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Cleavage of Disulfide Bonds Leads to Inactivation and Degradation of the Type IIa, But not Type IIb Sodium Phosphate Cotransporter Expressed in *Xenopus laevis* Oocytes

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Abstract. Tris(2-carboxyethyl)phosphine (TCEP) reduces (cleaves) disulfide bonds of the renal proximal tubule type IIa Na/Pi- cotransporter (rat NaPi IIa) and thereby inhibits its function. We tested the effect of TCEP on the murine type IIa Na/P_i-cotransporter and the corresponding IIb intestinal isoform both expressed in *Xenopus laevis* oocytes. After incubation with TCEP the function of NaPi IIa was inhibited and protein amount was decreased. Injection of the lysosomal inhibitor leupeptin prevented degradation of the protein. Exposure of oocytes to TCEP at 0°C led to a reduction in transport function without concomitant loss in Na/Pi IIa protein. In contrast to NaPi type IIa, the type IIb isoform was neither inhibited, nor degraded after incubation with TCEP. These results suggest that cleavage of disulfide bonds led to changes within the confirmation of the type IIa transporter that result in (i) inhibition of the transport activity and (ii) internalization and subsequent lysosomal degradation of transporter protein. Sequence comparisons suggest the involvement/presence of different disulfide bonds in type IIa and type IIb Na/P_i-cotransporters.

Key words: Phosphate transport — *X. laevis* oocytes — TCEP — Disulfide bonds — Degradation

Introduction

There are two known isoforms of the mammalian type II sodium/phosphate cotransporter: The type IIa transporter, expressed in the brush border membrane of the renal proximal tubule (Magagnin et al., 1993; Custer et al., 1994; Biber, Murer & Foster, 1998; Murer et al., 1998) and the type IIb transporter expressed mainly in the small intestine and in lung (Hilfiker et al., 1998, Traebert et al., 1999). The overall amino acid homology of the two transporters is 57% (Hilfiker et al., 1998).

The type IIa transporter is regulated by alteration of the brush border membrane content of cotransporter proteins. This regulation is induced for example by parathyroid hormone (PTH) or by re-feeding P_i to P_i-deprived animals; it involves lysosomal degradation of the transporter protein (Kempson et al., 1995; Pfister et al., 1997; Keusch et al., 1998; Pfister et al., 1998; Pfister et al., 1998). These regulatory phenomena were also observed in opossum kidney (OK) cell lines expressing a type IIa Na^+/P_i-cotransporter (intrinsic and/or transfected) (Markovich et al., 1995; Pfister et al., 1998). In contrast, treatment of type IIb transfected OK cells with PTH does not lead to degradation of the type IIb transporter (Z. Karim et al., *in preparation*).

Tris(2-carboxyethyl)phosphine (TCEP) is a mild reducing agent capable of reducing (cleaving) disulfide bonds (White et al., 1996; Gendeh et al., 1997). In studies with rat brush border membranes, containing the type IIa transporter (Custer et al., 1994), Na^+/P_i-cotransport activity was decreased and the protein was cleaved into two parts by exposure to different reducing agents and the involvement of S-S bonds was suggested (Biber et al., 1996; Xiao et al., 1997). In these studies high concentrations of EtSH (4% v/v) or dithiothreitol (DTT) (500 mM) were required to cleave the protein whereas already 10 mM TCEP induced this effect (Xiao et al., 1997). In the preceding paper, we investigated the role of cysteine residues in the rat NaPi type IIa; TCEP was found to inhibit NaPi type IIa mediated P_i-transport after expression in *Xenopus* oocytes (Lambert et al., 2000). We concluded from this study, that there might be more than one disulfide bridge in NaPi IIa. In the present study, we further investigate the effect of TCEP treatment on the transport activity and protein amount of mice type IIa and IIb Na/P_i-cotransporters expressed in *Xenopus* oocytes. The mouse IIa isoform is 98% iden-

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tical to the rat isoform (Hartmann et al., 1996). The data suggest that IIa (but not IIb) is inhibited, internalized and degraded after TCEP treatment. Thus, in the type IIa NaPi-cotransporter disulfide bond(s) are functionally important and apparently also codetermine the membrane residence (protein stability).

Materials and Methods

The TCEP was obtained from Pierce (Rockford, IL), other chemicals from Fluka (Buchs, CH).

Xenopus laevis Oocyte Expression and Transport Assay

The procedures for oocyte preparation as well as the $^{32}P_i$-uptake assay have been described in detail elsewhere (Werner et al., 1990). Briefly, in vitro synthesis and capping of cRNAs were done by incubating the cDNAs, previously linearized by Not I (Pharmacia) digestion, in the presence of 40 U of T7 RNA Polymerase (Promega) and Cap Analog (NEB). Oocytes were injected with either 50 nl of water or 50 nl of water containing 5 ng of cRNA. $^{32}P_i$-uptake was measured 3 days after injection.

Incubation with TCEP

Oocytes were incubated with different concentrations of TCEP in Barth's solution (in mM): 88 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 2.4 $NaHCO_3$ and 10 Hepes, pH 7.4 for 30 min. Prior to uptake or homogenization, the oocytes were washed extensively with Barth's solution. Uptake or homogenization was started 5 min after removing the TCEP.

Immunoblot of Oocytes Homogenates

Yok-free homogenates were prepared 3 days after injection (H_2O or cRNA). Pools of 8 oocytes were lysed together with 160 μl of homogenization buffer [1% Elugent (Calbiochem) in 100 mM NaCl, 20 mM Tris/HCl, pH 7.6], by pipetting the oocytes up and down (Turk et al., 1996). To pellet the yolk proteins, samples were centrifuged at 16,000 × *g* for 3 min at room temperature (RT). 10 μl of supernatants in 2× loading buffer (4% SDS, 2 mM EDTA, 20% glycerol, 0.19 M Tris/HCl pH 6.8, 2 mg/ml bromphenol blue) were separated on a SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was then processed according to standard procedures (Sambrook, Fritsch & Maniatis, 1989) using rabbit polyclonal antibodies raised against synthetic peptides from the NH_2-termini of NaPi type IIa or IIb. The specificity of the antibodies was demonstrated previously (Custer et al., 1994; Hilfiker et al., 1998). Immunoreactive proteins were detected with a chemiluminescence system (Pierce).

Immunocytochemistry

The fixation of *X. laevis* oocytes has been previously described (Hayes et al., 1994). Briefly, the eggs were immersed during 30 min in PBS containing 3% paraformaldehyde and 0.1% glutaraldehyde. After rinsing in PBS (4°C), the eggs were frozen onto thin cork slices using liquid nitrogen cooled liquid propane. Sections of 5 μm were cut at −20°C using a cryomicrotome, and mounted on chromalumgelatin coated glass slides. For immunostaining, after preincubation in PBS containing 3% milk powder and 0.3% Triton X-100, the sections were first incubated overnight either with a rabbit anti NH_2- terminal NaPi IIa or NaPi IIb primary antibody, followed by incubation with a swine anti-rabbit IgG-conjugated fluorescein isothiocyanate (FITC) secondary antibody (Dakopatts). Finally, sections were coverslipped using DAKO Glycergel (Dakopatts) plus 2.5% 1,4-diazabicyclo(2.2.2)octane (Sigma) as a fading retardant.

Results

We studied the effect of TCEP on murine type IIa and IIb Na/P_i-cotransporters expressed in *Xenopus* oocytes in terms of function and specific protein content. We first performed type IIa mediated P_i uptake and specific cotransporter protein content measurements using oocytes incubated with different TCEP concentrations for 30 min (Fig. 1*A* and *B*). This incubation time was chosen because shorter incubation lead to only small effects, whereas longer incubation physically damaged the oocytes. Transport activity was already slightly reduced with 1 mM TCEP, at 3 mM the inhibition was about 60% and the maximal effect of about 80% inhibition was reached at 10 mM (Fig. 1*A*). These results paralleled those reported for the rat isoform in the preceding paper. The Western-blot also showed a dose-dependent decrease in protein amount. However, the effect on the protein amount was hardly detectable after incubation with up to 3 mM TCEP; a clear decrease could be detected after treatment with 10 mM TCEP (Fig. 1*B*). Thus, transport inhibition seems not to parallel protein content, i.e., protein degradation (*see below*).

The decrease in protein amount could be related to membrane retrieval and degradation of the transporter. To confirm, that the decreased activity was not just caused by less protein, we incubated oocytes that expressed the IIa transporter for 30 min either at room temperature (RT) or at 0°C (on ice) in the presence and absence of 10 mM TCEP (Fig. 2*A* and *B*). Incubating the cells at different temperatures had no influence on the transport activity of the oocytes. The inhibition after TCEP treatment was roughly the same with oocytes treated at 0°C or at RT (Fig. 2*A*). However, hardly any decrease of protein amount could be detected in oocytes treated with TCEP at 0°C, whereas the same treatment at RT led to a clear reduction in the protein amount (Fig. 2*B*). Thus, the decrease of transport activity is not due to lower protein amount, but to TCEP-induced changes in the protein itself. If membrane retrieval is allowed (20°C) 'inhibited' transporter molecules are apparently internalized and degraded.

To test whether the degradation of the transporter after TCEP treatment is mediated by leupeptin sensitive proteases (e.g., a lysosomal protease), we injected the

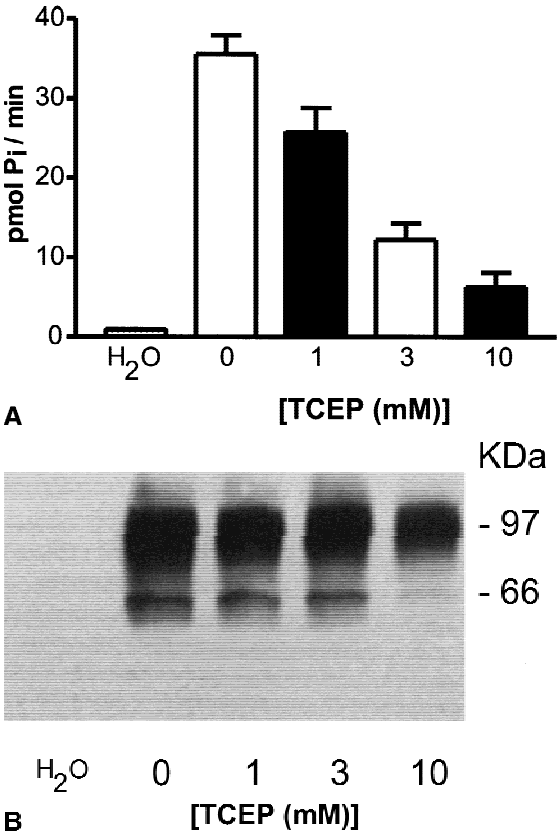

Fig. 1. Effect of 10 mM TCEP on transport activity (*A*) and protein amount (*B*) of the type IIa Na/P_i-cotransporter expressed in *Xenopus* oocytes. (*a*) Oocytes that express the type IIa cotransporter were incubated with the indicated concentrations of TCEP for 30 min. After washing and 5 min incubation in Barth's solution, the cells were incubated for 20 min at 25°C, in the presence of $^{32}P_i$. After extensive washing, single oocytes were transferred to vials and the incorporated $^{32}P_i$ measured. The bars represent the mean ± SE of 8 oocytes of a representative experiment. (*b*) Oocytes that express the wild-type IIa cotransporter were incubated with the indicated concentrations of TCEP for 30 min. After washing and 5 min incubation in Barth's solution oocytes were prepared for Western blot (*see* Materials and Methods). Yolk-free homogenate was separated in a 9% SDS-PAGE gel and analyzed by immunoblotting with a rabbit anti-mouse IIa (NaPi-7) polyclonal antibody. The 100 kDa band most likely corresponds to the fully glycosylated protein, whereas the band at about 66 kDa represents the core- or nonglycosylated form of the transporter (Hayes et al., 1994).

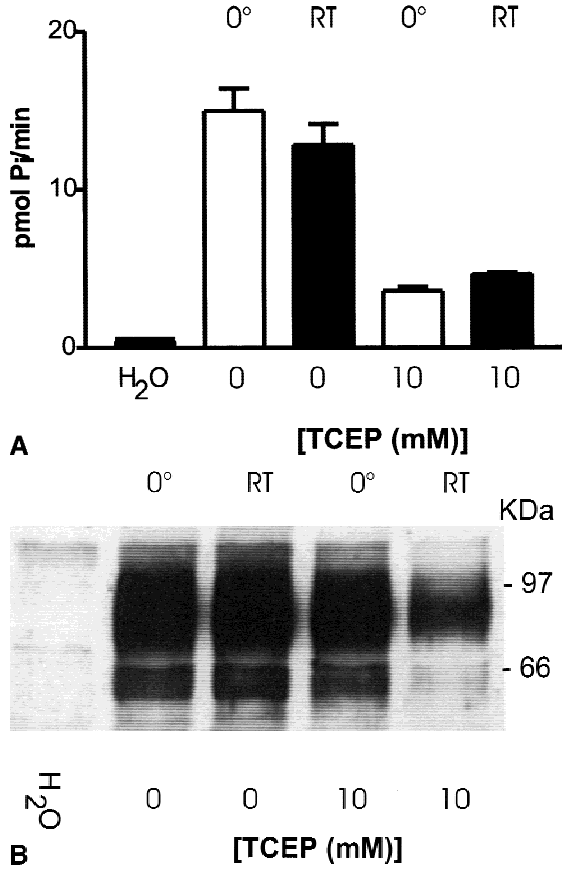

Fig. 2. Influence of different temperatures on the TCEP effect on type IIa mediated transport activity (*A*) and protein amount (*B*). (*A*) $^{32}P_i$-uptake measurements of oocytes that expressed type IIa transporter treated without TCEP at 0°C (1) or at RT (2) or treated with TCEP at 0°C (3) or at RT (4). (*B*) Western-blot of type IIa transporter protein expressed in oocytes treated without TCEP at 0°C (1) or at RT (2) or treated with TCEP at 0°C (3) or at RT (4).

oocytes with 50 nl of water with or without 0.2% leupeptin. After 30-min incubation time the oocytes were either treated with or without TCEP (Fig. 3). Comparing oocytes not treated with TCEP, injection of leupeptin led to a slight increase of the detectable protein amount after 1 hr of incubation time. Though the degradation of the type IIa Na/P_i-cotransporter due to TCEP treatment could not be completely blocked by injection of leupeptin, the degradation rate was clearly reduced. This suggests a similar degradation pathway of NaPi type IIa expressed in oocytes as observed in vivo (rats) and in vitro (OK-cells) after PTH treatment (Pfister et al., 1997; Keusch et al., 1998; Pfister et al., 1998).

To investigate, whether the TCEP effect is 'specific', we compared the TCEP effect on type IIa and type IIb Na/P_i-cotransporters. We performed Western-blots (Fig. 4), transport measurements (Fig. 5*A* and *B*) and immunohistochemical staining (Figs. 6*A–F*). After TCEP treatment, the amount of type IIa protein was reduced (*see* above, Figs. 1*B*, 2*B*, 3) but no change of type IIb protein amount could be detected (Fig. 4). Similar results were obtained by measuring transport function: The transport activity of oocytes that expressed type IIa was about 70% reduced by TCEP (Fig. 5*A*), whereas hardly any inhibition could be measured in type IIb expressing oocytes (Fig. 5*B*). Figure 6*A* and *B* shows oocytes expressing type IIa transporter treated without (*A*) or with (*B*) TCEP. Without TCEP treatment type IIa specific signal was located mainly in the plasma mem-

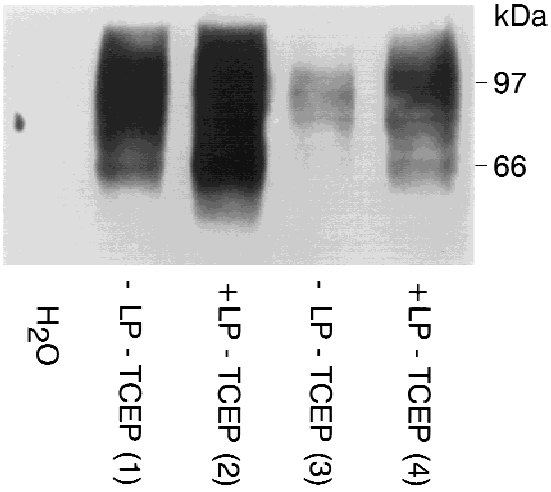

Fig. 3. Inhibition of TCEP induced degradation of the type IIa cotransporter by injection of leupeptin. Oocytes that expressed type IIa transporter were injected with 50 nl of 0.2% leupeptin (LP; 2,4) or 50 nl water (1,3). After 30 min both groups were incubated with (3,4) or without (1,2) 10 mM TCEP for 30 min and homogenates of these oocytes were separated on SDS-PAGE. In oocytes injected with leupeptin (band 4), the TCEP-induced degradation of the transporter was inhibited, compared to oocytes treated with TCEP but without leupeptin (band 3).

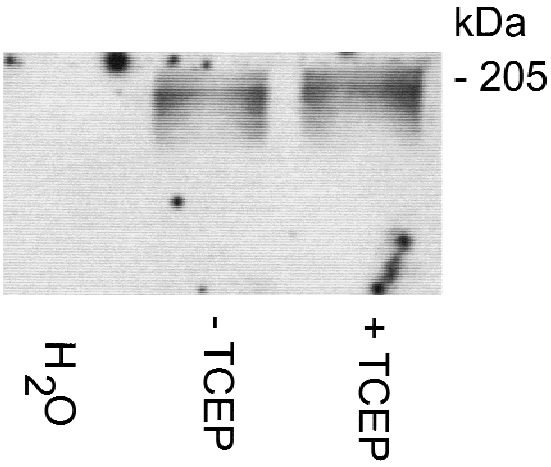

Fig. 4. Effect of TCEP on the protein amount of the type IIb NaP_i-cotransporter. Western-blots of type IIb expressing oocytes treated with or without TCEP at 20°C for 30 min are shown. The protein runs at a molecular mass of about 200 kDa. In preparations of intestinal brush border membranes, the same protein migrates at a molecular mass of only 100 kDa (Hilfiker et al., 1998). The difference can be explained by differences in the sample preparation from oocytes and brush border membranes.

brane, with only very little staining of intracellular compartments. After incubation with TCEP, the membrane staining was reduced and the protein was mainly located in intracellular compartments. Figure 6*C* and *D* shows oocytes that expressed the type IIb cotransporter treated

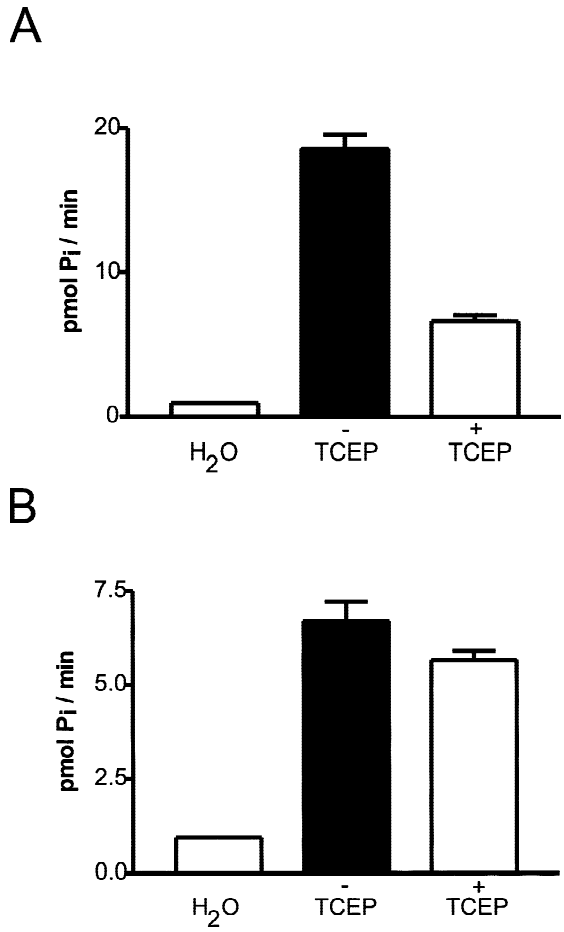

Fig. 5. Different effect of TCEP on transport activity of type IIa and IIb NaP_i-cotransporters: P_i transport activity of type IIa (*A*) or type IIb (*B*) after treatment with or without TCEP at 20°C for 30 min.

without (*C*) or with (*D*) TCEP. The signal pattern did not change due to incubation with TCEP. This again confirmed the different response of the type IIa and type IIb Na^+/P_i-cotransporters to TCEP treatment.

Discussion

We have observed a dual action of TCEP probably related to reduction of S-S bonds within the type IIa Na^+/P_i-cotransporter by TCEP. First, the transport activity is strongly diminished, probably related to an associated change in the protein conformation. Secondly, TCEP treatment produced a redistribution of the type IIa Na^+/P_i-cotransporter within the oocyte, which led to accumulation in intercellular compartments associated with its degradation. These results are compatible with increased retrieval of the transporter from the oocyte membrane and accumulation in lysosomes. Both effects were not observed in type IIb expressing oocytes.

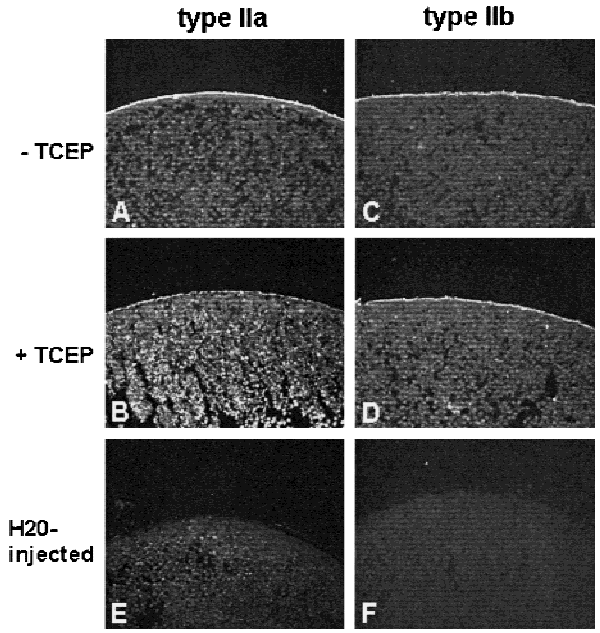

Fig. 6. Immunostaining of oocytes that express type IIa or IIb NaP_i-cotransporters: Cryosections of oocytes that expressed NaPi type IIa treated without (*A*) or with (*B*) 10 mM TCEP at 20°C for 30 min. (*C*) and (*D*) show the corresponding sections for type IIb. (*E*) and (*F*) show control oocytes stained with an anti- type IIa antibody (*E*) or with an anti- type IIb antibody (*F*).

Based on these results we conclude that S-S bonds within the type IIa transporter are essential for a transport competent conformation (Lambert et al., 2000). A cleavage of S-S bridges might lead to structural alterations that are then recognized at intracellular domains of the molecule, thereby leading to its internalization and degradation. The mechanism of this membrane retrieval is however unknown. It should be mentioned that protein kinase C activation induced an internalization of the type IIa transporter in oocytes (Forster et al., 1999). Also in OK-cells and in kidney proximal tubules, the type IIa transporter is internalized and degraded as part of its regulatory behavior (Pfister et al., 1997; Keusch et al., 1998; Pfister et al., 1998; Pfister et al., 1998; Lotscher et al., 1997). It is unknown whether the reduction of S-S bonds is involved in above regulation of the type IIa transporter in kidney cells. It might be speculated that reducing S-S bonds and different 'physiological' regulatory phenomena may lead to comparable conformational changes in the type IIa transporter molecule, which lead to a reduction in transport activity associated with membrane retrieval and degradation of the transporter protein.

An explanation for the different effect of TCEP on type IIa and IIb Na^+/P_i-cotransporter might be related to different intra- or intermolecular disulfide bond architecture of the two transporter isoforms. Type IIa may have a disulfide bond between cysteine residues 306 and 334 and another one between C225 and C520 has been proposed (Fig. 7) (Lambert et al., 2000). So far *nothing* is known about disulfide bonds in type IIb. The conservation of cysteine residues 306 and 334 in type IIb (the number of the positions are different between the isoforms, we use the position numbers of type IIa) suggest also a conserved disulfide bond (Fig. 7). The NH_2- as well as the COOH- terminus of both transporters are most probably intracellularly located, which makes the formation of disulfide bonds from cysteine residues in these parts of the transporter rather unlikely (Lambert et al., 1999). These considerations result in only 3 cysteine residues in type IIa (C225, C491 and C520, Fig. 7), that are absent in IIb. Also type IIb contains 3 cysteine residues (C313, C319 and C555, Fig. 7), absent in type IIa (again without the termini). These residues are most likely the candidates for the differences of the disulfide architecture.

For the type IIa transporter we postulated in addition to the above mentioned S-S bond (between C306 and C334) a second bond involving most likely C225 and C520 (Lambert et al., 2000); residue C491 is intracellularly located and not a candidate for S-S bond formation (Lambert et al., 1999). As these residues are absent in the type IIb transporter the S-S bond between C225 and C520 is not conserved between the two isoforms. Two of the type IIb specific cysteine residues are located in the predicted second extracellular loop, a third in transmembrane domain 8. The lack of a TCEP effect on the type IIb transporter function would therefore suggest that S-S bonds in type IIb (in contrast to type IIa) are not important for keeping a transport competent and membrane stable conformation. Furthermore, the conservation of S-S bond C306 to C334 suggests that it does not solely 'alone' determine the reported type IIa specific behavior (response to TCEP), but that a second bond (between C225 and C520) also participates in stabilizing the conformation of the type IIa Na^+/P_i cotransporter.

It is unknown whether the cysteine residues in type IIa and type IIb transporters contribute to intramolecular S-S bonds (homo-/heteromultimer). In our experiments with oocytes, sample preparation for Western blots with or without DTT did not result in different band pattern (*data not shown*) for type IIa. Furthermore, the fact that treatment with TCEP did not result in different protein pattern (in protein samples separated in the absence of reducing agents) suggests that at least extracellular located cysteine residues are not involved in forming multimeric complexes. However, although unlikely, we cannot exclude the participation of intracellular cysteine residues in the formation of multimeric complexes for both type IIa and type IIb.

It should also be indicated that in Western blots of brush border membranes isolated from normal rats after treatment with DTT the type IIa transporter falls in 2 parts, preceding a cleavage site between cysteine residue

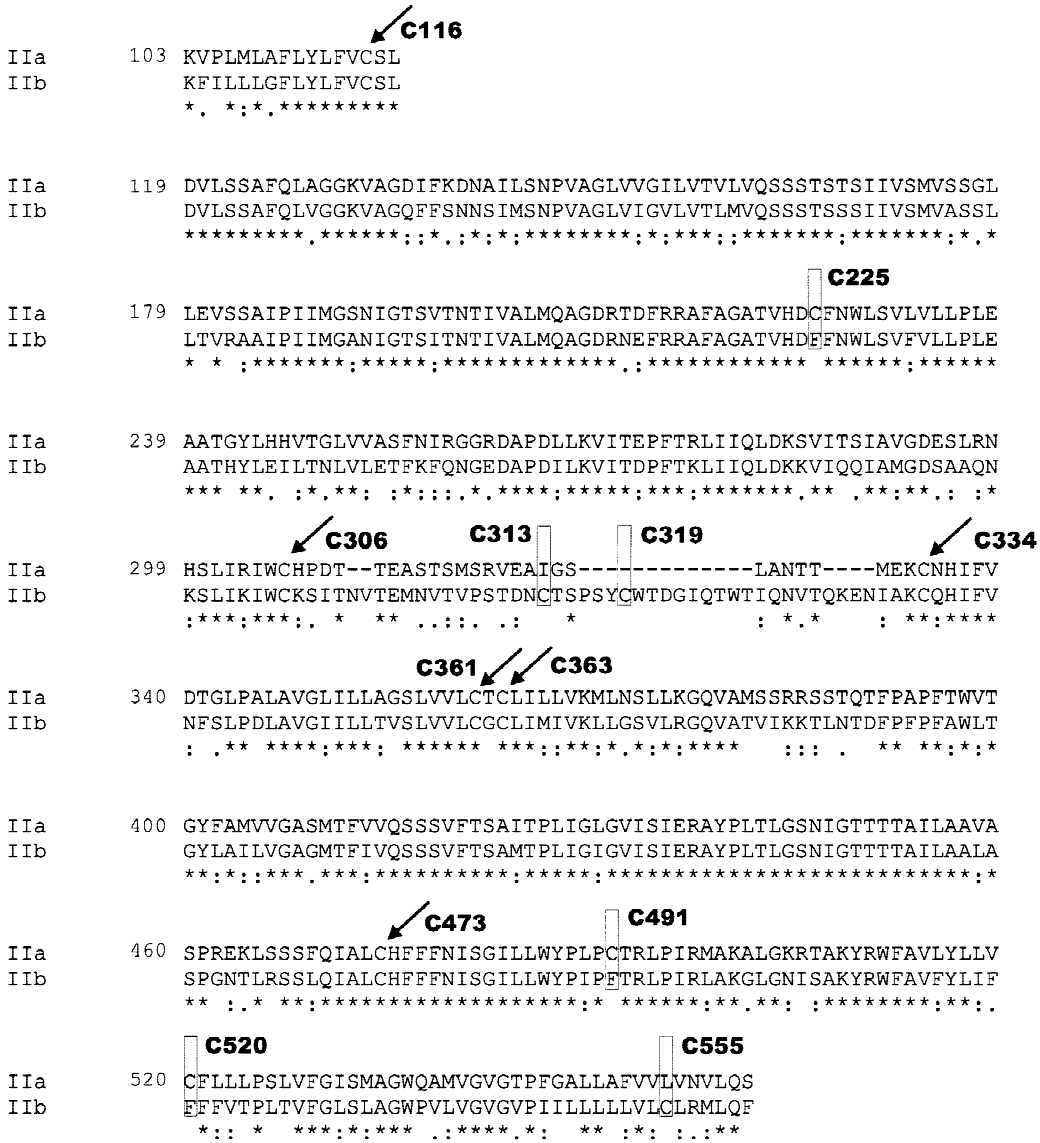

Fig. 7. Comparison between the amino acid sequences of type IIa and IIb NaP_i-cotransporters. The amino acid sequences of both transporters without the termini are compared. Identical amino acids are marked with a star; amino acids that share high or low similarities are marked with one or two points. Conserved cysteine residues are indicated by an arrow, differential cysteine residues between the IIa and IIb transporter are marked with a box. The position numbers given are based on the position in the IIa transporter. For comparison with our current model, *see* Fig. 8 in the preceding paper.

306 and 334 (Biber et al., 1996; Paquin et al., 1999). This also supports the existence of this S-S bond. It is however unknown whether this observed cleavage is an artifact related to membrane isolation/preparation or whether is occurs in situ.

Taken together, reducing S-S bonds in the type IIa Na^+/P_i cotransporter may lead to a conformational change associated with transport inhibition and membrane stability of the transporter protein. As the type IIb transporter does not show this behavior, the reduction in S-S bonds—or at least similar conformational changes—might be associated with physiological regulation of the type IIa transporter. Sequence comparisons between type IIa and IIb transporter proteins provide evidence for the importance of a second disulfide bond in the type IIa molecule (C225 and C520).

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References

Biber, J., Custer, M., Magagnin, S., Hayes, G., Werner, A., Lotscher, M., Kaissling, B., Murer, H. 1996. Renal Na/Pi-cotransporters. *Kidney Int.* **49:**981–985

Biber, J., Murer, H., Forster, I. 1998. The renal type II Na^+/phosphate cotransporter. *J. Bioenergetics & Biomembranes.* **30:**187–194

Custer, M., Lotscher, M., Biber, J., Murer, H., Kaissling, B. 1994. Expression of Na-P(i) cotransport in rat kidney: localization by RT-PCR and immunohistochemistry. *Am. J. Physiol.* **266:**F767–774

Forster, I., Traebert, M., Jankowski, M., Stange, G., Biber, J., Murer, H. 1999. Protein kinase C activators induce membrane retrieval of type II Na-phosphate cotransporters expressed in *Xenopus* oocytes. *J. Physiol.* **517.2:**327–340

Gendeh, G.S., Young, L.C., de Medeiros, C.L., Jeyaseelan, K., Harvey, A.L., Chung, M.C. 1997. A new potassium channel toxin from the sea anemone *Heteractis magnifica:* isolation, cDNA cloning, and functional expression. *Biochemistry* **36:**11461–11471

Hartmann, C.M., Hewson, A.S., Kos, C.H., Hilfiker, H., Soumounou, Y., Murer, H., Tenenhouse, H.S. 1996. Structure of murine and human renal type II Na^+-phosphate cotransporter genes (Npt2 and NPT2). *Proc. Natl. Acad. Sci. USA* **93:**7409–7414

Hayes, G., Busch, A., Lotscher, M., Waldegger, S., Lang, F., Verrey, F., Biber, J., Murer, H. 1994. Role of N-linked glycosylation in rat renal Na/Pi-cotransport. *J. Biol. Chem.* **269:**24143–24149

Hilfiker, H., Hattenhauer, O., Traebert, M., Forster, I., Murer, H., Biber, J. 1998. Characterization of a murine type II sodium-phosphate cotransporter expressed in mammalian small intestine. *Proc. Natl. Acad. Sci. USA* **95:**14564–14569

Kempson, S.A., Lotscher, M., Kaissling, B., Biber, J., Murer, H., Levi, M. 1995. Parathyroid hormone action on phosphate transporter mRNA and protein in rat renal proximal tubules. *Am. J. Physiol.* **268:**F784–F791

Keusch, I., Traebert, M., Lotscher, M., Kaissling, B., Murer, H., Biber, J. 1998. Parathyroid hormone and dietary phosphate provoke a lysosomal routing of the proximal tubular Na/Pi-cotransporter type II. *Kidney Int.* **54:**1224–1232

Lambert, G., Forster, I.C., Biber, J., Murer, H. 2000. Cysteine residues and the structure of the rat renal type IIa sodium/phosphate cotransporter type IIa. *J. Membrane Biol.* **176:**133–141

Lambert, G., Traebert, M., Hernando, N., Biber, J., Murer, H. 1999. Studies on the topology of the renal type II NaPi-cotransporter. *Pfluegers Arch.* **437:**972–978

Lotscher, M., Kaissling, B., Biber, J., Murer, H., Levi, M. 1997. Role of microtubules in the rapid regulation of renal phosphate transport in response to acute alterations in dietary phosphate content. *J. Clin. Inv.* **99:**1302–1312

Magagnin, S., Werner, A., Markovich, D., Sorribas, V., Stange, G., Biber, J., Murer, H. 1993. Expression cloning of human and rat renal cortex Na/Pi cotransport. *Proc. Natl. Acad. Sci. USA* **90:**5979–5983

Markovich, D., Verri, T., Sorribas, V., Forgo, J., Biber, J., Murer, H. 1995. Regulation of opossum kidney (OK) cell Na/Pi cotransport by Pi deprivation involves mRNA stability. *Pfluegers Arch.* **430:**459–463

Murer, H., Forster, I., Hilfiker, H., Pfister, M., Kaissling, B., Lotscher, M., Biber, J. 1998. Cellular/molecular control of renal Na/Pi-cotransport. *Kidney Int.* S2–S10

Paquin, J., Vincent, E., Dugre, A., Xiao, Y., Boyer, C.J., Beliveau, R. 1999. Membrane topography of the renal phosphate carrier NaPi-2: limited proteolysis studies. *Biochim. Biophys. Acta* **1431:**315–328

Pfister, M.F., Hilfiker, H., Forgo, J., Lederer, E., Biber, J., Murer, H. 1998. Cellular mechanisms involved in the acute adaptation of OK cell Na/P-i-cotransport to high- or low-P-i medium. *Pfluegers Arch.* **435:**713–719

Pfister, M.F., Lederer, E., Forgo, J., Ziegler, U., Lotscher, M., Quabius, E.S., Biber, J., Murer, H. 1997. Parathyroid hormone-dependent degradation of type II Na+/Pi cotransporters. *J. Biol. Chem.* **272:**20125–20130

Pfister, M.F., Ruf, I., Stange, G., Ziegler, U., Lederer, E., Biber, J., Murer, H. 1998. Parathyroid hormone leads to the lysosomal degradation of the renal type II Na/Pi cotransporter *Proc. Natl. Acad. Sci. USA* **95:**1909–1914

Sambrook, J., Fritsch, E.F., Maniatis, A.M. 1989. Molecular Cloning—A Laboratory Manual. C. Nolan, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Traebert, M., Hattenhauer, O., Murer, H., Kaissling, B., Biber, J. 1999. Expression of type II Na-Pi cotransporter in alveolar type II cells *Am. J. Physiol.* **277:**L868–L873

Turk, E., Kerner, C.J., Lostao, M.P., Wright, E.M. 1996. Membrane topology of the human Na^+/glucose cotransporter SGLT1. *J. Biol. Chem.* **271:**1925–1934

Werner, A., Biber, J., Forgo, J., Palacin, M., Murer, H. 1990. Expression of renal transport systems for inorganic phosphate and sulfate in *Xenopus laevis* oocytes. *J. Biol. Chem.* **265:**12331–12336

White, C.E., Hunter, M.J., Meininger, D.P., Garrod, S., Komives, E.A. 1996. The fifth epidermal growth factorlike domain of thrombomodulin does not have an epidermal growth factorlike disulfide bonding pattern. *Proc. Natl. Acad. Sci. USA* **93:**10177–10182

Xiao, Y., Boyer, C.J., Vincent, E., Dugre, A., Vachon, V., Potier, M., Beliveau, R. 1997. Involvement of disulfide bonds in the renal sodium/phosphate cotransporter NaPi-2. *Biochem. J.* **323:**401–408