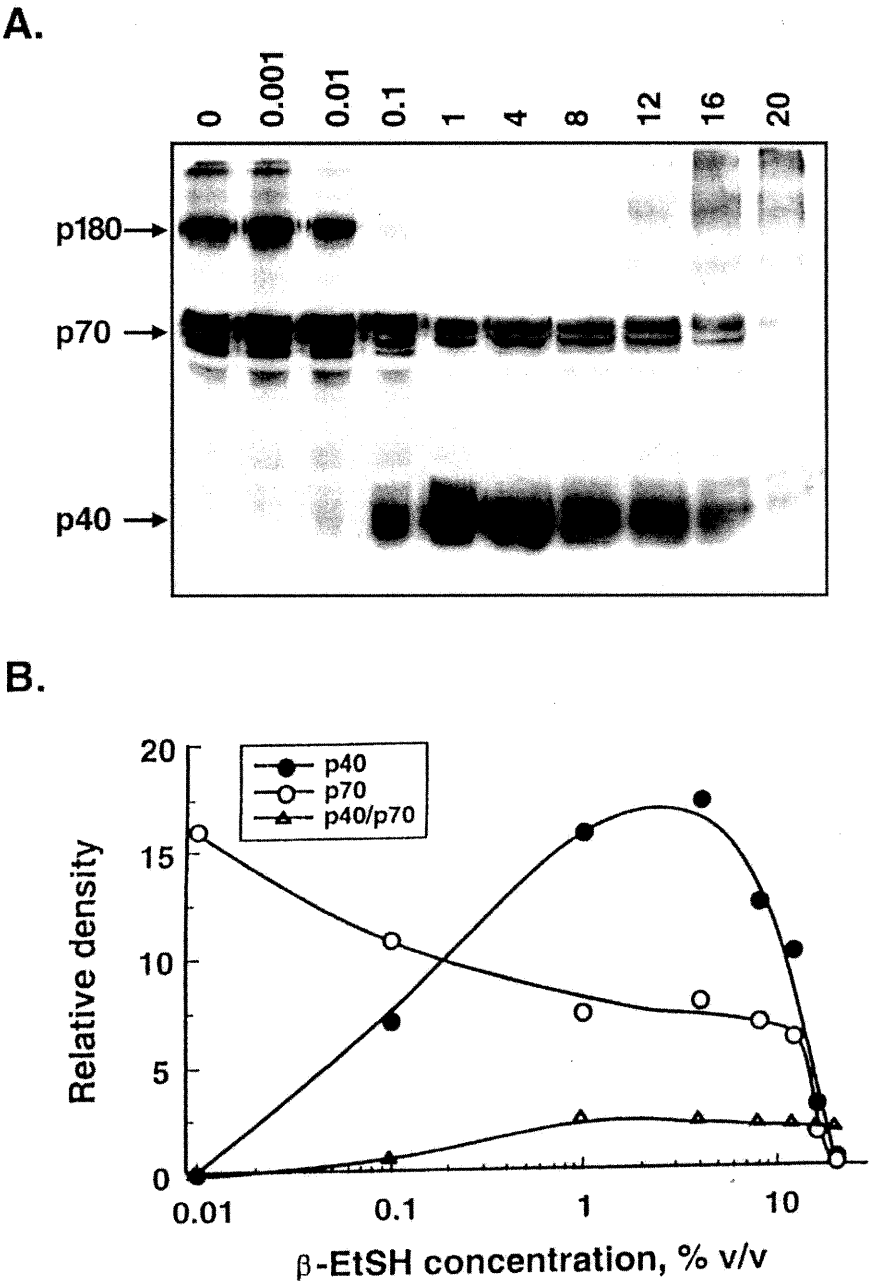
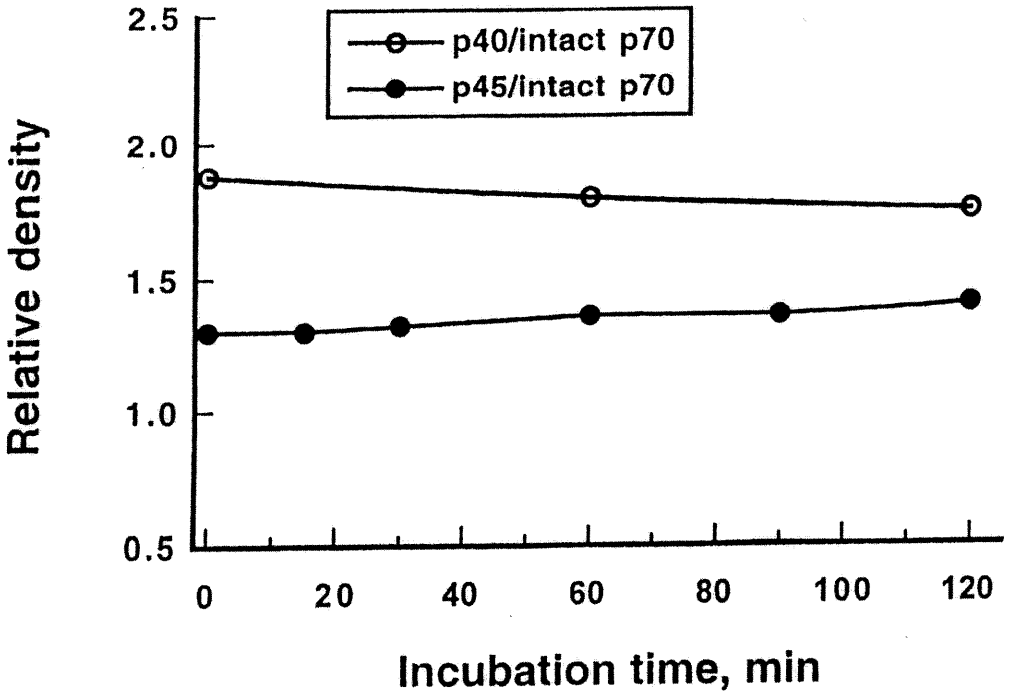
1.0
0.5
0.0
p < 0.01
p < 0.01
p < 0.01
5d
15d
A
B.
Na/Glucose cotransport, pmol/μg/5s
0.20
0.15
0.10
0.05
0.00
p < 0.01
p < 0.01
5d
15d
A
C.
Na/Pi cotransport, pmol/μg/5s
2.0
1.5
1.0
0.5
0.0
p<0.05
S C
JC
D.
Na/Pi cotransport, pmol/μg/5s
1.5
1.0
0.5
0.0
p<0.01
NPD
LPD


A.
0
0.001
0.01
0.1
1
4
8
12
16
20
p180
p70
p40
B.
p40
p70
p40/p70
Relative density
20
15
10
5
0
0.01
0.1
1
10
β-EtSH concentration, % v/v


p40/intact p70
p45/intact p70
Relative density
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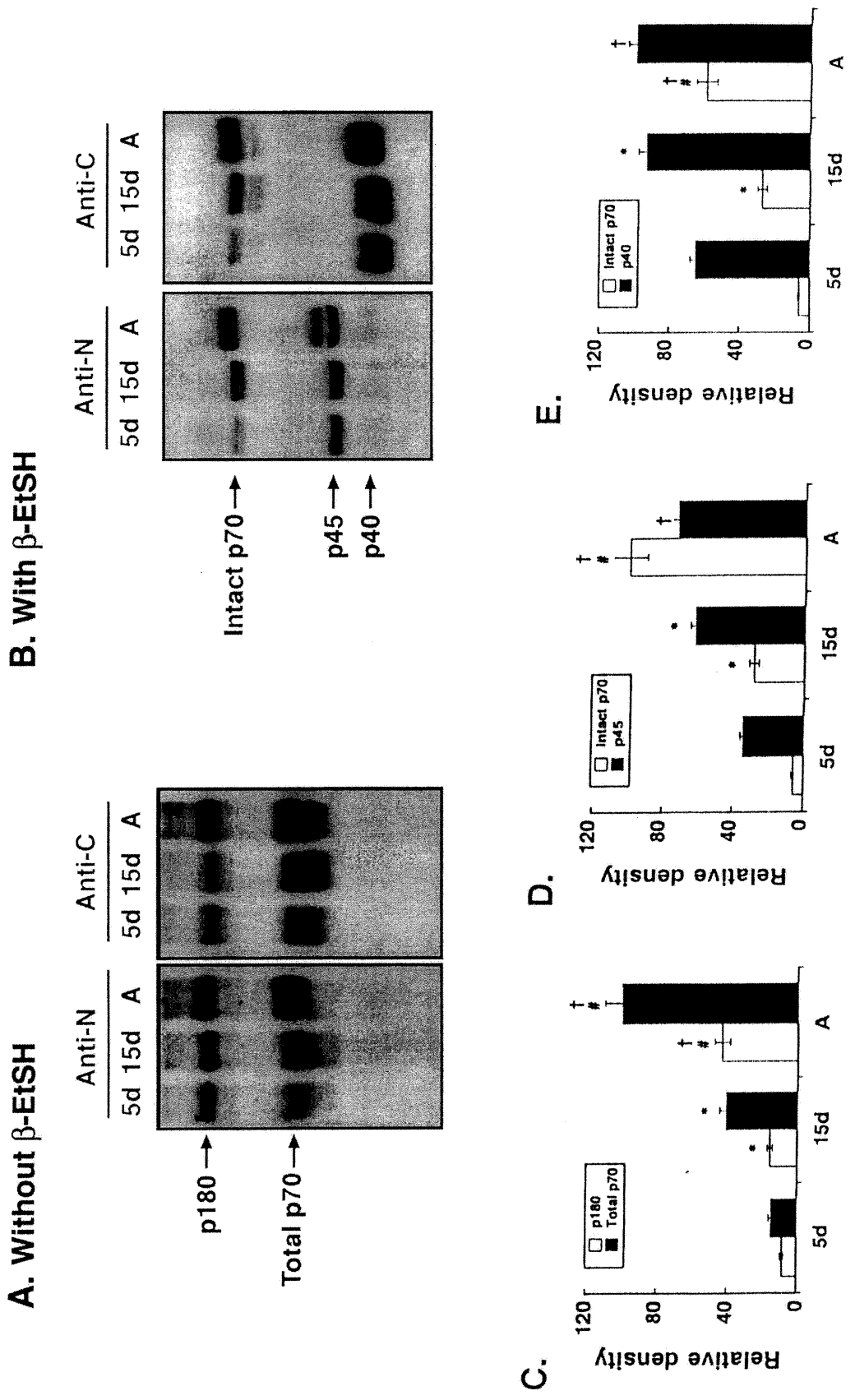
1.5
1.0
0.5
0
20
40
60
80
100
120
Incubation time, min


A. Without β-EtSH
Anti-N
Anti-C
5d 15d A
5d 15d A
p180
Total p70
B. With β-EtSH
Anti-N
Anti-C
5d 15d A
5d 15d A
Intact p70
p45
p40
C.
p180
Total p70
Relative density
120
80
40
0
5d
15d
A
D.
Intact p70
p45
Relative density
120
80
40
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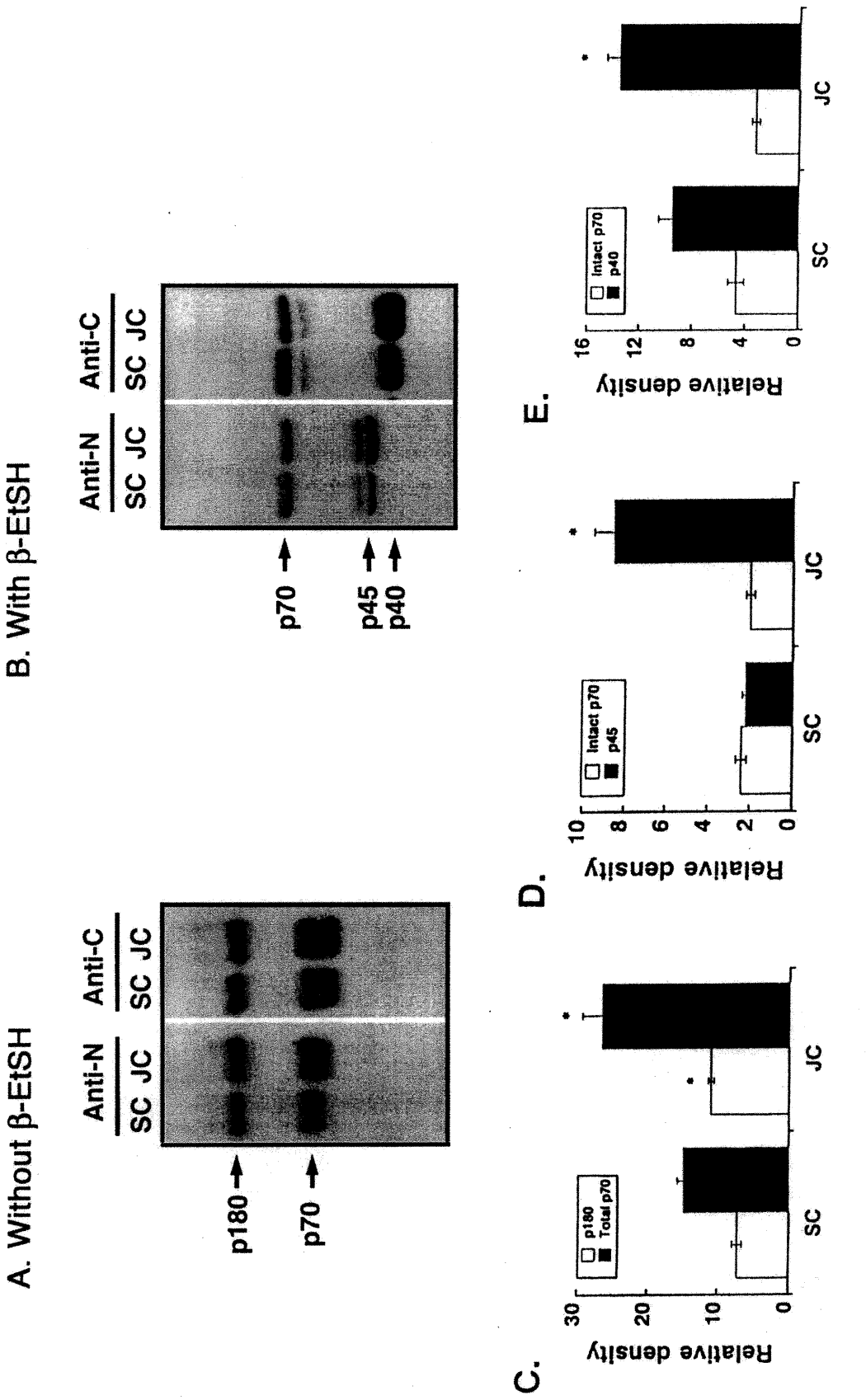
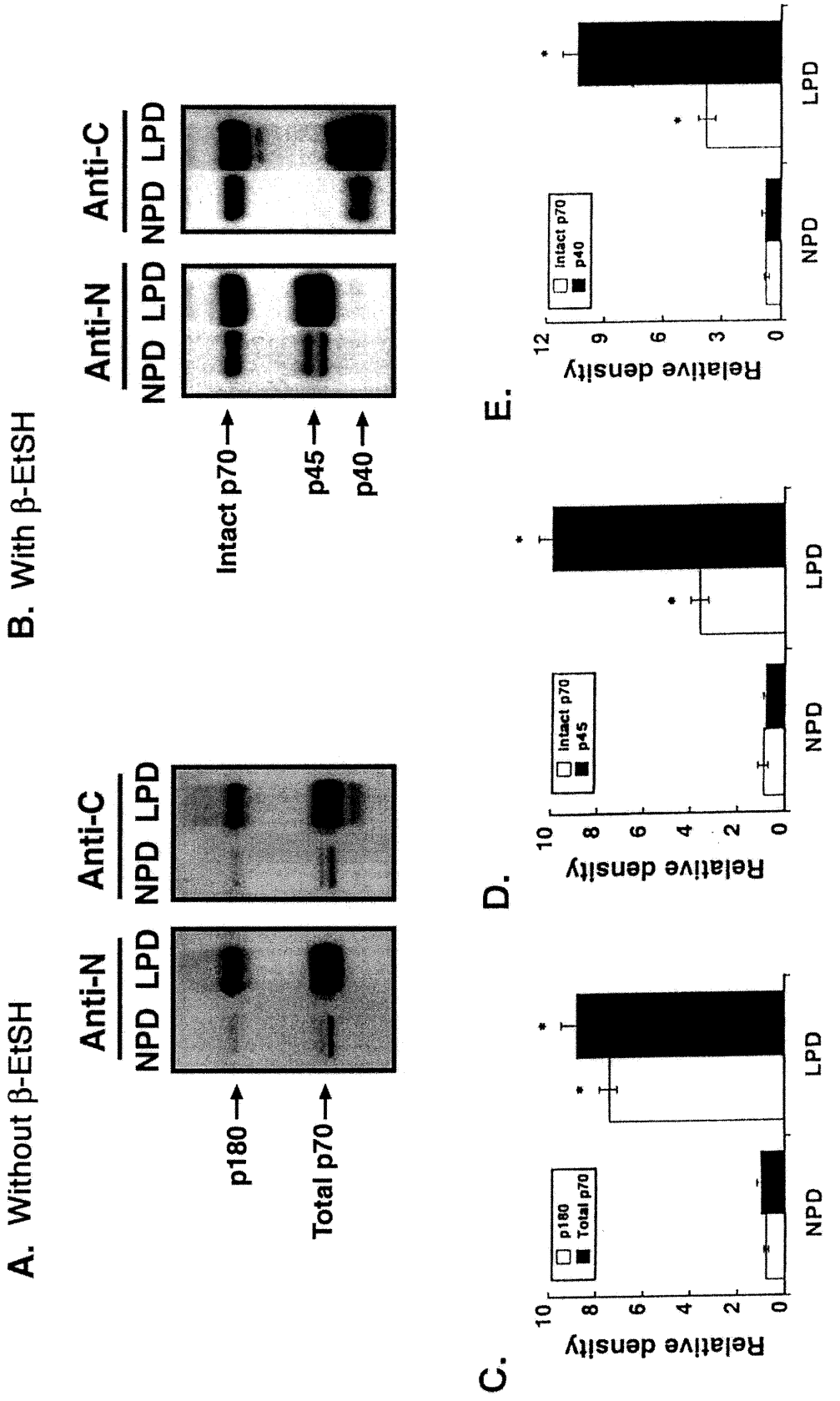
5d
15d
A
E.
Intact p70
p40
Relative density
120
80
40
0
5d
15d
A


A. Without β-EtSH
Anti-N
Anti-C
SC JC SC JC
p180
p70
B. With β-EtSH
Anti-N
Anti-C
SC JC SC JC
p70
p45
p40
C.
Relative density
p180
Total p70
SC
JC
D.
Relative density
Intact p70
p45
SC
JC
E.
Relative density
Intact p70
p40
SC
JC


A. Without β-EtSH
Anti-N
Anti-C
NPD LPD
NPD LPD
p180
Total p70
B. With β-EtSH
Anti-N
Anti-C
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NPD LPD
Intact p70
p45
p40
C.
p180
Total p70
Relative density
NPD
LPD
D.
Intact p70
p45
Relative density
NPD
LPD
E.
Intact p70
p40
Relative density
NPD
LPD

Effect of ischemia–reperfusion on the renal brush-border membrane sodium-dependent phosphate cotransporter NaP_i-2

Yansen Xiao, Richard R. Desrosiers, and Richard Béliveau

Abstract: To understand the mechanisms underlying ischemia–reperfusion-induced renal proximal tubule damage, we analyzed the expression of the Na^+-dependent phosphate (Na^+/P_i) cotransporter NaP_i-2 in brush border membranes (BBM) isolated from rats which had been subjected to 30 min renal ischemia and 60 min reperfusion. Na^+/P_i cotransport activities of the BBM vesicles were also determined. Ischemia caused a significant decrease (about 40%, $P < 0.05$) in all forms of NaP_i-2 in the BBM, despite a significant increase (31 ± 3%, $P < 0.05$) in the Na^+/P_i cotransport activity. After reperfusion, both NaP_i-2 expression and Na^+/P_i cotransport activity returned to control levels. In contrast with Na^+/P_i cotransport, ischemia significantly decreased Na^+-dependent glucose cotransport but did not affect Na^+-dependent proline cotransport. Reperfusion caused further decreases in both Na^+/glucose (by 60%) and Na^+/proline (by 33%) cotransport. Levels of NaP_i-2 were more reduced in the BBM than in cortex homogenates, suggesting a relocalization of NaP_i-2 as a result of ischemia. After reperfusion, NaP_i-2 levels returned to control values in both BBM and homogenates. These data indicate that the NaP_i-2 protein and BBM Na^+/P_i cotransport activity respond uniquely to reversible renal ischemia and reperfusion, and thus may play an important role in maintaining and restoring the structure and function of the proximal tubule.

Key words: kidney, ischemia, reperfusion, phosphate, transport.

Résumé : Pour comprendre les mécanismes en cause dans les dommages induits aux tubes proximaux rénaux lors de l'ischémie et de la reperfusion, nous avons analysé l'expression du cotransporteur de phosphate (Na^+/P_i) sous dépendance sodique, NaP_i-2, dans les membranes en bordure en brosse (MBB) isolées de rats soumis à une ischémie de 30 min et à une reperfusion de 60 min. Les activités de cotransport Na^+/P_i ont été aussi déterminées dans les vésicules de MBB. L'ischémie causait une diminution significative (environ 40%, $P < 0.05$) de toutes les formes de NaP_i-2 présentes dans les MBB malgré une hausse significative (31% ± 3%, $P < 0.05$) de l'activité de cotransport de Na^+/P_i. Après la reperfusion, l'expression de NaP_i-2 et l'activité de cotransport de Na^+/P_i retournaient aux niveaux des contrôles. Contrairement à l'activité de cotransport de Na^+/P_i, l'ischémie diminuait significativement le cotransport de glucose sous dépendance sodique alors qu'elle n'avait pas d'effet sur le cotransport de la proline dépendante du sodium. La reperfusion causait des diminutions supplémentaires des activités de cotransport Na^+/glucose (60%) et Na^+/proline (33%). Les niveaux de NaP_i-2 étaient plus fortement réduits dans les MBB que dans les homogénats de cortex suggérant une relocalisation du cotransporteur lors de l'ischémie. Après la reperfusion, les niveaux de NaP_i-2 revenaient à ceux des contrôles dans les MBB et les homogénats. Ces données indiquent que la protéine NaP_i-2 et que l'activité du cotransporteur de Na^+/P_i dans les MBB répondent de façon unique à une ischémie rénale réversible et qu'ils peuvent jouer un rôle clé dans la restauration et le maintien de la structure et la fonction des tubes proximaux.

Mots clés : rein, ischémie, reperfusion, phosphate, transport.

Introduction

Renal ischemia–reperfusion is the main cause of renal failure in many pathophysiological situations. Because of its high rate of metabolism, the renal proximal tubule is particularly sensitive to ischemia–reperfusion injury (Weinberg 1991). Acute renal ischemia causes disruption of the cytoskeleton and associated structures due to rapid depletion of intracellular ATP, leading to the destruction of the brush border membrane (BBM) (Leiser and Molitoris 1993; Schwartz et al. 1999). Such an alteration should consequently affect the reabsorptive function of the proximal tubules. Indeed, earlier functional analyses have shown that acute ischemia–reperfusion reduced, in a time-dependent fashion, the renal BBM vesicle Na^+-dependent glucose (Na^+/glucose), Na^+-dependent phosphate (Na^+/P_i), and several organic anion transport activities (Molitoris and Kinne 1987; Kim et al. 1995; Kim et al. 1996).

The mechanisms underlying ischemia–reperfusion injury to some proximal tubule transport functions have recently been determined, following identification of the molecular

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basis of the transport functions. For example, the polyuric and natriuretic effects of temporary ischemia were shown to be caused by reduced expression of aquaporins and sodium transporters in the proximal tubules as well as in other parts of the nephron (Kwon et al. 1999; Wang et al. 1997; Wang et al. 1998).

One crucial function of the proximal tubule is to maintain inorganic phosphate (P_i) homeostasis through Na^+/P_i cotransport across the BBM (Murer and Biber 1992). This process might also be important for the proximal tubule itself during ischemia–reperfusion, since P_i is directly involved in energy metabolism, which plays a key role in ischemia-induced pathogenesis (Leiser and Molitoris 1993). However, earlier limited functional analyses did not clearly show the effects of ischemia–reperfusion on these activities. Acute renal ischemia (less than 60 min) in rabbits had no effect on BBM Na^+/P_i cotransport, while reperfusion following acute renal ischemia in rats was significantly reduced (Silverman et al. 1989; Kim et al. 1995). Furthermore, the molecular mechanisms underlying these pathologic processes have not yet been described.

Three types of Na^+/P_i cotransport systems in renal proximal tubule have been identified and partly characterized (Murer et al. 1999; Boyer et al. 1996; Xiao et al. 1997). The type II Na^+/P_i cotransporter NaP_i-2 was determined to be the predominant Na^+/P_i cotransporter in the rat BBM (Murer et al. 1997). The aim of our study was to monitor the expression of NaP_i-2 protein using immunoblotting analysis of BBM isolated from rats subjected to acute renal ischemia–reperfusion injury. The transport activities of the BBM vesicles were also measured and compared to the protein content. This study helps us to better understand the molecular basis regulating P_i homeostasis during ischemia and reperfusion.

Materials and methods

Induction of ischemia–reperfusion

The animals were maintained and experiments conducted in accordance with the guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (Charles River, St-Constant, Quebec) weighing 300–350 g were maintained on standard chow for 5 days, then divided into four groups for each of three separate experiments. All rats received, by subcutaneous injection, 2.5 mg/kg of acepromazine as a tranquilizer, followed by 87 mg/kg of ketamine and 7 mg/kg of xylazine to induce anesthesia. An abdominal incision was performed on every animal. In each experiment, Group I (n = 3) served as a sham control group (anesthesia for 30 min, A30) for ischemia. Group II animals (n = 6) were subjected to 30 min left renal pedical clamping to induce unilateral ischemia (I) after removal of the renal capsules. Group III animals (n = 3) were anesthetized for 90 min (A90) and served as a sham control group for reperfusion. Group IV animals (n = 6) were subjected to left pedical clamping for 30 min followed by reperfusion (R) for 60 min by releasing the clamps. At the end of the ischemia–reperfusion treatments, all rats were killed by lethal inhalation of CO_2 gas followed by decapitation.

Preparation of BBM vesicles

The BBM vesicles (BBMVs) from kidney cortex were purified with a $MgCl_2$ precipitation method described previously (Booth and Kenny 1974). Briefly, kidney cortices were carefully dissected and homogenized in a solution of 50 mM mannitol and 20 mM Tris–HCl, pH 7.4. BBM were isolated and resuspended in a solution of 300 mM mannitol and 20 mM Tris–HCl, pH 7.4, and stored at –80°C until analyzed. Protein concentration was determined with the Bradford method using bovine serum albumin (BSA) as standard (Bradford 1976). The isolated BBM were 9 ± 0.5-fold enriched over cortex homogenates as determined by analyses of the alkaline phosphatase activities.

Analyses of transport activities

The activities of Na^+-dependent P_i, D-glucose, and L-proline transport of the BBMVs were analyzed with a rapid filtration method described previously (Hopfer et al. 1973). Five microlitres of BBMV (about 80 μg protein) was incubated at 25°C for 5 s in a reaction medium composed of substrates and trace isotopes, followed by the addition of 1 mL ice-cold stop solution containing 150 mM KCl and 5 mM Tris–HCl, pH 7.4. For the Na^+/P_i cotransport assay, the reaction medium was composed of 150 mM NaCl or KCl, 200 μM [^{32}P]-KH_2PHO_4 (1 μCi/assay), and 25 mM Tris–HCl, pH 7.4. Similar media were used for the Na^+/D-glucose or Na^+/L-proline cotransport assays, except that 50 μM D-[^{3}H]-glucose (0.5 μCi/assay) or 50 μM L-[^{3}H]-proline (0.5 μCi/assay) were used, respectively, instead of [^{32}P]-KH_2PHO_4. Samples from each group of animals were measured in triplicate.

Gel electrophoresis

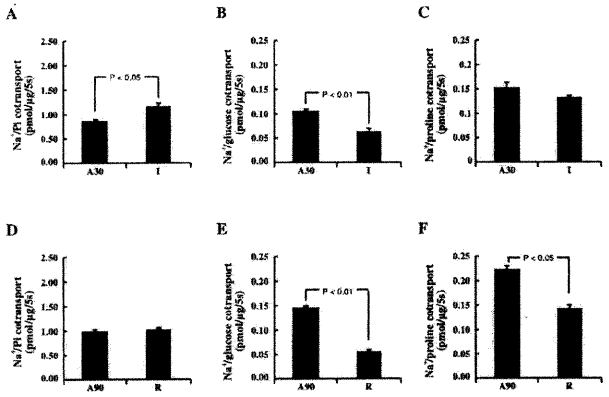
Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was done in the discontinuous system described by Laemmli (Laemmli 1970) using a Mini-Protean II apparatus (Bio-Rad, Mississauga, Ont.). The BBM samples were solubilized in a loading buffer containing 20% (w/v) glycerol, 1% (w/v) SDS, and 62.5 mM Tris–HCl, pH 6.8, with or without 4% β-mercaptoethanol (β-EtSH). Twelve micrograms of BBM proteins were loaded into each well in the 7.5% polyacrylamide gels.

For two-dimensional gel electrophoresis (2-D), the first dimension was isoelectric focusing (IEF) and the second was SDS-PAGE. BBM samples (100 μg protein) were solubilized in 2% SDS and mixed with an immobilized pH gradient (IPG) Drystrip (Pharmacia Biotech, Montréal, Que.) reswelling buffer containing 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) Brij-58, 0.3% (w/v) dithiothreitol, 2% (v/v) carrier ampholyte (Bio-lyte, Bio-Rad) pH 3–10, a trace amount of Bromophenol blue, and protease inhibitors in a final volume of 125 μL. Brij-58 was added because it is the most efficient non-ionizing detergent for NaP_i-2, as assessed by our preliminary experiments. This mixture was incubated with 7-cm IPG Drystrips at 25°C overnight. IEF was run with a standard protocol as described by the manufacturer. Following IEF, strips were equilibrated in SDS-PAGE sample buffer and loaded onto 12% polyacrylamide gels.

Western blotting analysis

NaP_i-2 proteins were detected with two antibodies raised individually against the N- and C-termini of NaP_i-2, as previously described (Boyer et al. 1996; Xiao et al. 1997). Briefly, proteins electrotransferred to the polyvinylidene difluoride (PVDF) membranes were blocked overnight at 4°C in a washing buffer of 150 mM NaCl, 0.3% (w/v) Tween-20, and 50 mM Tris–HCl, pH 7.5. The membranes were incubated with anti-C (1: 20 000) or anti-N (1: 10 000) antibody for 1 h at 25°C and then with a horseradish peroxidase-linked secondary antibody (1: 1 000) from donkey (Amersham Pharmacia Biotech, Montréal, Que.) for another hour. PVDF membranes were incubated with enhanced chemiluminescence solutions and exposed to X-ray films. For densitometric analysis, protein bands were scanned with a Personal Densitometer SI scanner (Molecular Dynamics, Sunnyvale, Calif.) and quantified with the IPLab Gel H program (Sigma Analytics Corporation, St. Louis, Mo.).

Fig. 1. Effect of ischemia and reperfusion on Na^+/P_i, Na^+/D-glucose, and Na^+/L-proline cotransport in renal BBMV. Rats were submitted to kidney ischemia for 30 min (A, B, C) or ischemia for 30 min followed by 60 min reperfusion (D, E, F). The Na^+/P_i, Na^+/D-glucose, and Na^+/L-proline cotransport activities were measured using a rapid filtration method. A30, sham-operated rats as control for ischemic rats; I, ischemic kidneys; A90, sham-operated rats as control for reperfused rats; R, reperfused kidneys. Results represent mean ± SE of three experiments.



Statistical analysis

The results are expressed as mean ± SE of three assays, and analyzed with the Student's *t*-test. Only significant differences ($P < 0.05$) are indicated in the figures.

Results

Effect of ischemia–reperfusion on transport activities in BBM

The Na^+/P_i cotransport activity of the isolated BBMVs was analyzed first to determine the effects of ischemia–reperfusion at the functional level. The results showed that, unexpectedly, ischemia led to a 31% increase ($P < 0.05$) in this activity as compared with the sham control (Fig. 1A). The Na^+/D-glucose and Na^+-dependent amino acid cotransport activities, which have been used to assess the effects of ischemia on proximal tubule function in previous studies (Kim et al. 1995; Silverman et al. 1989), were also analyzed in our study. In contrast with Na^+/P_i cotransport, the Na^+/D-glucose cotransport activity of the BBMV from ischemic rats was significantly decreased by 45% (Fig. 1B), while the Na^+/L-proline cotransport did not change significantly (Fig. 1C). After 60 min of reperfusion, the Na^+/P_i cotransport activity of the reperfused rats returned to a level comparable to that of control rats (Fig. 1D), whereas the Na^+/D-glucose and Na^+/L-proline cotransport activities were further suppressed to 60% and 33%, respectively (Fig. 1E and 1F).

Expression of NaP_i-2 protein following ischemia–reperfusion

Previous Western blot analyses showed that NaP_i-2 displays different protein patterns on reducing and non-reducing SDS-PAGE (Boyer et al. 1996; Xiao et al. 1997; Biber et al. 1996). In non-reducing SDS-PAGE, NaP_i-2 was detected as two major proteins of 180 kDa (p180) and 70 kDa (p70) when probed with the anti-N as well as the anti-C antibody. In reducing SDS-PAGE, the anti-N antibody recognized two major proteins of 70 kDa (p70) and 45 kDa (p45) while the anti-C recognized two proteins of 70 kDa (p70) and 40 kDa (p40) (Boyer et al. 1996; Xiao et al. 1997; Biber et al. 1996). All forms of NaP_i-2 proteins were immunodetected and quantified by densitometry so we could determine the effects of ischemia–reperfusion on NaP_i-2 proteins.

Fig. 2A and 2B show the effects of ischemia on the abundance of NaP_i-2 in BBM as analyzed under reducing electrophoresis conditions. It is obvious that ischemia led to an overall diminution of all reduced forms (p70, p45, and p40) of NaP_i-2. Densitometric analysis showed that the

Fig. 2. Immunoblot analysis of NaP_i-2 p70, p45, and p40 expression in renal BBM from rats subjected to renal ischemia and reperfusion. BBM proteins (12 µg per lane) from rats subjected to renal ischemia and reperfusion were analyzed by SDS-PAGE under reducing conditions in the presence of 4% β-EtSH followed by electrotransfer to PVDF membranes. Immunodection with antibodies directed against the N-terminal region (A) or C-terminal region (B) of NaP_i-2 protein to visualize NaP_i-2 p70 and p45 or NaP_i-2 p70 and p40 forms was performed. Quantitative analysis of NaP_i-2 protein expression was assessed using densitometry (C). Data for p70 were the average of changes in its expression derived from densitometric analysis of both antibodies directed against the anti-N (Panel A) and the anti-C (Panel B) regions of the protein. Data for p45 were from its immunodetection in Panel A and those for p40 were from Panel B. Results represent mean ± SE of three experiments. * Significantly different ($P < 0.05$, $n = 3$, Student's *t*- test) from the corresponding control (dashed line).

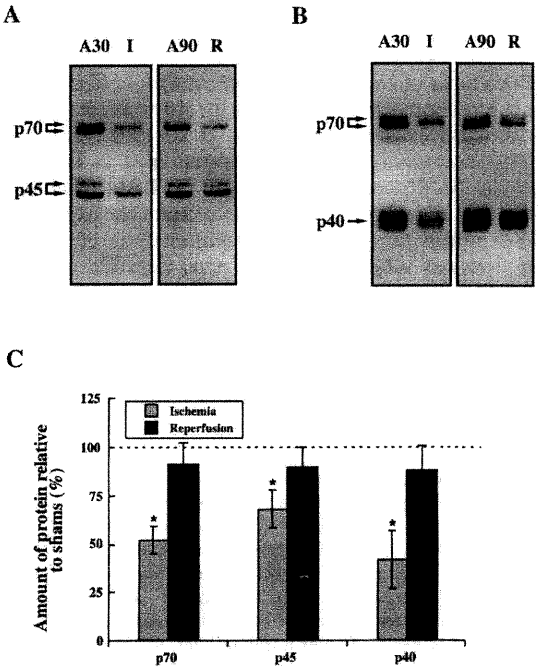
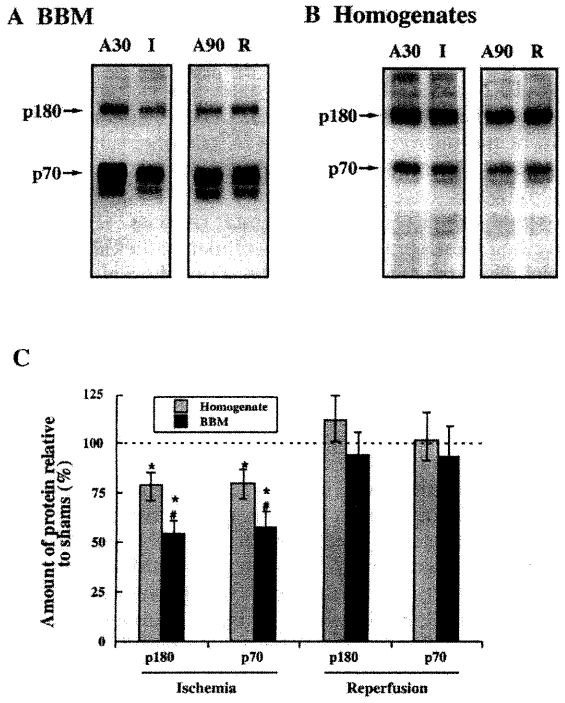


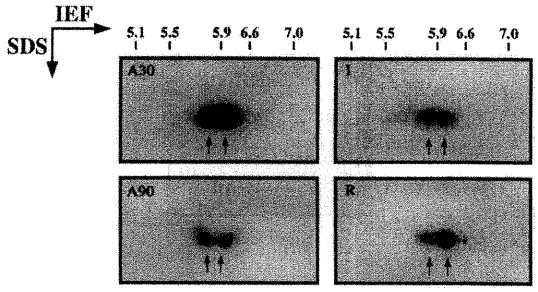
Fig. 3. Immunoblot analysis of NaP_i-2 p180 and p70 expression in BBM and renal cortex homogenates from rats subjected to renal ischemia and reperfusion. (A) Proteins of BBM (12 μg per lane) and (B) cortex homogenates (60 μg per lane) from rats subjected to renal ischemia and reperfusion were analyzed by SDS-PAGE under non-reducing conditions, followed by electrotransfer to PVDF membranes and immunodetection with antibody directed against the C-terminal region of the NaP_i-2 protein. Under these conditions, only p180 and p70 were detected in BBM and cortex homogenates. (C) Assessment of NaP_i-2 protein levels were determined by densitometric analysis of immunodetected p70 and p180 forms in Panels A and B. Results represent mean ± SE of three experiments. * Significantly different ($P < 0.05$, $n = 3$, Student's t-test) from the corresponding control (the dashed line). # Significant difference ($P < 0.05$, $n = 3$, Student's t-test) in the amounts of p180 or p70 between homogenates and BBM.



amounts of these forms were decreased significantly by 48%, 32%, and 58%, respectively (Fig. 2C). After 60 min reperfusion, however, the amounts of each form returned to the levels found in shams, being 91%, 90%, and 88% of the control values (Fig. 2C). The degree of change to each individual form was not significantly different from the others, neither during ischemia nor after reperfusion, suggesting a uniform effect of ischemia–reperfusion on these forms.

The effects of ischemia–reperfusion on NaP_i-2 proteins were also compared between BBM and cortex homogenates using electrophoresis under non-reducing conditions and Western blotting analysis. The advantage of the non-reducing conditions was to analyze the effects of ischemia on expression levels of high molecular weight forms of NaP_i-2 (p70 and p180). Ischemia decreased the amounts of NaP_i-2 proteins in both BBM and cortex homogenates, but

Fig. 4. Two-dimensional electrophoresis of NaP_i-2 proteins in BBM from rats subjected to ischemia and reperfusion. BBM proteins (100 μg per gel) from sham (A30 or A90), ischemic (I), and reperfused (R) rats were subjected to IEF electrophoresis as the first dimension in immobilized pH gradient gel strips. After IEF, the gel strips proceeded to reducing SDS-PAGE as the second dimension, followed by electrotransfer and immunodetection with the antibody that was raised against the C-terminal region of NaP_i-2 protein. The isoelectric points (P_i) of NaP_i-2 protein were measured using BioRad IEF standard proteins for calibration. The arrows designate the two major spots of the NaP_i-2 p70 form.



the degree of changes in these two preparations were different (Fig. 3A and 3B). Densitometric analysis showed that in BBM, ischemia led to 45% and 42% decreases of p180 and p70, respectively, whereas in the homogenate, ischemia led to only 21% and 20% decreases, respectively (Fig. 3C). After reperfusion, the levels of p180 and p70 in both the BBM and homogenate returned to control values.

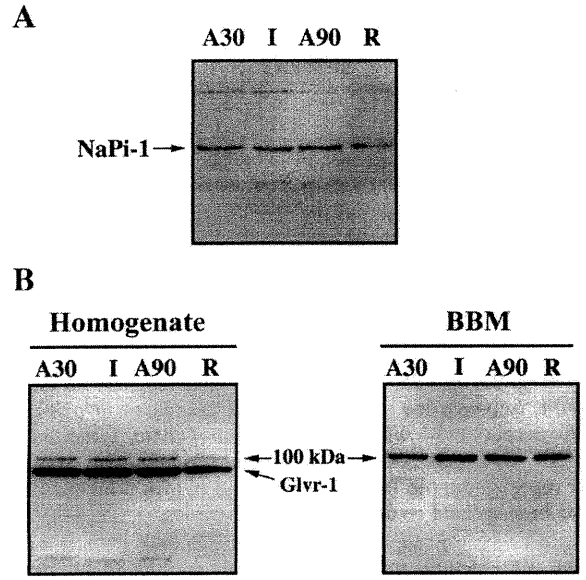
Two dimensional gel electrophoresis of NaP_i-2 following ischemia–reperfusion

The two-dimensional electrophoretic (2-D) profiles of NaP_i-2 proteins in BBM from sham controls, ischemic and reperfused kidneys are displayed in Fig. 4. Only the p70 form was clearly detected with anti-C under the 2-D conditions. As shown in Fig. 4, this polypeptide appeared as two major spots on the immunoblots of every group of rats. The isoelectric point (P_i) of these two spots are 5.8 and 6.0, respectively, as calibrated against a set of IEF standard proteins. Although the relative densities of the two spots varied among different groups of rats, the P_i values remained unchanged. The total amount of p70 in the 2-D immunoblot was strongly reduced by 30 min ischemia as compared to sham rats, whereas after reperfusion, the amount of p70 was almost returned to control level. These quantitative changes revealed by 2-D analysis are generally consistent with those observed in Fig. 2 and Fig. 3. In addition, they show that phosphate uptake modulation during ischemia does not result from changes in different forms of NaP_i-2, such as their relative amounts.

Effects of ischemia–reperfusion on the expression of NaP_i-1 and Glvr-1

Other types of Na^+/P_i cotransporters, such as NaP_i-1 and Glvr-1, exist in renal proximal tubules (Biber et al. 1993, Delisle et al. 1994, Boyer et al. 1998) and we examined the effects of ischemia–reperfusion on the expression of these cotransporters. Densitometric analysis of immunoblots

Fig. 5. Immunoblot analysis of type I (NaP_i-1) and type III (Glvr-1) Na^+/P_i cotransporters in BBM or cortex homogenates from rats subjected to renal ischemia and reperfusion. (A) BBM proteins (20 μg per lane) from rats subjected to renal ischemia and reperfusion were analyzed by SDS-PAGE, electrotransferred to PVDF membranes and were probed with an antibody against NaP_i-1 protein. (B) Homogenate proteins (20 μg per lane) or BBM proteins (60 μg per lane) from rats subjected to renal ischemia and reperfusion were analyzed by SDS-PAGE, electrotransferred to PVDF membrane and were probed with an antibody against Glvr-1.



revealed that ischemia and reperfusion significantly decreased the abundance of type I cotransporter NaP_i-1 by 44% and 39%, respectively (Fig. 5A).

The type III Na^+/P_i cotransporter Glvr-1 has previously been detected in OK cells and in crude membranes of rat kidney cortex as a polypeptide of 85 kDa (Boyer et al. 1998), but the subcelluar distribution of this protein in the proximal tubular epithelial cells has not been established. Our study compared the expression of Glvr-1 in kidney cortex homogenate and BBM. A polypeptide of 85 kDa was detected in the homogenate but not in the BBM of any group of rats, indicating that Glvr-1 was not expressed in the apical membrane of proximal tubules (Fig. 5B). An unknown protein of 100 kDa was detected both in homogenates and in BBM. This protein was not enriched in the isolated BBM, compared with homogenates, when the same amount of protein was applied onto gels (data not shown). In cortex homogenates, densitometric analysis showed that Glvr-1 expression was unaffected by ischemia (Fig. 5B).

Discussion

Our study analyzed the effects of reversible renal ischemia and reperfusion on the expression of NaP_i-2 proteins in rat renal BBM. Our results demonstrated a remarkable decrease in the amounts of different forms of this protein after 30 min of ischemia. Reperfusion for 60 min, however, led to the recovery of all forms of NaP_i-2 to almost control levels. Functional analysis indicated that the BBM Na^+/P_i cotransport activity was somehow increased by ischemia, but returned to the control level after reperfusion.

The most striking initial event in acute ischemia-induced proximal tubule damage is the rapid disruption of the cytoskeletal system (Leiser and Molitoris 1993), due to the activation of actin depolymerization factor (Schwartz et al. 1999). Such a drastic event would presumably suppress the expression of membrane proteins whose intracellular trafficking is highly dependent on the cytoskeletal system. NaP_i-2 may be one of this type of protein. Previous studies have shown that marked alterations in the polymerization dynamics and stability of both actin microfilaments and microtubules are involved in the modulation of Na^+/P_i cotransport in opossum kidney cells (Papakonstanti et al. 1996). In those cells, NaP_i-4, another member of the type II Na^+/P_i cotransporter that is nearly identical (structurally and functionally) to NaP_i-2, is the predominant BBM Na^+/P_i cotransporter (Sorribas et al. 1994). In addition, the turnover of NaP_i-2 protein in BBM has been convincingly shown to be modulated through microtubules, most likely as a component of the microtubule cargoes in proximal tubular epithelial cells (Lotscher et al. 1997; Lotscher et al. 1999). Microtubules have been demonstrated to be disrupted in proximal tubules during ischemia (Abbate et al. 1994). Based on these studies, the intracellular trafficking of NaP_i-2 is tightly associated with both the actin cytoskeleton and the microtubules. Thus, the decreased expression of all forms of NaP_i-2 protein in the BBM by ischemia, as observed in the present study, could be a direct consequence of ischemia on these cytoskeletal systems.

After reperfusion for 60 min, the levels of NaP_i-2 forms in BBM recovered almost to control levels, probably due to the re-establishment of the cytoskeletal systems that facilitate the relocalization of membrane proteins (Leiser and Molitoris 1993). Compared to the rate of complete recovery of the cytoskeleton (Schwartz et al. 1999; Abbate et al. 1994), the restoration of NaP_i-2 to the BBM within 60 min is a rapid process. The details of this process are unknown so far, but an early recovery of NaP_i-2 protein and Na^+/P_i cotransport activity in the BBM would benefit the recovery of the proximal tubular epithelial cell, by pumping in P_i ions for the needed energy supply (ATP).

The ischemia-induced decrease in the amount of NaP_i-2 is also observed in the kidney cortical homogenate, although to a lesser extent than in the BBM. The decrease in the total amount of NaP_i-2 is probably due to lysosomal degradation, a mechanism involved in the rapid downregulation of NaP_i-2 protein through factors such as parathyroid hormone and a high P_i diet (Katai et al. 1997; Keusch et al. 1998; Traebert et al. 2000). Blebbing of BBM into the proximal tubule lumen (Leiser and Molitoris 1993) could also be responsible for the loss of some NaP_i-2 proteins from the cortical homogenate. The difference (about 20%) in the extent of decrease of NaP_i-2 between the BBM and the cortex homogenate suggests that BBM NaP_i-2 proteins are more vulnerable to change than NaP_i-2 proteins in other compartments such as subapical vesicles and endosomes (Katai et al. 1997; Custer et al. 1994).

Although decreases in activities such as Na^+/D-glucose cotransport and organic anion transport in proximal tubules

have been observed during renal ischemia previously as well as presently (Molitoris and Kinne 1987; Kim et al. 1995; Kim et al. 1996), the increased Na^+/P_i cotransport activity accompanied by a decreased amount of the predominant renal BBM Na^+/P_i cotransporter, NaP_i-2, was unexpected. To explain the discrete effect of ischemia on BBM Na^+/P_i cotransport activity, we examined the possible involvement of ischemia-induced posttranslational modifications on NaP_i-2 with 2-D electrophoresis, as well as the expression of other types of renal Na^+/P_i cotransporters. However, results from the 2-D analysis did not show a change in the isoelectric points of the two isoforms of NaP_i-2 p70 in the BBM from ischemic rats. Although there is a slight difference in the relative ratios of the two p70 isoforms among different groups of rats, it could not clearly explain the differences in the transport activity since the levels of both isoforms decreased and their contributions to P_i transport remains to be established. Analysis of the expression of NaP_i-1 and Glvr-1 also excluded the possibility of these Na^+/P_i cotransporters increasing the Na^+/P_i cotransport activity, since NaP_i-1 is decreased by both ischemia and reperfusion, while Glvr-1 is not expressed in the BBM. The absence of Glvr-1 in the BBM suggests that it might be expressed in the basolateral membrane, since this protein was detected in the proximal tubular cells (Boyer et al. 1998). The absence of Glvr-1 in the BBM from ischemic rats also implies that, at least within 30 min, ischemia did not cause the redistribution of Glvr-1 from the basolateral membrane to BBM. In contrast, the basolateral membrane protein Na^+/K^+-ATPase was found to be redistributed to the BBM during ischemia (Molitoris et al. 1992). This suggests that ischemia has selective effects on different membrane proteins.

Perhaps the increased BBM Na^+/P_i cotransport activity during ischemia could be explained by ischemia-induced modifications of the membrane properties. Previous studies have revealed that ischemia sharply reduced the BBM cholesterol-to-phospholipid ratios and increased BBM lipid peroxidation, leading to an increase in membrane fluidity (Molitoris and Kinne 1987; Molitoris et al. 1985; Montagna et al. 1998). Increases in BBM membrane fluidity are known to specifically increase Na^+/P_i cotransport activity and decrease Na^+/glucose cotransport activity (Molitoris et al. 1985; Friedlander et al. 1988; Levi et al. 1989; Friedlander et al. 1990). Thus, the increased Na^+/P_i cotransport activity observed in our study could be better explained by the effect of ischemia on BBM fluidity. However, the underlying mechanisms are not known and need further investigation.

So far, the phosphate uptake taking place during kidney ischemia or reperfusion has been poorly studied. The physiological role for the increase in Na^+/P_i cotransport activity that we reported in this study in renal BBM during ischemia remains to be established. The increase in Na^+/P_i cotransport activity in BBM during ischemia suggests, however, that the proximal tubule reabsorption of filtered phosphate should not be compromised and that the fractional excretion of phosphate should not increase in spite of a marked reduction of NaP_i-2 level in these apical membranes under these injury conditions.

In summary, this study shows that reversible renal ischemia significantly decreased the abundance of NaP_i-2 protein in rat renal BBM, while reperfusion led to a rapid recovery of NaP_i-2 protein in BBM. The Na^+/P_i cotransport activity of the BBM was increased by ischemia but, upon reperfusion, returned to levels seen in sham rats. The contrasting effects of ischemia upon the BBM NaP_i-2 protein and Na^+/P_i cotransport activity suggests that P_i transport plays a key role during ischemia–reperfusion in restoring and maintaining both normal energy metabolism and the structure and function of the proximal tubules.

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IV. DISCUSSION

IV. DISCUSSION

Using biochemical approaches in combination with functional analytical methods, this thesis project investigated the basic structural components of NaPi-2 protein and their relationships, the functional unit of NaPi-2, the identification and implication of NaPi-2 cleavage, and the effect of ischemia/reperfusion on Na^+/Pi cotransport activity and NaPi-2 protein expression in BBM. Quality control of experiments has been carried out carefully throughout all studies. For the purity of the BBM, activity of alkaline phosphatase (Kelly and Hamilton, 1970) were measured and the enrichment of the BBM was 9-11 fold as compared to homogenate. Protein concentration of BBM was determined in duplicate or triplicate with Bradford method (Bradford, 1976). In Western blot experiments, the amounts of proteins loaded on gels were the same (10 to 20 •g per lane) in most cases unless otherwise specified. This amount is within the linear range of densitometric analysis of NaPi-2 (Fig. 2, MANUSCRIPT I). For LPD experiments, we have found that the alkaline phosphatase activity was not altered by LPD. For tissue distribution and age effect experiments (MANUSCRIPT III) as well as ischemia experiments, same amount of total BBM proteins were loaded on each lane of gels. We did not use a specific protein as internal control since we did not know if other proteins are affected, especially in the case of ischemia where numerous proteins were changed in a time-dependent manner (Leiser and Molitoris, 1993, Schwartz *et al.*, 1999). Densitometric analyses were done with a Personal Densiotometer SI scanner (Molecular Dynamics) and the images were analyzed with the IPLab Gel H program (Signal Analytics Corporation).

IV.1 The basic structure of NaPi-2 protein in rat renal BBM

Previous studies from our group as well as others suggested that the rat renal sodium dependent phosphate cotransporter, NaPi-2, might exist as a complete (p70) and/or a fragment (p40) of the whole polypeptide (Boyer *et al.*, 1996, Biber *et al.*, 1996). To better understand the structure-function relationship of this transporter, the present study first tried to demonstrate more clearly the structural links between the different NaPi-2 entities. Our hypothesis was that an N-terminal fragment of NaPi-2 might also exist in BBM.

To prove this point, we purified another antibody (anti-N) raised against the N-terminal sequence of NaPi-2. Together with the anti-C antibody used previously (Boyer *et al.*, 1996), we compared the electrophoretic profile of NaPi-2 protein under reducing and non-

reducing conditions. As a result, upon non-reducing SDS-PAGE, both antibodies detected protein bands with identical apparent sizes ranging from 70 to 90 kDa (p70). This polypeptide should be the monometric form of NaPi-2 that has a predicted size of 68.7 kDa (Magagnin *et al.*, 1993). The higher apparent size might be the reflection of the fact that NaPi-2 is N-glycosylated (Hayes *et al.*, 1994), while the breadth of the p70 band on Western blot might be due to different levels of glycosylation. This result is consistent with results of others under similar experimental conditions (Biber *et al.*, 1996). In the present study a band with a much higher apparent size of 180 kDa (p180) was also revealed by both antibodies. For the ease of discussion, this band is described later (see Section IV. 2).

Upon reducing SDS-PAGE in which 5% ß-EtSH was added to the sample preparation buffer, in addition to the p70, the anti-C detected the 40 kDa peptide which is the p40 as shown previously (Boyer *et al.*, 1996), while the anti-N detected a new peptide of 45-49 kDa and named p45. This result is nearly identical to another report from Murer's group (Biber *et al.*, 1996). Again, the width of the bands suggests that both p45 and p40 are glycosylated. These data have interesting implications in the structure of NaPi-2. First, the existence of p45 and p40 which have sizes smaller than the monomeric form, p70, suggests that in rat renal BBM a significant amount of NaPi-2 is expressed in cleaved forms. Second, the fact that the sum of the sizes of p45 and p40 are close to the size of p70 indicates that p45 represents the N-terminal while the p40 represents the C-terminal product of NaPi-2 (p70) cleavage. Actually, the assumption that the p40 is derived from the C-terminal of p70 has been strongly demonstrated in our previous study by comparing the tryptic proteolysis profile of p40 and p70 (Boyer *et al.*, 1996).

The direct evidence for the origin of p40 came from the experiment in which the unreduced p70 was further analyzed under reducing conditions. As a result, p40 was detected upon reduction of p70. Using the same experimental procedure, p45 was detected with anti-N antibody (unpublished data). Thus, it is obvious that p45 and p40 are derived from p70 via the disruption of disulfide bond(s) between them. The dose-dependent appearance of p45 and p40 and disappearance of p70 with the treatment of the reducing agent ß-EtSH, DTT or TCEP strongly supported this point. This observation implies that alteration in the redox status of the microenvironment of BBM might modulate the association state of p45 and p40, and thus influence the Na^+/Pi cotransport activity.

Besides our and Biber's reports (Boyer *et al.*, 1996, Biber *et al.*, 1996), studies from other groups rarely mention peptides similar to p45 and p40 when describing immunodetection of rat renal BBM (for example, Arar et al., 1999). This may be for two reasons. First, each study used different concentrations of reducing agent during SDS-PAGE analysis and thus obtained different degrees of reduction of p70. Second, some studies may simply consider p45 and p40 as protein degradation products occurs during sample preparation. To see a complete reduction pattern of NaPi-2, we incubated the BBM with sample buffers containing different concentrations of ß-EtSH. Indeed, we found that the concentration of ß-EtSH affects the yields of p45 and p40 on immunoblots. The ratio of p45 or p40 to p70 increases with the increase of ß-EtSH concentration. But a saturation effect was found at ß-EtSH concentrations higher than 1% after which the ratio remained unchanged. This indicates that NaPi-2 in the rat renal BBM exists both in intact form (intact p70) and in cleaved form (cleaved p70), with the intact form being the integral NaPi-2 protein and the cleaved form being the p45 and p40. Therefore, the basic structural entities of NaPi-2 in the rat renal BBM are the three polypeptides, namely, p45, p40, and intact p70.

Regarding the second reason mentioned above, our results clearly demonstrated that p45 and p40 are not degradation products, since incubation of isolated kidney on ice as well as incubation of isolated BBM at 37°C did not change the ratio of cleaved p70 to intact p70. Furthermore, the addition of protease inhibitors during BBM purification or the incubation of purified BBM with protease inhibitors had no effect on the detection of p70, p45 and p40. These data strongly supports our point of view that p45 and p40 are not degradation artifacts created during the preparation of BBM.

IV.2 The functional unit of NaPi-2 protein as Na^+/Pi cotransporter

In addition to p70, the present study detected a protein of 180 kDa (p180) with both anti-N and anti-C antibodies on non-reducing SDS-PAGE. This p180 has been reported earlier under the same experimental conditions, although it was not further described (Biber *et al.*, 1996). Our data also showed that p180 disappears upon reduction and this process is dose-dependent. That is, as the concentration of the reducing agents increase, p180 disappears gradually. Concomitantly, those lower molecular weight forms of NaPi-2, the p45, p40, and p70 appear or increase in amount. Thus, p180 is most likely an oligomeric form of NaPi-2 composed of the three basic structures of NaPi-2. In fact, this has been confirmed by the

experiment in which the isolated p180 was completely converted into p45, p40, and p70 by treatment with ß-EtSH. As the molecular weight of p180 is close to the sum of two p70's, it is very likely that the p180 is the dimeric form of p70. Based on the above analysis, models for the structure of p180 could be proposed as the following: (1) p180 is composed of two intact p70s linked through disulfide bond (s), (2) p180 is composed of one intact p70 linked through disulfide bond(s) with one cleaved p70 consisting of disulfide bond-linked p45 and p40, (3) p180 is composed of two cleaved p70s linked through disulfide bond (s), each cleaved p70 consisting of disulfide bond-linked p45 and p40. Although much more investigation is needed to verify these models, they can serve as useful working hypotheses in further analysis of the structure-function relationship of NaPi-2.

Clearly, the above data indicate that disulfide bonds must play an important role in the formation of p180. One can speculate that through the formation of disulfide bonds, the oligomeric state of NaPi-2 is stabilized, and the function of NaPi-2 as a Na^+/Pi cotransporter is assured. The importance of disulfide bonds in the formation of oligomeric structure and function of a membrane protein has been demonstrated in many cases such as the type-C natriuretic peptide receptor (Iwashina *et al.*, 1994) and the GLUT1 glucose transporter (Zottla *et al.*, 1995). To see how disulfide bond is important for Na^+/Pi cotransport activity of NaPi-2, the present study analyzed Na^+/Pi cotransport activity of the isolated rat renal BBMV after treatment with reducing agents *in vitro*. In this way, a striking correlation between the Na^+/Pi cotransport activity and the amount of p180 was observed. When p180 was reduced by the addition of any of the three reducing agents (ß-EtSH, DTT and TCEP), detection of p70 was increased but p180 disappeared in correlation with a diminished phosphate uptake into the BBM vesicles. These data demonstrate p180 as the functional unit of NaPi-2. We also found that at a given concentration the reducing agents had a much weaker effect on the reduction of NaPi-2 when added to intact BBM vesicles than when added to solubilized membranes. The inter-polypeptide disulfide bonds thus seem to be protected in the intact membrane and become readily exposed only once the membrane proteins have been solubilized and unfolded by SDS.

Our results from the effect of reducing agents on Na^+/Pi cotransport study are somewhat at variance with those of Suzuki *et al.* (Suzuki *et al.*, 1990), who reported a slight but significant stimulation of phosphate transport in rabbit renal BBM vesicles pre-incubated with 5 mM DTT. Phosphate transport was also increased in vesicles reconstituted from

bovine renal BBM when 1 mM DTT was included in the buffer used to solubilize the proteins (Vachon *et al.*, 1995). The different effects of reducing agents observed in these studies could, however, be due to the use of different animal species and experimental conditions. For example, in the present study, the vesicles were incubated at least twice as long and, for most experiments, with higher concentrations of the reducing agents.

The target size (TS) of NaPi-2 was also analyzed to complement the above studies in the identification of the functional unit. Similar target sizes (TS's), ranging from 178 kDa to 199 kDa, were estimated from the radiation-induced disappearance of p40, p70 and p180 in immunoblots probed with anti-C antibody. These values, which are not significantly different from one another, strongly suggest that all three polypeptide species are parts of the same oligomeric structure. Because the oligosaccharide moiety of glycoproteins does not contribute to the molecular sizes determined with the radiation inactivation method (Beauregard, *et al.*, 1987, Jung *et al.*, 1988, Beliveau *et al.*, 1989), these TS values correspond to two to three times the size of a p70 subunit. Thus, this study at least partly supports our observation that NaPi-2 functions as a dimer in the BBM. The different polypeptides within the NaPi-2 oligomer seem to be closely associated with one another because, after a single radiation hit, the absorbed energy causes the fragmentation of all subunits. Disulfide bonds between p70 subunits might contribute to this energy transfer. The results obtained from vesicles pretreated with ß-EtSH before irradiation demonstrated the effects of reducing agents on the state of oligomerization of NaPi-2. Under these reducing conditions, NaPi-2 exists as a monomer but not an oligomer.

IV.3 Identification of the cleavage site

As discussed above, the rat renal BBM NaPi-2 exists also in cleaved but disulfide bond-linked forms. This cleavage must occur at a specific site since only two peptides are seen. Specific proteolytic cleavage is a process involved in regulation of many proteins, including membrane-associated receptors and enzymes (Denault *et al.*, 1996, Bolduc *et al.*, 1997). The effect of cleavage of NaPi-2 on transport activity of NaPi-2 is unknown. We investigated location of the cleavage site on the protein. As demonstrated in the deglycosylation study, the intact p70 as well as p45 and p40 are all N-glycosylated. The deglycosylated intact p70 migrated as a protein of 57 kDa, a value significantly lower than that calculated from the amino acid sequence of NaPi-2 (68.7 kDa) (Magagnin, *et al.*, 1993).

Similarly, a lower apparent MW of 60-65 kDa has also been reported after NaPi-2 was deglycosylated with N-glycosidase (Hayes *et al.*, 1994). The lower apparent MW of unglycosylated polypeptide backbone as compared to the theoretical value has also been observed for other membrane transporter proteins (Jacquemin *et al.*, 1994, Hagenbuch *et al.*, 1991). The reason for this discrepancy is unknown. It is most probably due to the complex migratory behavior of membrane proteins in SDS-PAGE gels.

Our data showed that once deglycosylated, p40 and p45 appear to be 30 and 35 kDa respectively. The sum of these values is very close to the predicted size of NaPi-2, suggesting p45 and p40 as cleavage products of NaPi-2. By matching the deglycosylated sizes of p45 and p40 with the amino acid sequence of NaPi-2 using the protein analysis software MacVector, this cleavage site can be estimated to be in the second extracellular loop between transmembrane domains TM3 and TM4. A more precise estimation of the cleavage site could be done based on the sites of N-glycosylation of NaPi-2. Site-directed mutagenesis experiments indicated that NaPi-2 is N-glycosylated at only two out of four potential N-glycosylation sites, the Asn 298 and the Asn 328 (Hayes *et al.*, 1994). Thus, the cleavage site was localized to somewhere between these two sites which are only 29 amino acids apart.

To locate the exact site of the cleavage by protein microsequencing, the p40 was purified upon the reduction of the isolated p180 with ß-EtSH. After N-deglycosylation, a band of p40 was identified with Western blot and Coomassie blue stain. N-terminal amino acid sequence analysis of this band indicated that the first five amino acids of the p40 N-termini are VEAIG, which corresponds to residues 320-324 of NaPi-2. Although a sequence of GLDRP was also detected, this signal represented less than 30% of the total signal and this sequence does not exist in any of the type II cotransporters that could be detected with anti-C, nor in the N-terminals of the newly identified NaPi-2 α, β, or γ isoforms (Tatsumi *et al.*, 1998). This GLDRP sequence could arise from the endoglycosidases themselves, or other BBM proteins that behaved like NaPi-2 during the purification procedure, or it could correspond to an amino acid released by random chemical cleavages of glycosidase, NaPi-2 or other polypeptides during sequencing. Overall, the sequencing results indicate that the cleavage site in p70 that yields p40 and p45 is the peptide bond between Arg 319 and Val 320. This cleavage site, located between the two N-glycosylation sites at the largest extracellular loop of NaPi-2 as we predicted with deglycosylation experiment, exposed to a variety of proteases, including vesicular, secretory and ecto-enzymes. Convertases of the furin

family are candidate proteases for the cleavage of NaPi-2 since they cleave at arginine and lysine residues and they are involved in the bioactivation of a variety of secretory and membrane proteins (Denault *et al.*, 1996, Bolduc *et al.*, 1997).

Since the two sites are located between TM3 and TM4 of NaPi-2 (Magagnin *et al.*, 1993), the p45 must be a polypeptide possessing the three N-terminal TMs of NaPi-2, while the p40 must be the one possessing the five C-terminal TMs of NaPi-2. Interestingly, a recent study suggested that the type II cotransporter is a cotransporter that originates from two ancient independent genes encoding an N-terminal three-TM polypeptide and a C-terminal five-TM polypeptide respectively (Kohl *et al.*, 1996). This coincidence implies that the cleaved NaPi-2 might also be effective as Na^+/Pi cotransporter.

Based on the procedure of p40 purification, we were able to estimate that NaPi-2 proteins account for about 0.005% of the total BBM proteins for rats fed a normal Pi diet. This percentage could reach as high as 0.05% considering that LPD increased the expression of NaPi-2 by more than 10 folds (Fig. 8, Review of Literature).

IV. 4 Mediation of Na^+/Pi cotransport by different forms of NaPi-2

The ideal method to determine which form of NaPi-2 plays a more important role in Na^+/Pi cotransport would have been the expression of different forms in appropriate cell lines. Unfortunately, transfection of NaPi-2 in OK cells, which is the best-studied in vitro model for Na+/Pi cotransport, resulted in the expression of the intact form (p70) only (personal communication with Dr. Biber from the Institute of Physiology, University of Zurich, Switzerland). Since Na^+/Pi cotransport activity is influenced by many physiological processes as described in the REVIEW of LITERATURE section of this thesis (refer to sections I.9 to I.16), and most importantly, different forms of NaPi-2 can be detected in the kidney, it should be easier to clarify the role of each form in renal Na^+/Pi cotransport. Based on this rationale, we compared variations in renal Na^+/Pi cotransport activity with changes in the levels of different forms of NaPi-2 in three different situations in rats, including developmental age, localization in the kidney cortex and dietary Pi. As summarized in Table 1 and discussed thereafter (Manuscript III), the results show that increased activity does not correlate with a unique protein expression pattern.

In general, our results indicated a higher transport activity in BBM obtained from adult compared to newborn rats, from juxtamedullary compared to superficial rat renal cortex, and from LPD compared to NPD fed rats. The stimulating effect of LPD on renal Na^+/Pi cotransport had already been very well established (Murer *et al.*, 1992) and our results are in agreement with this. Results from our developmental study showed that Na^+/Pi cotransport activity increases with growth in the following ascending order: 5-day old suckling rats, 15-day old suckling rats and adult animals. These findings are also consistent with those of others demonstrating higher transport activities in rats at end-weaning stages (2-3 weeks after birth) and in adulthood than in rats at early-weaning stages (the second week after birth) (Taufiq *et al.*, 1997). In adult rats, Na^+/Pi cotransport activity was reported to decline slowly with age (Sorribas *et al.*, 1996). These data thus suggest a pattern of developmental regulation of rat renal Na^+/Pi cotransport in which the transport activity increases rapidly after birth, reaches its peak value at end-weaning stage, and then returns to its adult level.

The Na^+/Pi cotransport activities of SC and JC kidney subcompartments have been compared by others previously (Levi, 1990, Loghman-Adham, 1992). Contrary to the observation in those studies, our present study indicated a higher transport activity in JC than in SC. We believe that this discrepancy is due merely to the difference in the criteria used to dissect JC and SC. In the present study, much thinner (1.5 mm) sections were taken for SC while thicker (2 mm) sections were taken for JC. Our results are supported by the fact that NaPi-2 protein is expressed more abundantly in the JC and in SC sections (Custer *et al.*, 1994). This variance would not affect the interpretation of our results since our purpose is to compare transport activity and protein expression in BBM from different sections.

Western blot analysis of the BBM proteins showed that monomeric and dimeric forms of NaPi-2 polypeptides as well as intact and cleaved p70 constituents (Fig. 1, MANUSCRIPT III) are expressed in every situation. These data suggest that all forms, including the intact and cleaved p70's and monomer and dimer of NaPi-2, are involved in Pi transport. Table 1 shows that higher amount of dimers (p180) and monomers (total p70) were always associated with higher Pi transport activity, indicating that the overall level of NaPi-2 proteins determines transport activity. However, there was a striking disproportionate relationship between expression and activity levels. For instance, a 3.6-fold change in activity was accompanied by an 11.8 (i.e. 5.1 + 6.7)-fold change in expression during development (adult / 5-day old), or by a 9.8 (i.e. 5.2 + 4.6)-fold change in expression for JC / SC (Table 1). Thus, change in

expression was on average 3 to 4-fold more pronounced than that in activity. This implies that NaPi-2 proteins must form multimers to function as Na^+/Pi cotransporters in the renal BBM. Such an arrangement of NaPi-2 supports the results of previous radiation inactivation studies suggesting that the active transporter target sizes range from 190 to 220 kDa (Xiao *et al.*, 1997, Delisle *et al.*, 1994, Jette *et al.*, 1996).

The lower apparent molecular weights of all forms of NaPi-2 protein in the young rats as seen on Western blots suggests that the level of NaPi-2 glycosylation is low at that age. This may partly explain why less NaPi-2 is expressed in the young than in the adult BBMs, since the newly-synthesized unglycosylated NaPi-2 protein is less efficiently expressed in the BBM (Hayes *et al.*, 1994, Hernando *et al.*, 1999). However, once expressed in the BBM, it should mediate Na^+/Pi cotransport as efficiently as the full-glycosylated NaPi-2 (Hayes *et al.*, 1994).

LPD increases the expression of NaPi-2 proteins in BBM via a complex mechanism involving increased rate of *de novo* protein synthesis, increased NaPi-2 mRNA stability, and possibly an increased rate of gene transcription (Ritthaler et al., 1999, Levi et al., 1994, Kido et al., 1999). The present data showed that LPD for twelve hours resulted in more than 40-fold increase in BBM NaPi-2 expression. This change is more drastic than those found in the situations of age and subcompartmental localization since the expression/activity ratio reached a value as high as 13 (i.e. [25.5 + 22.2] / 3.6) in this case. This means that some of the NaPi-2 proteins in the cotransporter complex are not functioning efficiently, suggesting that some inhibitory components might have been added to the oligomeric assemblages of NaPi-2 to inactivate transport activity. In fact, earlier studies demonstrated that the radiation inactivation size of the rat renal BBM Na^+/Pi cotransporter increased from 255 to 335 kDa upon LPD of the animals (Béliveau et al., 1990).

The identities of the inhibitory components are unknown, but recent data has described some isoforms of NaPi-2, namely NaPi-2 α and γ, that can function as inhibitors (Tatsumi *et al.*, 1998). These two isoforms, resembling the p45 and p40 moieties of p70 polypeptide, were detected in the kidney and found to decrease Pi transport when coexpressed with NaPi-2 itself (Tatsumi *et al.*, 1998). Unfortunately, our antibodies would not distinguish p45 and NaPi-2 α isoform, or p40 and NaPi-2 γ isoform, since both members of each pair bear the same amino acid sequence against which the antibodies were raised (Boyer *et al.*, 1996, Xiao *et al.*, 1997).

Thus, it is not known whether and to what extent these isoforms were increased in the present study. Nor is it known whether the NaPi-2 isoforms exist in newborn rats or in adult rats exposed to NPD, but it seems that they have the potential to help the animals adapt to variations in dietary Pi. They could be part of a dynamic mechanism that has evolved to rapidly regulate the transport function in response to dietary or hormonal fluctuations, since a tight regulation of phosphate retention is important to prevent acidosis. The binding of inhibitory components would exquisitely complement the already demonstrated processes of mobilization / demobilization of transporter molecules at the BBM (Levi *et al.*, 1996, Murer *et al.*, 1996, Murer *et al.*, 1997). In any case, it will be interesting to determine the stoichiometry of the inhibitory isoforms versus the multimers when appropriate antibodies become available.

We also analyzed levels of the reduced forms (intact p70, p45 and p40) of the transporter to reveal any effect on transport activity. So far, variations in the relative amounts of intact versus cleaved p70 do not appear to influence transport activity. Indeed, growth and subcompartmental localization exhibited almost exactly opposite responses regarding the variation in the level of reduced species. For example, for a similar increase in activity there was a 9.5- and 1.9-fold rise in the level of intact and cleaved p70, respectively, in the first situation, while the inverse was found for the latter (Table 1, MANUSCRIPT III). These results clearly demonstrate that all reduced NaPi-2 proteins are likely responsible for Pi transport. The intermediate values obtained with dietary phosphate (Table 1, MANUSCRIPT III) do not contradict this conclusion. Our deduction that cleaved p70 must be active, agrees with the study showing that coexpression of p45 and p40 corresponding segments of the flounder type II Na^+/Pi cotransporter resulted in full expression of Pi transport function in *Xenopus* oocytes (Kohl *et al.*, 1998).

Although there are types of Na^+/Pi cotransporters other than type II, they could not be responsible for changes in transport activity in renal BBM. This is because the type I cotransporter (NaPi-1) is expressed constitutively and distributed uniformly throughout the kidney cortex (Biber *et al.*, 1993), while the type III (Glvr-1) cotransporter was found not to be expressed in the rat renal BBM (MANUSCRIPT IV).

In summary, the present study demonstrates that different protein expression patterns of NaPi-2 can lead to an increased Na^+/Pi cotransport activity in the rat kidney. The absence

of a unique protein profile suggests that a complex mechanism is involved in the modulation of the cotransport function.

IV.5 Effect of renal ischemia on the expression of NaPi-2 and Na^+/Pi cotransport

In the above studies we have discussed the basic structure, functional unit, regulation by age and dietary Pi, and renal cortical distribution of rat renal BBM NaPi-2 protein. As a rapidly regulated protein, NaPi-2 is also involved in many pathophysiological processes, such as in XLH, in acid-base imbalance, acidosis, and nephrotoxicity induced by high metals (Tenenhouse *et al.*, 1994, Tenenhouse *et al.*, 1995, Herak-Kramberger *et al.*, 1996, Ambuhl *et al.*, 1998). Renal ischemia is a common clinical problem complicated by water and solute imbalance. To understand the role of NaPi-2 in this process, we examined the effect of acute (reversible) renal ischemia on the expression of BBM NaPi-2 in rats in the last part of this thesis project.

Our results demonstrated that 30 min of unilateral renal ischemia resulted in marked decrease in the amounts of different NaPi-2 protein forms, including p180, p70, p45 and p40. Interestingly, reperfusion for 60 min caused the recovery of all forms of NaPi-2 nearly to control levels. However, BBM Na^+/Pi cotransport activity was increased by ischemia, an alteration that cannot be explained by changes in NaPi-2 abundance. After reperfusion the transport activity returned to the control level

We also noticed that anesthesia induced by ketamine + xylazine increased all transport activities. The reason is not clear. The increase in Na^+/Pi cotransport can't be explained by the stimulating effect of these two anesthetics on the level of serum PTH (Schultz *et al.*, 1995), a hormone that actually decreases Na^+/Pi cotransport by removal of NaPi-2 protein from the BBM (Kempson *et al.*, 1995, Lotscher *et al.*, 1996, Zhang *et al.*, 1999). Effects of these anesthetics on Na^+/glucose and Na^+/proline cotransport remain to be established. Whatever the reason, these changes have little influence on the interpretation of our results since the effects of anesthetics were controlled in all transport experiments.

The reduction in the abundance of BBM NaPi-2 protein by acute renal ischemia is easy to understand. At the beginning of ischemia, activation and relocalization of the actin depolymerization factor results in rapid disruption of the cytoskeletal system of the proximal tubules (Leiser and Molitoris, 1993, Schwartz *et al.*, 1999, Ashworth *et al.*, 2001). Such a

drastic event would presumably suppress the expression of membrane proteins whose membrane insertion is highly dependent on the cytoskeletal system. Previous studies have shown that marked alterations in the polymerization dynamics and stability of both actin microfilaments and microtubules are involved in the modulation of Na^+/Pi cotransport in OK cells (Papakonstanti *et al.*, 1996). In those cells NaPi-4, a member of the type II Na^+/Pi cotransporter that is nearly identical both structurally and functionally to NaPi-2, is the predominant BBM Na^+/P cotransporter (Sorribas *et al.*, 1994). In addition, the turnover of NaPi-2 protein in BBM has been convincingly shown to be modulated through microtubules, most likely as a component of the microtubule cargos in proximal tubular epithelial cells (Lotscher *et al.*, 1997, Lotscher *et a*l., 1999). Microtubules have been demonstrated to be disrupted in proximal tubules during ischemia (Abbate *et al.*, 1994). These studies suggest that the intracellular trafficking of NaPi-2 is tightly associated with both the actin cytoskeleton and microtubules. Thus, the decreased expression of all forms of NaPi-2 protein in BBM by ischemia, as observed in the present study, could be a direct consequence of ischemia on these cytoskeletal systems.

Another reason of NaPi-2 reduction in the BBM might be the internalization of the microvillar membrane. Shortly after the beginning of ischemia, the microvilli whose special structure is directly supported by cytoskeletal system may collapse into the cytoplasm (Leiser and Molitoris, 1993, Schwartz *et al.*, 1999), leading to the internalization of NaPi-2 protein. It is also possible that NaPi-2 is freed from the membrane compartment into the cytoplasm, as observed for alkaline phosphatase and γ-glutamyltransferase (Khundmiri *et al.*, 1997).

The level of total NaPi-2 proteins in the renal cortex can be reflected by their presence in the cortical homogenate. This includes the NaPi-2 proteins in the BBM, in the subapical compartment, and, in the case of renal ischemia, in the tubule lumen (Leiser and Molitoris 1993, Katai *et al.*, 1997). Our results regarding the detection of NaPi-2 in cortical homogenate indicate that ischemia also reduced the abundance of total cortical NaPi-2, although to a lesser extent than in the BBM alone. The decrease in the total amount of NaPi-2 is probably due to lysosomal degradation that accounts for the rapid downregulation of NaPi-2 by PTH and high Pi diet (Katai *et al.*, 1997, Keusch *et al.*, 1998, Traebert *et al.*, 2000). Blebbing of BBM into the proximal tubule lumen (Leiser and Molitoris 1993) could also be responsible for the loss of some NaPi-2 proteins from the cortical homogenate. The difference (about 20%) in the decrease of NaPi-2 between the BBM and the cortex homogenate suggests that BBM NaPi-2

proteins are more vulnerable to change than NaPi-2 proteins in other compartments such as subapical vesicles and endosomes (Katai *et al.*, 1997, Custer *et al.*, 1994).

Reperfusion for 60 min restored the abundance of all BBM NaPi-2 forms to nearly control levels. This could be caused by the re-constitution of the cytoskeletal system that facilitates the re-insertion proteins to the BBM (Leiser and Molitoris, 1993). Compared to the rate of complete recovery of the cytoskeleton (Schwartz *et al.*, 1999, Abbate *et al.*, 1994), the restoration of NaPi-2 to the BBM within 60 min is a rather rapid process. The details of this process are unknown, but an early recovery of NaPi-2 protein and Na^+/Pi cotransport activity in the BBM would benefit the global recovery of the proximal tubular epithelial cell, by pumping in Pi ions for the needed energy supply (ATP).

The increase in the Na^+/Pi cotransport activity with a decrease in BBM NaPi-2 abundance during ischemia is totally unexpected. Since in many cases it has been shown that ischemia inhibited transport activities such as Na^+/glucose cotransport and organic anion (Molitoris and Kinne, 1987, Kim *et al.*, 1995, Kim *et al.*, 1996). The present study also indicated that the Na^+/glucose and Na^+/proline transport was suppressed. Possible explanation for this discrete effect of ischemia on BBM Na^+/Pi cotransport would be the involvement of ischemia-induced posttranslational modifications on NaPi-2 or the existence of other types of renal Na^+/Pi cotransporters. However, 2-D analysis of the BBM did not show any significant change in the isoelectric points of the two isoforms of NaPi-2 p70. The slight difference in the relative ratios of the two p70 isoforms among different groups of rats could not explain the differences in the transport activity, since the levels of both isoforms decreased and their individual contributions to Pi transport remains to be established.

Analysis of the expression of NaPi-1 and Glvr-1 also excluded the possibility of these Na^+/Pi cotransporters in increasing Na^+/Pi cotransport activity, since NaPi-1 is decreased by both ischemia and reperfusion, while Glvr-1 is not expressed in the BBM at all. The absence of Glvr-1 in the BBM suggests that it might be expressed in the basolateral membrane, since this protein was detected in the proximal tubule cells (Boyer *et al.*, 1998). This observation alone could be interesting since the polarized expression of Glvr-1 in PT epithelia has never been demonstrated. The absence of Glvr-1 in the BBM from ischemic rat also implies that, at least within 30 min, ischemia did not cause the redistribution of Glvr-1 from the basolateral membrane to BBM. In contrast, the basolateral membrane protein Na^+/K^+ ATPase was found

redistributed to the BBM during ischemia (Molitoris *et al.*, 1992). This suggests that ischemia has selective effects on different membrane proteins.

At this point, we can only speculate that increased BBM Na^+/Pi cotransport activity could be due to ischemia-induced modifications of membrane properties such as membrane content and fluidity. Previous studies revealed that ischemia sharply reduced the BBM cholesterol-to-phospholipid ratios and increased BBM lipid peroxidation, likely leading to an increase in membrane fluidity (Molitoris and Kinne 1987, Molitoris *et al.*, 1985, Montagna *et al.*, 1998). Increase in BBM membrane fluidity has been known to specifically increase Na^+/Pi cotransport activity and decrease Na^+/glucose cotransport activity (Molitoris *et al.*, 1985, Friedlander *et al.*, 1988, Levi *et al.*, 1989, Friedlander *et al.*, 1990). Thus, the increased Na^+/Pi cotransport activity observed in the present study could be better explained by the effect of ischemia on BBM fluidity. However, the underlying mechanisms are not known and further investigation is obviously needed in future studies.

In summary, we demonstrated that reversible renal ischemia significantly reduced the abundance of NaPi-2 proteins in BBM, which is rapidly restored upon reperfusion. The Na^+/Pi cotransport activity of the BBM was unexpectedly increased by ischemia but returned to sham levels after reperfusion. The opposite effects of ischemia on the BBM NaPi-2 proteins and Na^+/Pi cotransport activity suggest a unique role for Pi transport during ischemia/reperfusion in restoring and maintaining the normal energy metabolism and thus the normal structure and function of the proximal tubules.

V. CONCLUSION

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V.1 The dimeric form (p180) is a functional unit of NaPi-2

NaPi-2 proteins in the rat renal BBM exist both as oligomers and as monomers. The oligomer (p180) as shown by nonreducing SDS-PAGE is most likely the dimeric form of the monomer the p70 (both intact and cleaved). The correlation between the Na^+/Pi cotransport activity and the amount of p180 clearly demonstrates that p180 is a functional unit in Na^+/Pi cotransport. The target size of NaPi-2, 190 kDa, as revealed by radiation fragmentation study, also supports the suggestion that the dimeric form is the active form.

V.2 Supermolecular assemblage of NaPi-2

NaPi-2 is expressed in BBM both in intact form and in cleaved form. The intact form is composed of the whole amino acid sequence of NaPi-2, i.e., the intact p70. The cleaved form is the two terminal segments of NaPi-2 as detected under complete reducing SDS-PAGE, the N-terminal segment p45 and the C-terminal segment p40. Thus, the basic structural components of NaPi-2 protein are p45, p40, and intact p70. Consequently, the functional unit of NaPi-2 is composed of these basic components, and disulfide bonds play an important role in the construction of this functional unit. A model of the functional unit (p180) could be described as the following: 1) two intact p70s associated via disulfide bond(s), 2) one intact p70 associated via disulfide bond(s) with one cleaved p70, the latter is composed of one p45 and one p40 linked via disulfide bond(s), 3) two cleaved p70s associated via disulfide bond(s), each of the cleaved p70s is composed of a p45 and a p40 linked via disulfide bond(s).

V.3 Cleaved p70s are also involved in Na^+/Pi cotransport

The expression of cleaved p70s in BBM from the juxtamedullary cortex is significantly higher than that from the superficial cortex in rat kidney. The BBM Na^+/Pi cotransport activity is also higher in juxtamedullary cortex than in superficial cortex. Thus, the cleaved p70s are also involved in Na^+/Pi cotransport. The higher Na^+/Pi cotransport activity in association with the greater expression of cleaved p70s due to LPD also supports this point. In addition, the cleaved p70s are also involved in the formation of the functional unit, the p180. Thus, the cleaved forms of NaPi-2 are also active as Na^+/Pi cotransporters, and the cleavage is unlikely an inhibitory process. However, comparison between the transport

activity and the expression of different forms of NaPi-2 proteins indicates that there is not a unique expression pattern controlling the transport of Pi by NaPi-2.

V.4 Identification of the cleavage site

The cleavage of NaPi-2 is not an experimental artifact. It occurs probably at the level of BBM, since the BBM is enriched in peptidase. The fact that only two sites are used for N-linked glycosylation and both p45 and p40 are glycosylated led to the estimation that the cleavage site is between Asn 298 and Asn 328 in the middle of the largest extracellular loop of NaPi-2. The exact site of cleavage was found to be between Arg 319 and Val 320 by NH_3-terminal sequencing of the purified p40. The purification procedure also allowed us to estimate that NaPi-2 proteins represent approximately 0.005% of the total BBM proteins for rats fed a normal Pi diet and 0.05% for rats fed with a low Pi diet for 3 days.

V.5 Ischemia decreases the abundance of NaPi-2 in BBM

Reversible (30 min) renal ischemia significantly decreased the amount of NaPi-2 in BBM, while reperfusion rapidly restored to control level. However, ischemia increased BBM Na^+/Pi cotransport and reperfusion normalized this activity rapidly. In contrast, Na^+/glucose and Na^+/proline transport activities were dramatically suppressed by ischemia and reperfusion. Thus Na^+/Pi cotransport and NaPi-2 proteins play a unique role in maintaining and restoring a normal function of proximal tubule epithelial cells during ischmia and reperfusion.

V.6 Posttranslational modification of NaPi-2

The present study has demonstrated that renal Na^+/Pi cotransport is under the control of posttranslational regulation. One type of the posttranslational regulation is the formation and disruption of disulfide bond(s) between different forms of NaPi-2 proteins. Breaking the disulfide bond(s) leads to a functional downregulation of Na^+/Pi cotransport. Another type of posttranslational regulation is the cleavage of NaPi-2 into p45 and p40. This cleavage does not significantly affect Na^+/Pi cotransport activity.

VI. FUTURE PROJECTS

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Future studies on the molecular mechanism of renal sodium-dependent phosphate cotransport should be focused on the following:

1. The increased expression of p45 and p40 by LPD suggests that the mechanism underlying NaPi-2 cleavage must also be more active during LPD. This mechanism might be the increased expression or function of a proteolytic enzyme. Thus it would be interesting to identify this enzyme and investigate how it is regulated by LPD.

2. The relative amounts of the different forms of NaPi-2 have not been determined precisely. This needs to be established under well-defined conditions, as NaPi-2 changes rapidly by dietary Pi and hormones. It would also be interesting to look at the ratio of different forms of NaPi-2 proteins in *Hyp* mice compared to normal control mice. We have noticed that the mouse type II Na^+/Pi cotransporter that is nearly identical to NaPi-2 is also cleaved in a way similar to that of NaPi-2.

3. Compare the expression of NaPi-2 mRNA and NaPi-2 proteins in rats of different ages with fine age intervals, using immunohistochemistry and Western blotting methods.

4. Investigate the mechanism of increased Na^+/Pi cotransport activity in BBM of ischemic rats as observed in this thesis project.

VII. REFERENCES

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VIII. ANNEX

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VIII.1 Contribution of co-authors

Christian Boyer:

Participated in the production of the two antibodies, and primary characterization of NaPi-2 in manuscript I. Participated in the primary organization of the second part of manuscript II.

Richard Béliveau

My research director. He directed all the experiments in all the manuscripts. He participated in the design and correction of all the articles.

Richard Desrosiers:

Made major suggestion and correction on manuscript IV.

André Dugré:

He participated in the study on the topography of NaPi-2 in manuscript II.

Joanne Paquin:

Performed the final writing of manuscript II. Participated in the writing of manuscript III.

Michel Potier

Dr. Potier from l`Hopital Sanit-Justine provided the irradiator for the target size study in manuscript I.

Vincent Vachon:

Verbal correction and scientific suggestions for manuscript I.

Éric Vincent:

Participated in the transport assay, immunodetection of NaPi-2 in manuscript I. Participated in the identification of the cleavage site.

Yansen Xiao:

In manuscript I: participated in the production of both anti-N and anti-C antibody; directly proved the origin of p45 and p40; performed the deglycosylation and radiation inactivation studies; involved in the design of most experiments; wrote the primary version of the manuscript. In manuscript II: design the method of p40 purification and participated in the purification experiments; did some verifying experiments in the topography study. In manuscript III: did all the experiments; proposed the structural model, and wrote the primary versions of the manuscript. In manuscript IV: did all experiments and wrote the manuscript nearly independently.