Kidney International, Vol. 54 (1998), pp. 1224–1232

Parathyroid hormone and dietary phosphate provoke a lysosomal routing of the proximal tubular Na/Pi-cotransporter type II

Isabelle Keusch, Martin Traebert, Marius Lötscher, Brigitte Kaissling, Heini Murer, and Jürg Biber

Institute of Physiology and Institute of Anatomy, University of Zürich, Zürich, Switzerland

Parathyroid hormone and dietary phosphate provoke a lysosomal routing of the proximal tubular Na/Pi-cotransporter type II.

Background. A decrease of proximal tubular reabsorption of phosphate (Pi), which can be provoked by parathyroid hormone (PTH) or by a high Pi-diet, has been shown to correlate with a decrease of the number of type II Na/Pi-cotransporters residing in the brush border membrane. While both PTH and a high Pi-diet lead to an internalization of type II cotransporters, the further cellular routing of internalized cotransporters has not been established unequivocally.

Methods. To prevent lysosomal degradation, rats were treated with leupeptin prior to the injection of PTH or feeding acutely with a high Pi-diet. Kidney cortex were recovered and used for immunohistochemistry. In parallel, brush border membranes and lysosomes were isolated and analyzed by Western blotting.

Results. Under both conditions (PTH and high Pi-diet), a strong overlap of internalized type II cotransporters with the late endosomes/lysosomes was observed by immunohistochemistry. In agreement, the content of type II Na/Pi-cotransporters was increased in lysosomes isolated from the corresponding tissues.

Conclusions. These results suggest that in proximal tubular cells type II Na/Pi-cotransporters internalized due to the action of PTH and acute high Pi-diet are routed to the lysosomes, and likely do not enter a recycling compartment.

Phosphate homeostasis is effectively balanced via the control of renal proximal tubular reabsorption of phosphate (Pi). Transport of Pi through the proximal apical membrane is largely performed by the type II Na/Pi cotransporter [1–4]. Renal expression of other Na/Pi cotransporters also has been described, however, the role of other cotransporters such as type I [5] and type III [6] in the renal handling of Pi is not well established.

Parathyroid hormone (PTH) and phosphorous content of the diet are two important factors controlling proximal tubular phosphate (Pi) absorption [7, 8]. As shown recently, changes of the rate of proximal Pi-reabsorption provoked by these two factors can be explained by changes of the number of type II Na/Pi cotransporters residing in the brush border membrane [2, 9–11]. PTH causes a decrease of the expression of luminal cotransporters leading to a transient accumulation of cotransporters in a subapical compartment [2, 9]. Inhibition of Na/Pi cotransport by PTH has also been demonstrated in opossum kidney (OK) cells [12], and there is recent evidence that in these cells inhibition of Na/Pi cotransport by PTH occurs via a decrease of type II cotransporters at the apical membrane [13, 14]. Proximal tubular capacity is also influenced by the dietary content of phosphate [7, 8]. Both up-regulation and down-regulation of Na/Pi cotransport induced by a low Pi-diet or a Pi-rich diet, respectively, correlate with the extend of apical expression of type II cotransporters [10, 11]. Changes of proximal Na/Pi cotransport and the number of apical type II Na/Pi cotransporters have been shown to occur as soon as a few hours after feeding the animals with a diet of altered Pi content [10, 15]. For example, in rats fed with a high Pi-diet for several days (chronic adaptation), within a few hours a low Pi-diet (acute adaptation) results in a decreased rate of Pi reabsorption that correlated with a decrease of the expression of type II cotransporters at the luminal membrane. Since after the acute low Pi-diet an increased intracellular type II Na/Pi cotransporter associated immunostaining was observed, it has been suggested that acute down-regulation mediated by a high Pi-diet is due to an internalization of type II Na/Pi cotransporters [16].

Studies in OK cells have found that type II Na/Pi cotransporters internalized upon a treatment with PTH or a high medium Pi content are directed to the lysosomes [14]. Most likely internalized cotransporters do not recycle, since recovery of Na/Pi cotransport after removal of PTH is entirely dependent on protein *de novo* synthesis [14, 17]. However, under *in vivo* conditions the routing of internalized type II Na/Pi cotransporters has not yet been described unequivocally. Based on two observations it has been

Key words: transport, phosphate, diet regulation, proximal tubule, lysosomes, parathyroid hormone.

Received for publication November 4, 1997
and in revised form May 13, 1998
Accepted for publication May 13, 1998

suggested that, similarly to OK cells, in proximal tubular cells internalized type II cotransporters may be routed to the lysosomes as well. (*1*) Immunohistochemical analysis of kidney slices obtained from rats treated either with PTH or fed acutely with a high Pi-diet diet provided evidence for an association of intracellular type II cotransporter with the lysosomes [9, 16]. (*2*) After a low Pi-diet the presence of degradation products of type II cotransporter in purified lysosomes has been reported [18].

To demonstrate whether internalized type II Na/Pi cotransporters under *in vivo* conditions are directed to the lysosomes, rats were treated with leupeptin to block lysosomal degradation prior to an injection of PTH or an acute feeding with a Pi-rich diet. Kidney cortex tissue was examined by immunohistochemistry or used to isolate lysosomes. The results provide evidence that in proximal tubular cells, type II Na/Pi cotransporters internalized upon by PTH or by a high Pi-diet are largely directed to the lysosomes and most likely do not enter a recycling compartment.

METHODS

Experimental animals

All results were obtained from experiments performed with Sprague-Dawley rats weighing approximately 200 g.

To study the effect of PTH, animals fed with a normal chow were anesthetized with thiopental (100 mg/kg body wt), and 6 mg leupeptin (Sigma) disolved in 0.5 ml PBS were injected into the vena cava. After 45 minutes, PTH (1-34 fragment; Bachem; 10 μg per 100 g body wt) was injected. After an additional 60 minutes the kidneys were either fixed by retrograde perfusion via the adominal aorta with a fixative buffer as described [16, 19] and subsequently used used for analysis by immunohistochemistry, or the kidneys were removed without prior fixation and used to isolate brush border membranes and lysosomes.

To regulate proximal Pi reabsorption acutely by a Pi-rich diet rats were fed for six days with a low Pi-diet that was given daily for four hours in order to train the animals to eat within a short period of time. Pi-diets (0.1% and 1.2% total phosphorous content, respectively) were obtained from Kliba Mühlen (Klingenberg, Switzerland). On the day of experiment rats were first injected intraperitoneally with 6 mg leupeptin (in 0.5 ml of PBS) and after 45 minutes fed for 3.5 hours either with a low Pi-diet (controls) or high Pi-diet. Afterwards the rats were anesthetized and kidneys were either fixed and used for immunofluorescence as described [16, 19] or removed directly and used to isolate brush border membranes and lysosomes.

Isolation of lysosomes and brush border membranes

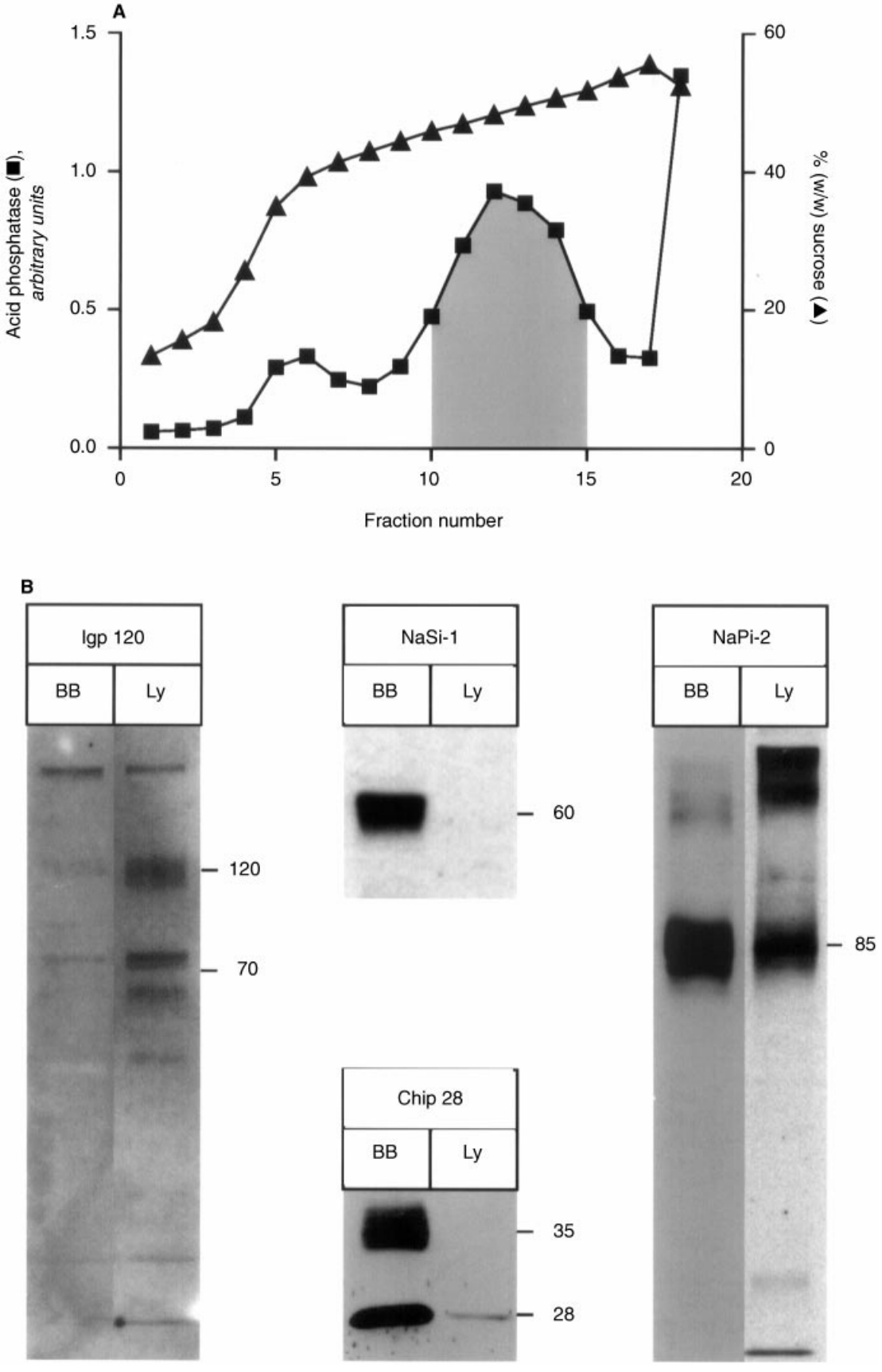
From kidney cortex slices lysosomes were isolated according to Maunsbach [20]. Homogenization of finely minced kidney cortex tissue was performed in 300 mM sucrose, 1 mM EDTA, 1 mM Tris/HCl (pH 7.4) using a potter Elevehjem (10 strokes at low speed). The homogenate was centifuged at 150 $\times$ g for 10 minutes and from the resulting supernatant a crude lysosmal fraction was obtained by centrifugation at 9,000 $\times$ g for three minutes. Afterwards lysosomes were purified by use of a linear sucrose gradient (1.2 M to 2.2 M in 1 mM EDTA, 1 mM Tris-HCl; pH 7.5). The distribution pattern of the lysosomes was determined by the activities of acid phosphatase and glucosaminidase. Fractions between 46% and 53% (wt/wt) sucrose were pooled (Fig. 1), diluted sixfold with cold water and lysosomes were collected by centrifugation at 100,000 $\times$ g for 45 minutes. Brush border membranes were isolated from combined supernatants and pellets obtained during the purification of crude lysosomes. After homogenization with a polytron homogenizer $MgCl_2$ was added up to 10 mM and brush border membranes were prepared as described [21].

Immunodetections by Western blots and immunofluorescence

Immunofluorescence using cyostat sections of fixed kidney cortex tissue and Western blot analysis with purified membranes were performed as described [19]. Prior to gel electrophoresis membrane solutions were denaturated in 0.1 M Tris-HCl (pH 6.9); 2% SDS; 1 mM EDTA and 10% glycerol (2 min, 95°C) and 15 to 20 μg of total protein was loaded. Protein was determined according to Bradford [22] using the BioRad dye reagent. Antibody binding was visualized by ECL using HRP-conjugated secondary antibodies (Amersham). Finally developed blots were stained with ponceau rouge in order to ensure for equal loadings. To quantify Western blots, films (Kodak X-Omat AR) have been scanned and analyzed by the software package ImageQuant (Molecular Dynamics).

For immunofluorescence cryostat sections of kidney cortex (5 μm thick) were processed and analyzed by confocal fluorescence imaging (Zeiss LSM 310; Zeiss, Oberkochen, Germany) as described [16]. Slices were first incubated for 10 minutes in PBS containing 3% defatted milk powder and 0.02% Triton X-100 to block nonspecific staining. Primary antibodies were applied overnight at 4°C in the blocking solution. After three washes with PBS secondary antibodies [swine anti-rabbit IgG conjugated with FITC, 22 μg/ml; Dakopatts, Glostrup, Denmark; and goat anti-mouse IgG conjugated with indobarbocyanine (Cy3), 7.5 μg/ml; Jackson ImmunoResearch, West Grove, PA, USA] were applied in PBS/3% milk powder for 30 minutes at room temperature.

For Western blots and immunofluorescence the following primary antibodies were used: (*a*) a polyclonal antiserum directed directed against the N-terminus of the rat type II Na/Pi cotransporter [19]; (*b*) late endosomes and lysosomes were detected with an anti(lgp120) antibody provided by Dr. I. Melman [23] or with an anti(lampII) antibody provided by Dr. M. Jadot [24]; (*c*) polyclonal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Acid phosphatase (■), arbitrary units
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Fraction number
B
Igp 120
BB
Ly
120
70
NaSi-1
BB
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NaPi-2
BB
Ly
85
Chip 28
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Ly
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Fig. 1. Isolation and characterization of proximal tubular lysosomes. From kidney cortex tissue of rats treated with leupeptin lysosomes were isolated by sucrose density gradient centrifugation (*A*) and collected lysosomes (shaded aera) were analyzed by Western blots (*B*) using the primary antibodies indicated. In parallel Western blots were made with purified brush border membranes (BB). Gels were loaded with 16 μg of total protein. Numbers indicate the apparent molecular mass in kD.

anti(NaSi-1) antibodies as described [25]; and (*d*) a polyclonal anti(aquaporin 1) antiserum was provided by Dr. I. Sabolic [26].

Presentation of data

All treatments and subsequent analysis were performed twice using two different batches of animals. Qualitatively similar results were obtained in both experiments. The results presented were obtained from one experiment.

RESULTS

To investigate if internalized type II Na/Pi cotransporters are routed to the lysosomes under *in vivo* conditions, rats have been injected with leupeptin in order to block lysosomal degradation before a treatment with PTH or before feeding acutely with a Pi-rich diet. Fixed renal cortex tissue was examined by immunohistochemistry and unfixed tissue was used to isolate lysosomes and brush border membranes that were analyzed by Western blotting. Lysosomes were isolated by sucrose gradient centrifugation and their distribution was determined based on the activity of the acid phosphatase (Fig. 1A). The distribution pattern shown was obtained using kidney cortex of rats either injected or not injected with leupeptin and was not altered by PTH or by a high Pi-diet (data not shown). Thus, the same population of mature lysosomes [20] was analyzed under all conditions described. Lysosomes were recovered from fractions between 46% and 52% sucrose and analyzed by Western blots using an anti(lgp120)-antibody (Fig. 1B). Besides the expected band of 120 kD, two other proteins of an apparent molecular mass between 60 kD and 80 kD were detected as well, which is in agreement with the reported different molecular weights of antigens detected by this antibody [23]. A similar staining pattern was obtained by using an anti(lampII) antibody [24] (not shown). Only faint reactions were observed in isolated brush border membranes with the anti(lgp120) and anti(lampII) antibodies (Fig. 1B). These reactions may result from a small cross-contamination of purified brush border membranes with lysosomal membranes as a small enrichment of acid phosphatase activity in purified brush border membranes has been reported [21].

Purified lysosomal fractions were further analyzed by Western blots using antibodies against two proximal tubular apical membrane proteins, the Na/SO_4-cotransporter, NaSi-1 [25], and aquaporin-1 [26, 27]. As illustrated in Figure 1B, the Na/SO_4-cotransporter as well as the glycosylated form of aquaporin 1 were absent in the lysosomes, indicating that purified lysosomes were not cross contaminated by brush border membranes. For unknown reasons, a small amount of the unglycosylated form of aquaporin 1 was detected in purified lysosomes. In contrast, purified lysosomes contained significant amounts of the type II Na/Pi cotransporter (Fig. 1B). The majority of the type II cotransporter associated immunostaining was detected at a molecular mass of around 85 kD, similar to that in purified brush border membranes. In all lysosomal preparations a faint immunoreaction was also observed at a molecular mass of around 30 kD, most likely representing a degradation product of the type II cotransporter.

PTH provokes lysosomal accumulation of type II Na/Pi cotransporters

Rats were first injected intravenously with leupeptin and after 45 minutes with PTH. After an additional 60 minutes the kidneys were fixed and processed for immunohistochemistry using an anti(type II) and an anti(lgp120) antibody. In controls (pretreatment with leupeptin, no PTH; Fig. 2A) most of the type II cotransporter associated staining was found in the apical membrane. Some intracellular staining was detected that overlapped partly with lysosomes and partly was associated with the Golgi apparatus (data not shown and [16]). In kidney slices obtained from rats that were not pretreated with leupeptin the immunostaining was similar to that shown in Figure 2A (not shown). After 60 minutes of PTH infusion, type II cotransporter associated staining in the brush borders was decreased, and in parallel, intracellular type II mediated staining increased and to a large extent overlapped with the distribution pattern of the late endosomes/lysosomes (Fig. 2B). In cortex slices obtained from rats infused with PTH but not pretreated with leupeptin intracellular type II mediated staining was found to be weaker (Fig. 2C), but still clearly overlapped with the pattern of late endosomes and lysosomes.

Lysosomes and brush border membranes were isolated from kidney cortex tissue of rats pretreated with leupeptin, and afterwards with or without PTH and used for Western blot analysis. In agreement with the results obtained by immunohistochemistry (Fig. 2), PTH lead to a decrease of the content of the type II cotransporter in the isolated brush border membranes and in parallel to an increase in the lysosomes (Fig. 3).

Acute high Pi-diet leads to lysosomal accumulation of type II cotransporters

A diet of high phosphorous content (1.2%) given for a short period of time (2 to 4 hr) to rats chronically (several

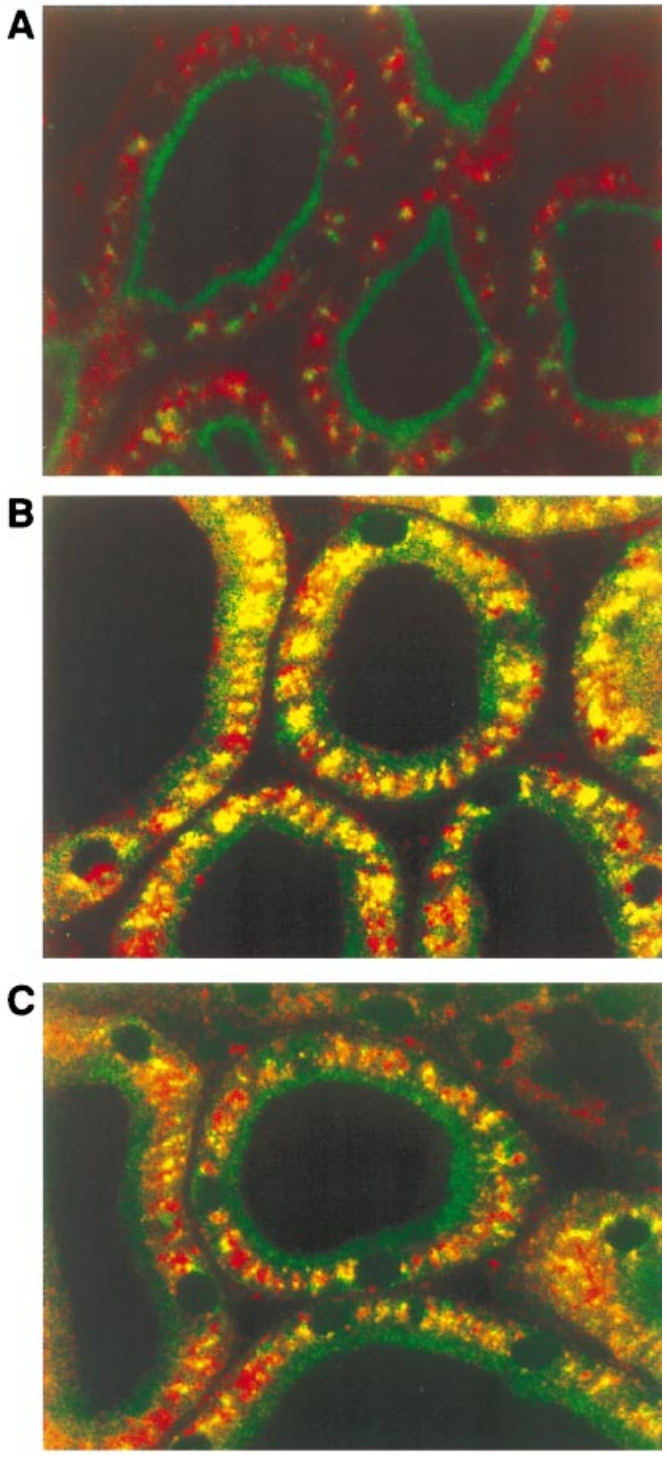

Fig. 2. Immunohistochemical localization of the type II Na/Pi cotransporter and late endosomes/lysosomes in S1 proximal tubular cells: Effect of parathyroid hormone. Cryostat sections were obtained from rats injected with leupeptin (*A*), from rats injected with leupeptin and parathyroid hormone (*B*), and rats injected with parathyroid hormone only (*C*). The sections were double-stained with an anti(type II) antibody (green) and with an anti(lgp120) antibody (red). In the presence of leupeptin a high degree of overlap (yellow) after the treatment with PTH is seen (B).

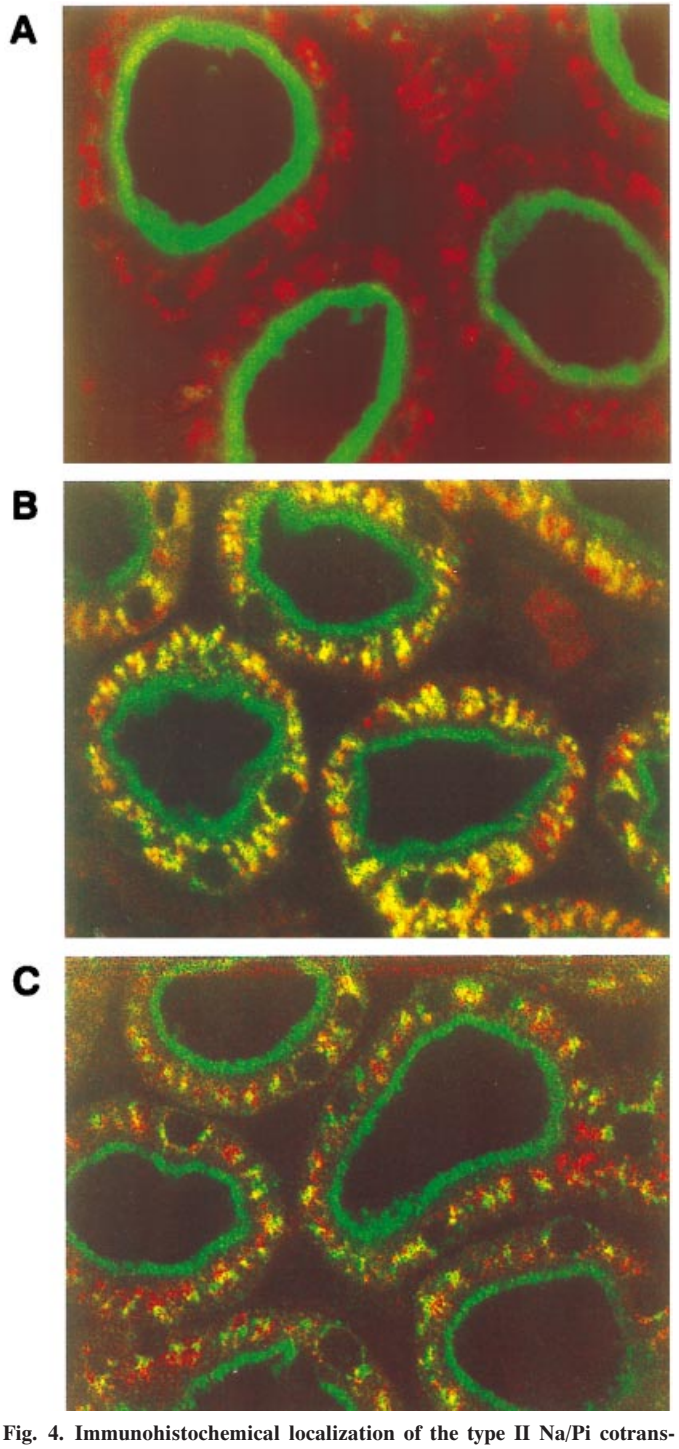

Fig. 4. Immunohistochemical localization of the type II Na/Pi cotransporter and late endosomes/lysosomes in S1 proximal tubular cells: Effect of an acute high Pi-diet. Rats were fed a low Pi-diet for six days and and then injected with leupeptin and fed for 3.5 hours with low a Pi-diet (*A*); injected with leupeptin and fed for 3.5 hours with a high Pi-diet (*B*) or fed acutely with a high Pi-diet without a prior injection of leupeptin (*C*). Cryosections were double-stained for type II Na/Pi cotransporters (green) and late endosomes/lysosomes using an anti(lampII) antibody (red). Regions of strong overlapping appear in yellow.

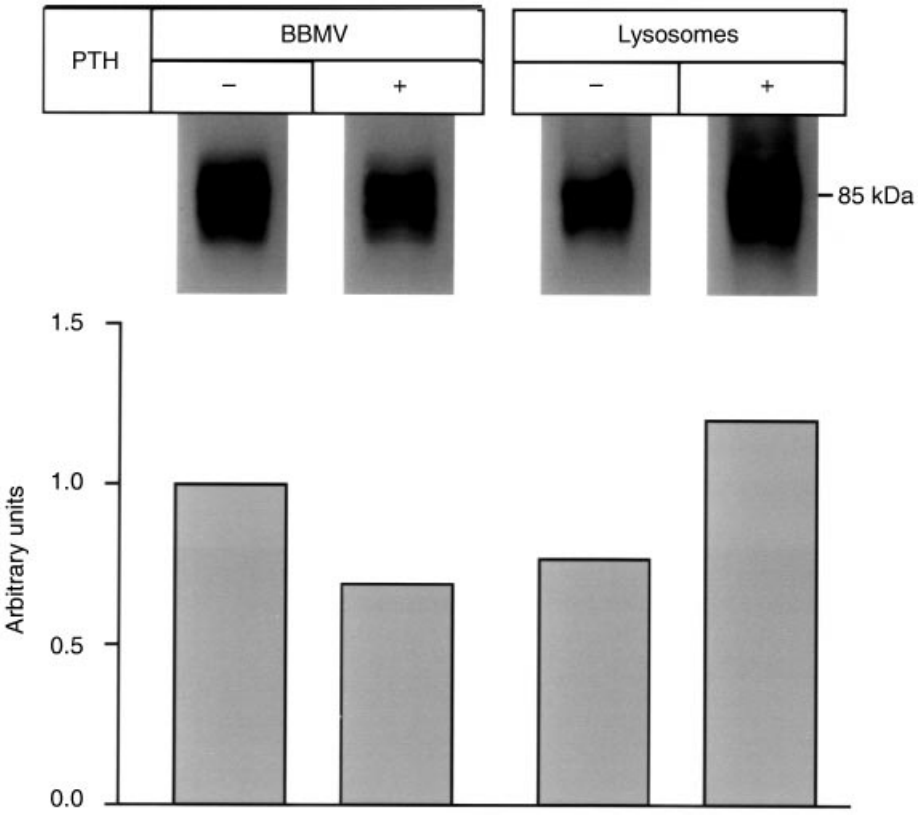

Fig. 3. Immunodetection of the type II Na/Pi cotransporter in lysosomes and brush border membranes isolated from kidney cortex of rats treated with leupeptin (−) or leupeptin plus PTH (+). Equal amounts of protein (20 μg) were loadad and analyzed with the anti(typeII) antibody. Bars indicate the relative amounts of the 85 kD band in the various fractions.

days) adapted to a low Pi-diet provokes a decrease of Pi reabsorption, which is paralleled by a reduction of the apical expression of apical type II Na/Pi cotransporters [10, 11, 15, 16]. To investigate the possibility of whether a high Pi-diet given acutely provokes a lysosomal routing of type II cotransporters, rats were injected intraperitoneally with leupeptin prior to the acute adaptation. As illustrated in Figure 4A, after a low Pi-diet given chronically for five days the type II cotransporter was expressed almost entirely in the brush border membrane and, in contrast to the control (chronic high Pi-diet, Fig. 2A), was only sparsely associated with intracellular structures. However, after a high Pi-diet given for 3.5 hours the brush border membrane staining was reduced and in the presence of leupeptin intracellular type II contransporter mediated immunostaining was almost entirely colocalized with the staining of late endosomes/lysosomes (Fig. 4B). In samples obtained from rats not injected with leupeptin, the high Pi-diet provoked a similar intracellular distribution of type II cotransporters (Fig. 4C), yet the extent of colocalization with the lysosomes was less pronounced.

From kidney cortex of rats fed chronically with a low Pi-diet and subsequently acutely with a high Pi-diet (or a low Pi-diet, which was used as a control) brush border membranes and lysosomes were isolated and analyzed by immunoblots (Fig. 5). In agreement with the data presented in Figure 4 and earlier data [10, 16], the intensity of the type II cotransporter band (signal around 85 kD) in brush border membranes was decreased by approximately half. In lysosomes purified from kidney cortex of rats fed chronically with a low Pi-diet the type II cotransporter was almost absent, but was strongly increased in lysosomes isolated from kidney cortex of rats fed acutely with the high Pi-diet.

DISCUSSION

Parathyroid hormone and the phosphorous content of the diet are two major regulators of renal proximal Pi reabsorption [7, 8]. A large body of evidence has been obtained that these regulators affect the relative abundance of the type II Na/Pi cotransporter in the brush border membrane and that the number of apical type II Na/Pi cotransporters directly correlates with the rate of proximal Pi reabsorption [2, 9, 11]. Observations made by immunohistochemistry suggested that PTH as well as a Pi-rich diet cause an internalization of type II Na/Pi cotransporters [9, 10, 16]. Under *in vivo* conditions the further intracellular routing, however, has not been analyzed in detail. In particular, the possibilities that internalized contransporters may enter a recycling pathway, as has been described for the regulation of the glucose transporter Glt4 by insulin [28] or that may be delivered to the lysosomes, has not been unequivocally established.

The results obtained in this *in vivo* study document that

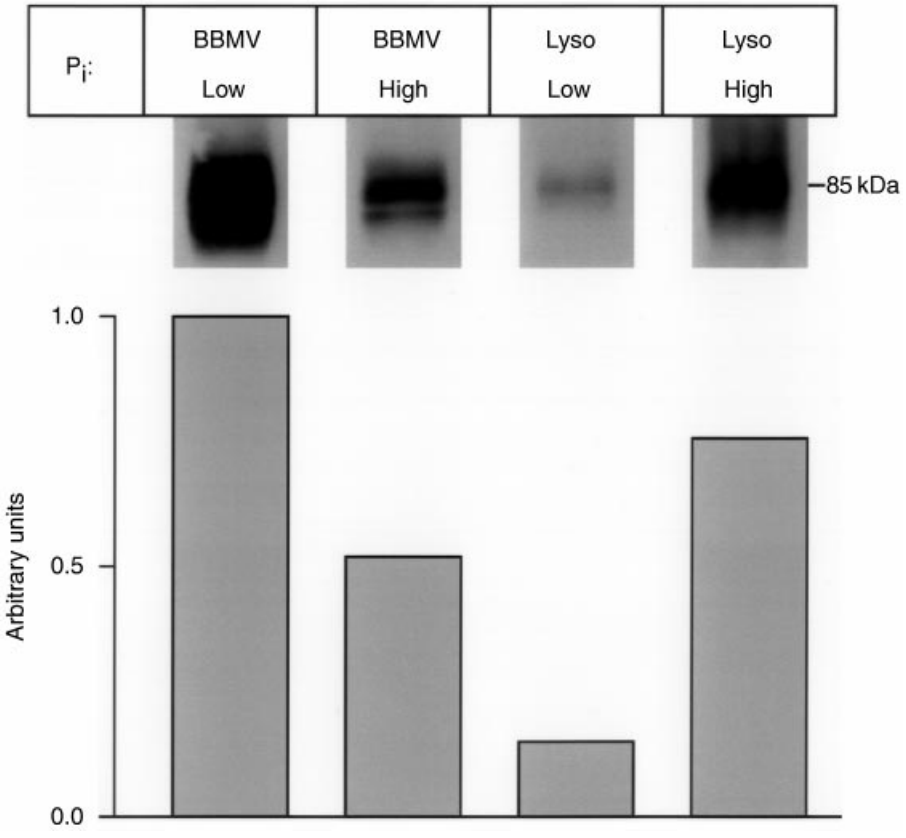

Fig. 5. Immunodetection of the type II Na/Pi cotransporter in lysosomes and brush border membranes: Effect of acute adaptation. Prior to sacrification rats (adapted for six days to low Pi-diet) were treated as described in Figure 4A and 4B. Bars represent the relative amount of the 85 kD band.

type II Na/Pi cotransporters internalized upon the action of PTH or low Pi-diet are mostly routed to the lysosomes, since (*a*) after internalization a high overlap of type II cotransporters with the late endosomes and lysosomes was observed and (*b*) the overlap of type II cotransporters with the lysosomes was markedley increased in the presence of leupeptin. Yet, based on light microscopy, the possibility that internalized cotransporters may to a certain extent reside in a subapical compartment from which they could be recruited and reinserted into the apical membrane cannot be excluded completely. However, in analogy to results obtained in studies on OK cells (see below), such a possibility seems rather unlikely. In agreement with the immunohistochemical observations, the amount of type II cotransporters in purified lysosomes as detected by Western blots was increased under all conditions of reduced brush border expression. Interestingly, in all lysosomal preparations (without or without injection of leupeptin) the type II cotransporter was detected at approximately 85 kD, which corresponds to the molecular mass detected in the brush border membrane. Only small amounts of degradation products were detected under all conditions. In contrast, it has been reported recently that after acute low Pi-diet degradation products of the type II cotransporter accumulated in the lysosomes [18].

Similar results to those described in the present *in vivo* study have been obtained by studies using opossum kidney (OK) cells in which regulation of Na/Pi cotransport by PTH and phosphate (extracellular phosphate concentration) has been demonstrated [12, 29]. Earlier studies suggested that PTH leads to an irreversible inhibition of Na/Pi cotransport, since upon removal of PTH the recovery of Na/Pi coransport was found to be dependent on protein *de novo* synthesis [17]. In agreement, we recently demonstrated that type II Na/Pi cotransporters are degraded due to the action of PTH and that recovery after removal of PTH is dependent on protein synthesis [13]. Furthermore, it could be demonstrated that degradation of type II cotransporters as provoked by PTH is inhibited by leupeptin, and results obtained by immunofluorescence and cell fractionations indicated that in OK cells type II cotransporters are routed to the lysosomes [14]. Similarly, the decrease of Na/Pi cotransport provoked by increasing the extracellular concentration of Pi is paralled by a decrease of the amount of apical type II contransporters due to internalization and subsequent routing to the lysosomes [30].

In all lysosomal preparations used in this study the Na/SO_4-cotransporter NaSi-1 and the glycosylated form of aquaporin-1 were not detected by Western blots. The later two proteins are expressed in the apical membrane of proximal cells and are not regulated by either PTH or a Pi-diet [10, 25–27]. The absence of these two apical proteins in the lysosomes suggests that the lysosomal routing of the type II cotransporter as induced by PTH or high Pi-diet

is not due to a generally increased membrane flow but rather includes specific signaling mechanism(s). Regulation of proximal apical Na/Pi cotransport by PTH has been associated with signaling pathways involving protein kinases A and C activities [2, 31]. The target protein(s) of these kinase activities, however, are not known. Moreover, the signaling mechanisms involved in the adaptive response of proximal tubular Pi reabsorption to dietary Pi are completely unknown. Regardless of the precise mechanisms, the present results suggest that both PTH as well as a high Pi-diet initiate an internalization of type II Na/Pi cotransporters and subsequent lysosomal routing. This type of regulation seems to be strikingly similar to the down-regulation of cell surface hormone receptors such as the epidermal growth factor receptor [32], and therefore it would be of interest to unravel any possible similarities such as the protein motifs that are involved. Several amino acid sequence motifs have been described that are likely to be involved in the lysosomal targeting of a variety of proteins [33]. In this respect it is of interest that within the primary amino acid sequence of the mammalian type II Na/Pi cotransporter, two potential lysosomal targeting motifs (GYXXM and YRWF) are contained, and additionally a dileucine based lysosomal targeting motif also seems to be evident. The precise role of such motifs in the lysosomal routing of type II Na/Pi cotransporters remains to be established.

In summary, this study demonstrates that in rat proximal tubules both PTH and high Pi-diet provoke an internalization and subsequent lysosomal routing of the type II Na/Pi cotransporter. These results provide evidence that internalized type II cotransporters do not enter a recycling compartment. Based on these studies it seems likely that other physiological regulators that result in a decrease of proximal Pi reabsorption may lead to a lysosomal routing of the type II cotransporter as well. The signaling mechanisms and the nature of the vesicular compartments involved in this kind of regulation of membrane transport are not known and remain to be described.

ACKNOWLEDGMENTS

Financial support was provided by grants from the Swiss National Foundation (31-46523.96 to H.M. and 31-34010.92 to B.K.). We thank Drs. I. Mellman, M. Jadot and I. Sabolic for providing us the antibodies against lgp120, lampII and aquaporin 1, respectively. We also thank Ch. Gasser for the excellent artwork.

Reprint requests to Dr. J. Biber, Institute of Physiology, University Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.
E-mail: Biber@Physiol.Unizh.CH

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