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Functional Studies on a Split Type II Na/P_i-Cotransporter

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Abstract. Analysis of rat and mouse proximal tubular brush-border membrane expression of the type IIa Na/P_i-cotransporter provides evidence for its cleavage in the large extracellular loop (ECL-2). To study functional properties and membrane distribution of this split NaP_i-IIa transporter we followed two strategies. In one strategy we expressed the transporter as two complementary parts (p40 and p45) in *Xenopus laevis* oocytes and as another strategy we cleaved the WT protein with trypsin. Both strategies resulted in a split NaP_i-IIa protein located in the plasma membrane. The two domains were tied together by a disulfide bridge, most likely involving the cysteines 306 and 334. Surface expression of the NaP_i-IIa fragments was dependent on the presence of both domains. If both domains were coexpressed, the transporter was functional and transport characteristics were identical to those of the WT-NaP_i-IIa protein. Corresponding to this, the transporter cleaved by trypsin also retains its transport capacity. These data indicate that cleavage of the type IIa Na/P_i-cotransporter at ECL-2 is compatible with its cotransport function.

Key words: *Xenopus laevis* oocytes — NaP_i-IIa — Na/P_i-cotransport — Disulfide bridge — Split transporter — Trypsin proteolysis

Introduction

Renal proximal tubular phosphate reabsorption is mainly mediated by the type IIa Na/P_i-cotransporter located in the brush-border membrane (Beck et al., 1998; Murer et al., 2000). The rat NaP_i-IIa isoform is a 637 amino-acid protein and on Western blots it is detected at an apparent molecular mass of 90–100 kDa (Magagnin et al., 1993; Biber et al., 1996). The current topology model, which is based on hydropathy analysis and epitope-tagging experiments, predicts eight helical transmembrane segments with both the N- and C-terminal ends located intracellularly. The third and the fourth transmembrane segments are connected by a large extracellular loop (ECL-2) that contains two N-glycosylation sites (Asp 298 and Asp 328) (Hayes et al., 1994) and two cysteines at positions 306 and 334, which form a disulfide bond (Fig. 1) (Lambert et al., 2000a). Evidence has been presented that in brush-border membranes the rat and murine type IIa Na/P_i-cotransporters exist in a cleaved isoform, which is separated in the large extracellular loop (Fig. 1) (Biber et al., 1996; Xiao et al., 1997).

A more detailed model of the nature and function of the Na/P_i-translocation pathway was recently obtained by cysteine-scanning experiments. These studies provided evidence that the intracellular loop 1 (ICL-1) and the extracellular loop 3 (ECL-3) together contribute to the Na/P_i-cotransport pathway (Fig. 1) (Lambert et al., 2000a; Kohler et al., 2001). Despite some hydrophobic nature, these loops, however, may not span the membrane entirely, but it appears more likely that they are embedded in a yet unknown arrangement of several transmembrane segments that form the Na/P_i-cotransport pathway. It is of note that all transport functions can be associated with a monomeric form of the NaP_i-IIa protein (Kohler et al., 2000).

One possible strategy to analyze the helix packing of a membrane protein under functional conditions is by crosslinking different transmembrane segments of the molecule. For example, cysteines can be introduced by site-directed mutagenesis, followed by the use of bireactive, cysteine crosslinking reagents (Kaback, Voss & Wu, 1997; Qiu, Nicoll & Philipson, 2001). For such an analysis, however, it would be ideal to have a split NaP_i-IIa-protein, which can then

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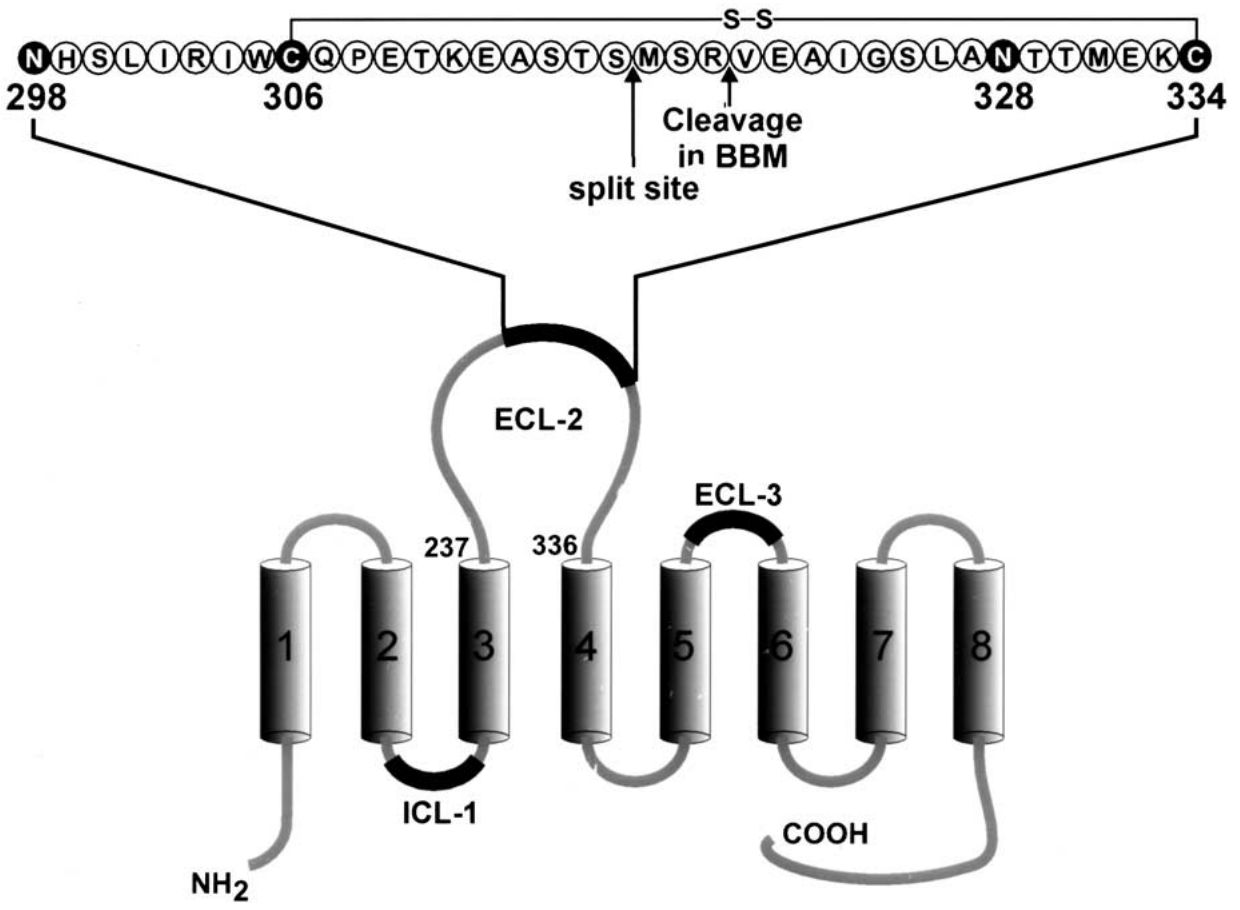

Fig. 1. Scheme of the secondary structure of the rat NaP_i-IIa cotransporter. The enlarged section of the extracellular loop ECL2 includes two glycosylation sites (298, 328) and two cysteines (306; 334) that are connected by a disulfide bond. The sites at which NaP_i-IIa is cleaved into p40 and p45 in mouse brush-border membranes (Paquin et al., 1999) and the split site used in this study are indicated.

be analyzed subsequently with or without cross-linking.

In this report we demonstrate that a functional NaP_i-IIa-cotransporter is formed in *Xenopus laevis* oocytes by coexpressing two nonoverlapping halves of the protein. Similarly, cotransport function is also retained after proteolytic cleavage at the large extracellular loop. Na/P_i-cotransport characteristics, such as apparent substrate-affinities, pH-dependence and electrogenicity are retained by the split NaP_i-IIa protein, suggesting that the relative arrangement of the transmembrane segments contributing to the transport pathway is identical to the one in the native protein.

Materials and Methods

Reagents

All restriction enzymes were obtained from Amersham Pharmacia Biotech and oligonucleotide primers from Microsynth (Switzerland). Other standard reagents were obtained from Fluka (Switzerland). Mutations were performed using the Quick-change Site Directed Mutagenesis Kit (Stratagene).

Solutions

Barth solution was (in mM), 1 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 2.4 $NaHCO_3$, 88 NaCl, 10 HEPES, Gentamycin 20 mg/l, adjusted to pH 7.4 with Tris. ND96 solution was (in mM), 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, adjusted to pH 7.4 with KOH.

Construction of cDNAs Encoding for Rat-NaP_i-IIa fragments

The cDNA encoding for the N-terminal fragment (transmembrane segments, TMs, 1 to 3; p40) was generated by introducing a stop codon into the native NaP_i-IIa-cDNA instead of the amino acid serine at position 316 (Fig. 1). The oligonucleotides used were: 5′-ttaccggagatgggacctgtagattcaccttcggagaaaacagagaccaactgtgg-3′ and 5′-ccaatggcctctaccctggacatctaagtggaagcctc-3′.

The cDNA encoding for the C-terminal fragment (TMs 4 to 8; p45) was generated by PCR, introducing an *Eco*RI restriction site upstream of the methionine at position 317 and including a *Bam*HI restriction site in pSPORT 1 (Life Technologies) downstream of the NaP_i-IIa-stop codon. The oligonucleotides used were: 5′-ctggaatt-

cccatcatgtccagggtagaggccattggcagccttgc-3′ (sense) and 5′ tgacgtcgcatg-cacgcgtacgtaagcttggatcctctagagcggccgccgactagtgag-3′ (antisense). The PCR products were digested with *Eco*RI and *Bam*HI and ligated into pSPORT 1 with T_4-DNA-Ligase.

The resulting DNA-constructs were sequenced to confirm that no other mutation was introduced.

Expression of Rat-NaP$_i$-IIa Proteins in *Xenopus laevis* Oocytes

The procedures for oocyte preparation and RNA injection have been previously described (Werner et al., 1990). Briefly, pSPORT 1 containing NaP$_i$-IIa-cDNAs was linearized at the 3′-end by digestion with *Not*I. In vitro synthesis and capping of RNAs were done by incubating the cDNAs in the presence of 40 U of T7 RNA Polymerase (Promega) and Cap Analog (New England Biolabs). Oocytes were injected with either 50 nl of water or 50 nl of water containing 5 ng of RNA. Afterwards, oocytes were incubated in Barth solution for 3–4 days, before the experiments were performed. All following experiments were performed with the same batch of oocytes obtained from the same frog and were repeated at least twice.

Western Blot Analysis

Yolk-free homogenates of a pool of three oocytes were obtained as follows: 60 μl of homogenization buffer (1% $C_{12}E_6$ (Calbiochem) in 100 mM NaCl, 20 mM Tris/HCl, adjusted to pH 7.4) were added. Afterwards, the samples were mixed, centrifuged at 16,000 × g for 3 min (22°C) and the supernatants were recovered (Turk et al., 1996). For gel-electrophoretic separation, 10 μl of the supernatants were mixed with 10 μl loading buffer (8% SDS, 2 mM EDTA, 20% glycerol, 0.19 M Tris/HCl, 200 mM 1,4-Dithiothreitol, pH 6.8) and denatured for 2 min at 95°C. Transferred proteins were processed as previously described (Custer et al., 1994) using rabbit polyclonal antibodies raised against synthetic peptides derived from the NH_2- or COOH-termini of the rat NaP$_i$-IIa. Immunoreactive proteins were detected with an enhanced chemiluminescence system (Pierce).

Immunocytochemistry

Eggs were immersed during 30 min in PBS containing 3% paraformaldehyde and 0.1% glutaraldehyde. After rinsing in PBS (4°C), single eggs were frozen onto thin cork slices with tissue-tek, using liquid propane cooled by liquid nitrogen (Hayes et al., 1994). Cryosections of 6 μm were cut at −21°C using a cryomicrotome (Leica) and mounted on chromalumgelatin-coated glass slides.

For immunostainings, the sections were preincubated for one hour in PBS containing 3% milk powder and 0.3% Triton X-100 and afterwards overnight in the same solution but containing anti-NaP$_i$-IIa antibodies. For visualization of the binding of the first antibody, sections were washed and incubated with swine anti-rabbit IgG conjugated to fluorescein isothiocyanate (Dakopatts, dilution 1:50). Finally, sections were mounted with a coverslip using DAKO glycergel (Dakopatts).

Trypsin Digestion

Approximately 12 oocytes were incubated with 200 μl trypsin solution (Sigma, No. T-8253, 0.014 mg/ml in Barth solution) for 10 min at 37°C. The trypsin cleavage was stopped by washing the oocytes two times with Pefablock-solution (Merck, 1 mg/ml in Barth solution), followed by four additional washing steps with Barth solution.

Transport Studies

Na^+-dependent transport of inorganic phosphate (P_i) was performed either by isotope flux measurements on groups of 10–12 oocytes as previously described by Werner et al., 1990 or by determining the electrogenic activity of individual oocytes associated with Na/P_i-cotransport. For the latter case, oocytes were superfused in a recording chamber and voltage-clamped using a conventional two-electrode voltage-clamp system (Forster et al., 1998). Data were acquired using a Digidata 1320A data acquisition system driven by pClamp 8.2 hard- and software (Axon Instruments, Union City, CA) and sampled at a rate corresponding to twice the recording bandwidth. The oocytes were voltage-clamped to a holding potential (V_h) = −50 mV for steady-state activation determinations and (V_h) = −60 mV for current-voltage (I/V) curve determinations. Dose-response data points were fit with a Michaelis-Menten function as previously described, using a nonlinear regression algorithm (Graphpad, San Diego, CA).

Results

Expression of Rat NaP$_i$-IIa Fragments in Oocytes of *Xenopus laevis*

We engineered cDNAs encoding for the NaP$_i$-IIa fragments p40 (AA 1–316; TMs 1 to 3) and p45 (AA 317–637; TMs 4 to 8). These two fragments were designed based on the observation that in isolated and gel-electrophoretically separated renal brush border membranes the NaP$_i$-IIa protein appears in a cleaved and an uncleaved form (Biber et al., 1996; Xiao et al., 1997). This cleavage has been shown to occur between the amino acids 319 and 320 (Fig. 1) (Paquin et al., 1999).

The p40- and p45-RNAs were injected into oocytes of *X. laevis* individually or together and protein expression was analyzed by Western blotting. If lysates of oocytes injected with p40-RNA were treated with a reducing agent (DTT), the N-terminal antibody detected two bands with molecular weights of approximately 40 and 45 kDa. The same two bands (most likely resulting from different degrees of glycosylation) were observed on Western blots of oocytes coinjected with p40- and p45-RNAs (Fig. 2*A*). In oocytes injected with the WT-RNA the usual WT-expression pattern (with a molecular weight of 80–100 kDa) was visible on the corresponding Western blots, but no signal for the p40 fragment. Similar results were obtained for the p45 fragment by using an antibody directed against the C-terminal end of NaP$_i$-IIa (*data not shown*). If lysates of p40/p45-coinjected oocytes were separated under nonreducing conditions, no bands at 40 and 45 kDa were detected. Instead, a band identical to the one observed in WT-injected oocytes was observed (Fig. 2*B*). These data suggest that the p40- and p45-fragments were cross-linked by a disulfide bridge.

To examine the localization of the split NaP$_i$-IIa products, immunofluorescence was performed. For

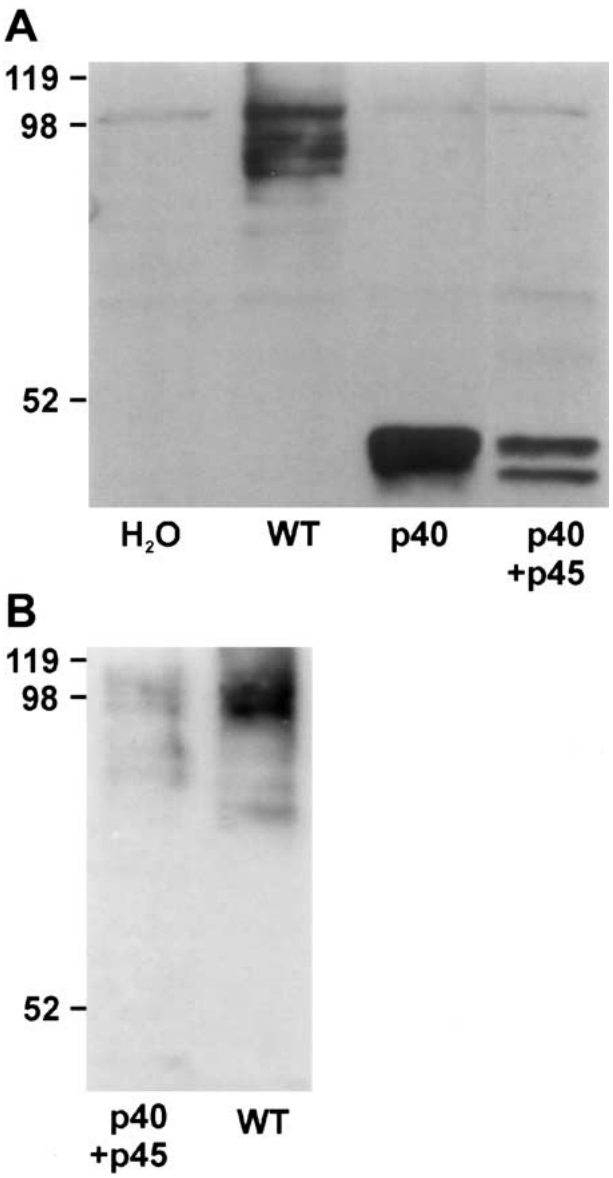

Fig. 2. Western blots of lysates of oocytes expressing WT, p40 and p40 and p45 together. 3 days after injection, pools of 3 oocytes for each group were lysed, liberated from yolk and solubilized in sample buffer containing DTT (*A*) or not containing any reducing agent (*B*). After separation by a 10% SDS-PAGE gel, Western blots were performed with a rabbit polyclonal antibody directed against the N-terminus of NaP_i-IIa.

oocytes injected with the p40- and p45-RNAs separately, no fluorescent signal in the plasmamembrane was observed, either by using an anti-N or an anti-C-terminal antibody (Fig. 3*D* and *H*). This suggested that, although the fragments were detectable by Western blots, these fragments were not delivered to the plasma membrane. However, in sections of oocytes coexpressing the p40 and the p45 fragments, both antibodies resulted in clear signals (Fig. 3*C* and *G*). Even though this signal was not as distinct as the one observed in oocytes expressing wild-type (WT) NaP_i-IIa (Fig. 3*B* and *F*), it was possible to clearly associate the signal with the plasma membrane when compared with a β-actin staining (*data not shown*).

Functional Characterization of the Split NaP_i-IIa Cotransporter

Oocytes injected either with cRNAs encoding for the WT, p40, p45 or coinjected with p40- and p45-RNAs, were tested for Na/P_i-cotransport function. Oocytes expressing either p40 or p45 alone did not show P_i-uptake (Fig. 4). However, coinjection of p40- and p45 cRNA resulted in P_i-transport significantly higher compared to control (water injected) oocytes, which was dependent on the presence of external Na^+-ions (Figs. 4 and 5*C*). In agreement with the lower signal as observed by immunofluorescence and Western blots (*see* Figs. 2*A*, *B* and 3*C* and *G*), the amount of P_i transport of p40/p45-coinjected oocytes was also lower compared to oocytes expressing the unsplit NaP_i-IIa cotransporter (Fig. 4). Since lower transport activity could also be due to altered kinetics of the reassembled NaP_i-IIa cotransporter, we determined the kinetics of Na/P_i-cotransport, such as pH-, P_i- and Na^+-dependency (Fig. 5*A–C*) and performed a complete functional characterization of the oocytes coexpressing p40 and p45 (*see* below). As illustrated in Fig. 5, dependencies on pH, Na^+- and P_i-concentrations of Na/P_i-cotransport into oocytes coinjected with p40- and p45-cRNA were not different from those observed in oocytes injected with WT NaP_i-IIa (*see also* Magagnin et al., 1993).

In addition, oocytes injected with cRNAs encoding the WT, p40 or p45 and oocytes coinjected with p40- and p45-cRNAs were assayed for electrogenic characteristics of Na/P_i-cotransport (Fig. 6). When challenged with 1 mM P_i in the presence of 100 mM Na^+, only oocytes that were injected with WT or coinjected with p40 and p45 gave measurable electrogenic responses under voltage-clamp conditions (V_h = −50 mV) as shown in Fig. 6*A*. The electrogenic response to an increasing dose of P_i increased in a monotonic fashion following a Michaelis-Menten characteristic as previously established for the WT NaP_i-IIa (Fig. 6*B*,*C*). When these data were fit with the Michaelis-Menten equation, the estimated apparent affinity constant for P_i was 0.06 ± 0.007 mM for the WT and 0.09 ± 0.009 mM for the coinjected oocytes (n = 3). Within the limits of experimental errors, these findings indicated that the apparent binding for P_i was not altered for the coinjected case when compared with the intact NaP_i-IIa protein.

As a further test for the electrogenic integrity of the split cotransporter we determined its voltage dependence. Fig. 6*D* shows representative tracings recorded in response to a series of voltage steps from V_h = −60 mV to potentials in the range −140 mV to +40 mV. Each tracing is the difference between the

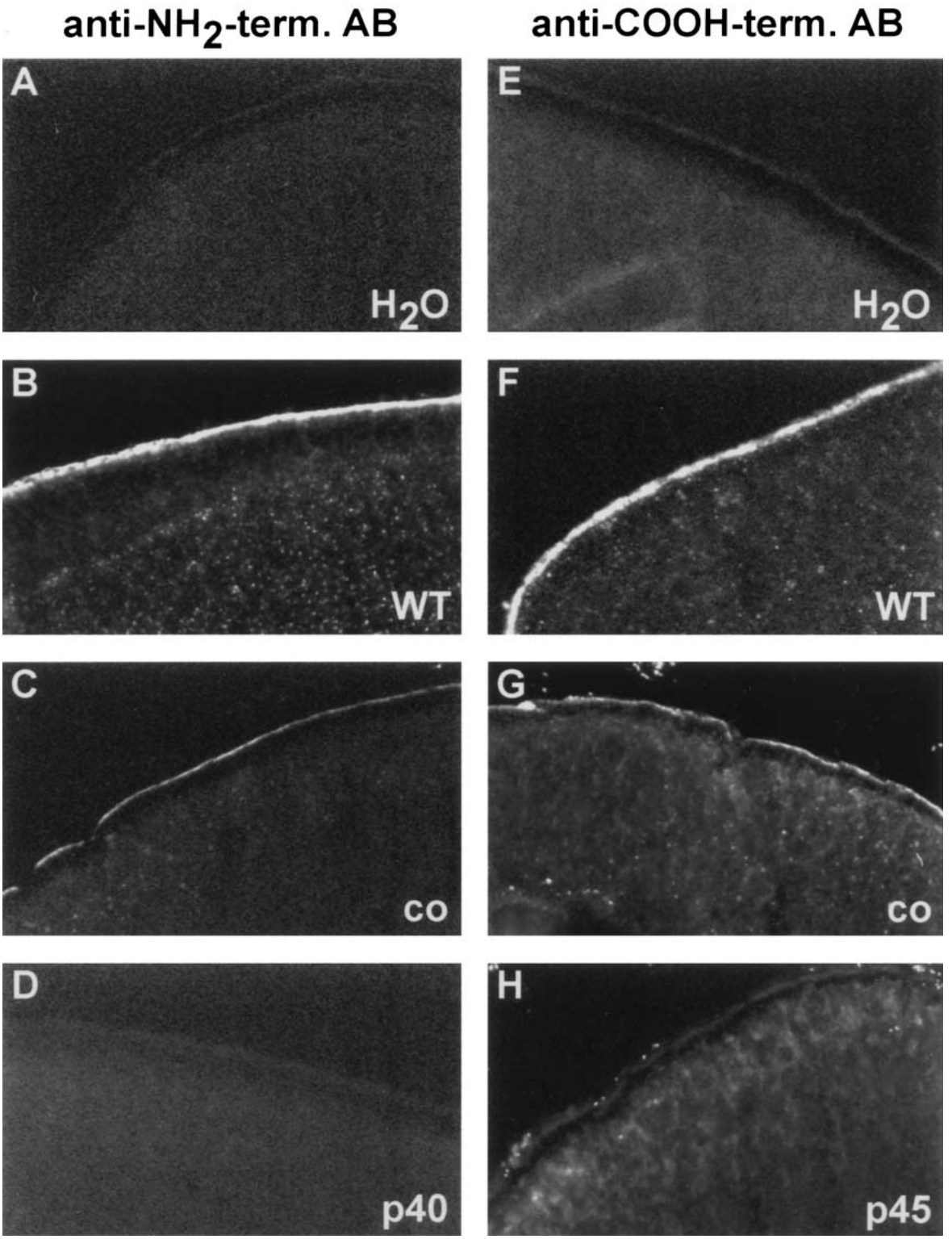

Fig. 3. Immunocytochemical detection of WT NaP_i-IIa and NaP_i-IIa-fragments (p40 and p45) in cryosections of oocytes. Oocytes injected with H_2O (*A*, *E*), or RNA for WT (*B*, *F*), p40 (*D*), p45 (*H*) or coinjected with p40- and p45-RNA (*C*, *G*), were fixed in paraformaldehyde. Cryosections were labeled with rabbit anti-NaP_i-IIa polyclonal antibodies directed against the N-terminal end (*A–D*) or the C-terminal end (*E–H*).

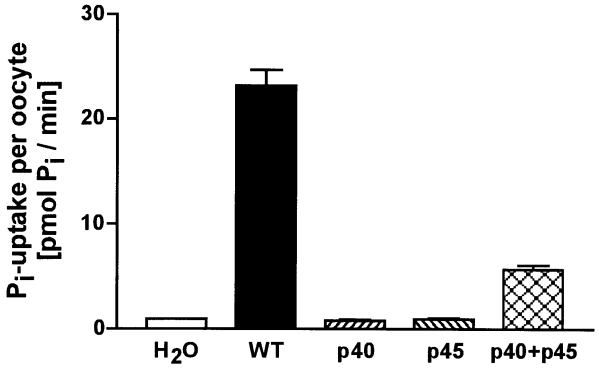

Fig. 4. P_i-uptake into oocytes injected with water or cRNA of WT NaP_i-IIa or the two NaP_i-IIa-fragments (p40, p45) individually or together. 3 days after injection, the cells were incubated in a solution containing [$^{32}P_i$]-P_i (1 mM) and Na^+ (100 mM) for 30 min. After several washing steps, $^{32}P_i$-contents in lysates of single oocytes were measured. The bars represent the mean ± SE ($n \geq 10$).

response in 100 mM Na^+ alone to that in the presence of 1 mM P_i. The relaxations to the new steady-state current level following each step represent presteady-state charge movements as previously reported for the WT construct (Forster et al., 1998). The steady-state current at each test potential, normalized to the current at −100 mV, is shown in Fig. 6*E*. For $V \geq 0$ mV we observed that the difference current was prone to contamination from endogenous chloride channels, however, in the range $-140 < V < -20$ mV, the *I-V* data show a typical monotonic decrease with hyperpolarization, as previously reported for the WT construct (Forster et al., 1998). Taken together, these findings confirmed that the electrogenic kinetics of the split construct were very similar to the intact, unsplit NaP_i-IIa protein.

Extracellular Proteolytic Cleavage of NaP_i-IIa

To introduce a cleavage after expression of the WT NaP_i-IIa, we treated NaP_i-IIa-expressing oocytes with trypsin. If Western blots of such treated oocytes were performed under nonreducing conditions, no difference between the cleaved and uncleaved (untreated oocytes) NaP_i-IIa protein was observed (Fig. 7*A*). However, if separated under reducing conditions, two additional protein fractions with a molecular weight of 40 and 45 kDa were detected in lysates of WT-expressing oocytes that had been treated with trypsin. The fact that not all of the NaP_i-IIa protein was cleaved by trypsin is explained by the observation that only a fraction of NaP_i-IIa cotransporters is inserted into the plasma membrane (Traebert et al., 2001). For this reason we assume that almost all of

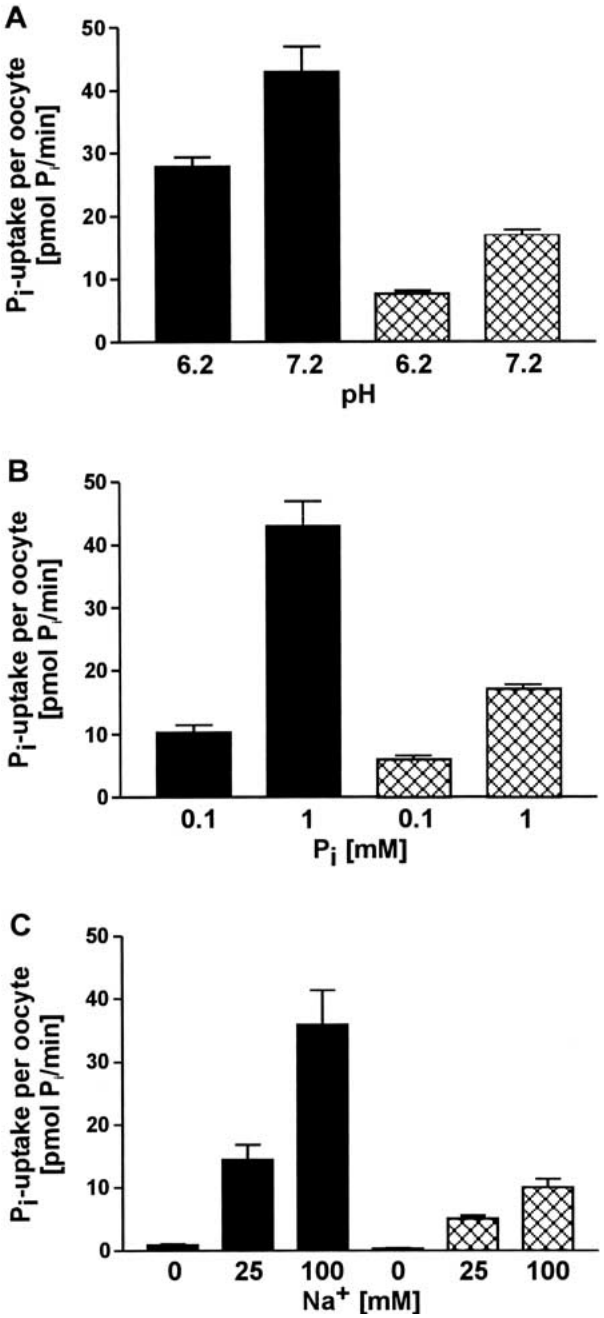

Fig. 5. pH- (*A*), P_i- (*B*) and Na^+-dependence (*C*) of NaP_i-IIa RNA-induced Na^+-dependent P_i-uptake in *Xenopus laevis* oocytes. Oocytes injected either with WT-(■) or coinjected with p40- and p45-RNA(⊠) were assayed for P_i-uptake 3 days after injection. In the study of Na^+-dependence, choline was used for isoosmotic ionic replacement. The bars represent the mean ± SE ($n \geq 10$).

the NaP_i-IIa protein located in the membrane was cleaved by trypsin.

P_i-uptake experiments were performed in parallel using the same oocytes as in the Western analysis. As illustrated in Fig. 8, Na/P_i-cotransport of trypsin-treated oocytes was not significantly different from

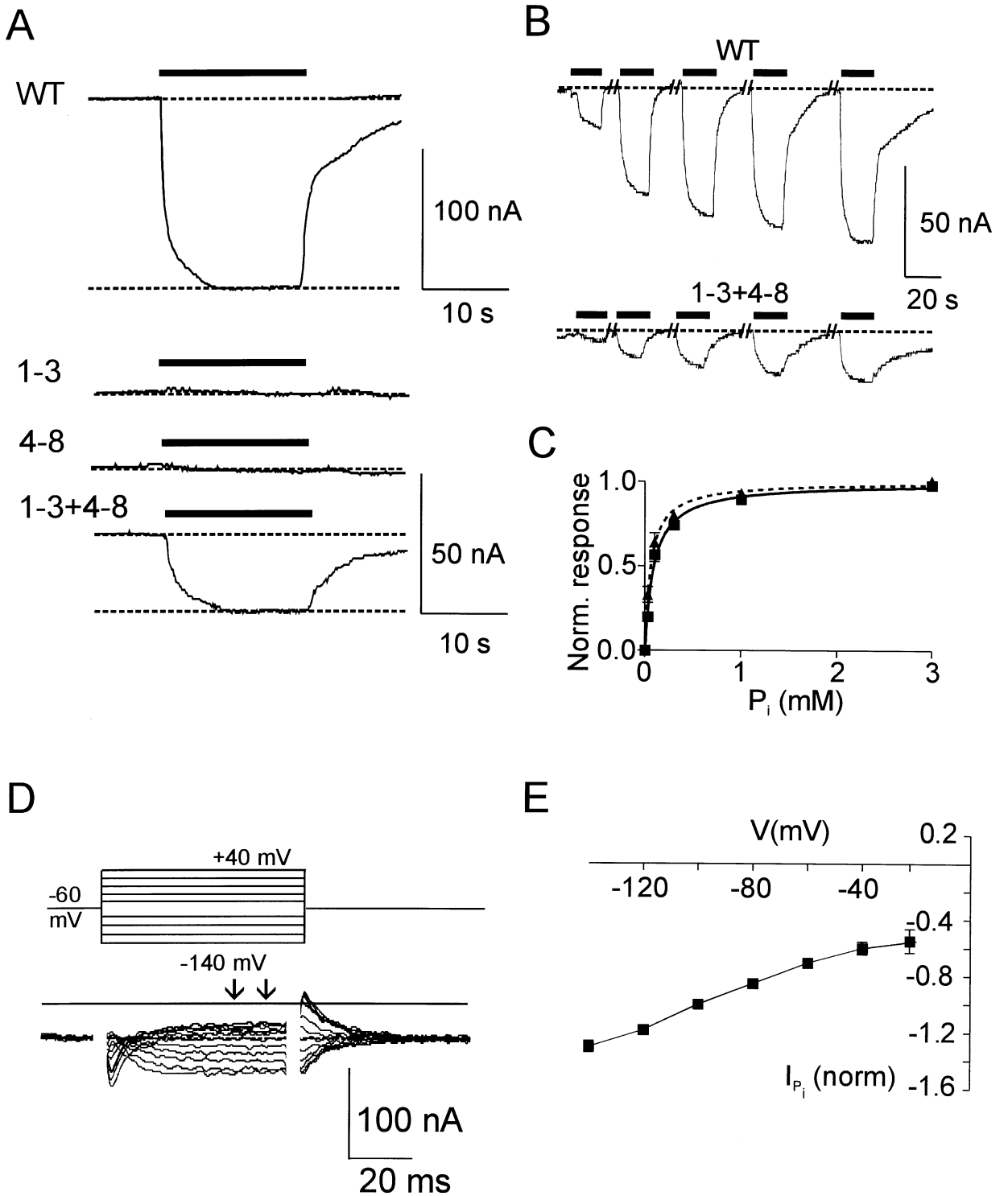

Fig. 6. Electrogenic properties of oocytes coinjected with p40 and p45 cRNAs. (*A*) Steady-state current induced by 1 mM P_i (applied during bar) for oocytes expressing WT, p40, p45 and coexpressing p40 and p45. The responses of the p40 and p45 were indistinguishable from water-injected oocytes (*data not shown*). (*B*) Representative recordings showing progressive increase in electrogenic response with increasing P_i concentration (from left to right: 0.03, 0.1, 0.3, 1, 3 mM; upper: WT; lower: p40- and p45-fragments coexpressed; for two oocytes from same donor-frog). (*C*) Michaelis-Menten fit to P_i-activation data. Each point is mean ± SE of 3 experiments using oocytes from different donor-frogs. Continuous line: fit to p40 + p45 data; broken line: fit to WT data. Each data set was first fit to estimate V_{max}, the data points were then normalized and pooled to account for different expression levels. (*D*) Representative response of an oocyte coinjected with cRNAs for p40 and p45 in response to voltage steps from $V_h = -60$ mV to voltages in the range of −140 to +40 mV (*upper*). Each trace is the difference between in ND96 + 1 mM P_i and ND96 alone. The mean current was determined between arrows and the *I-V* curve constructed as shown in (*E*), pooled from 3 cells.

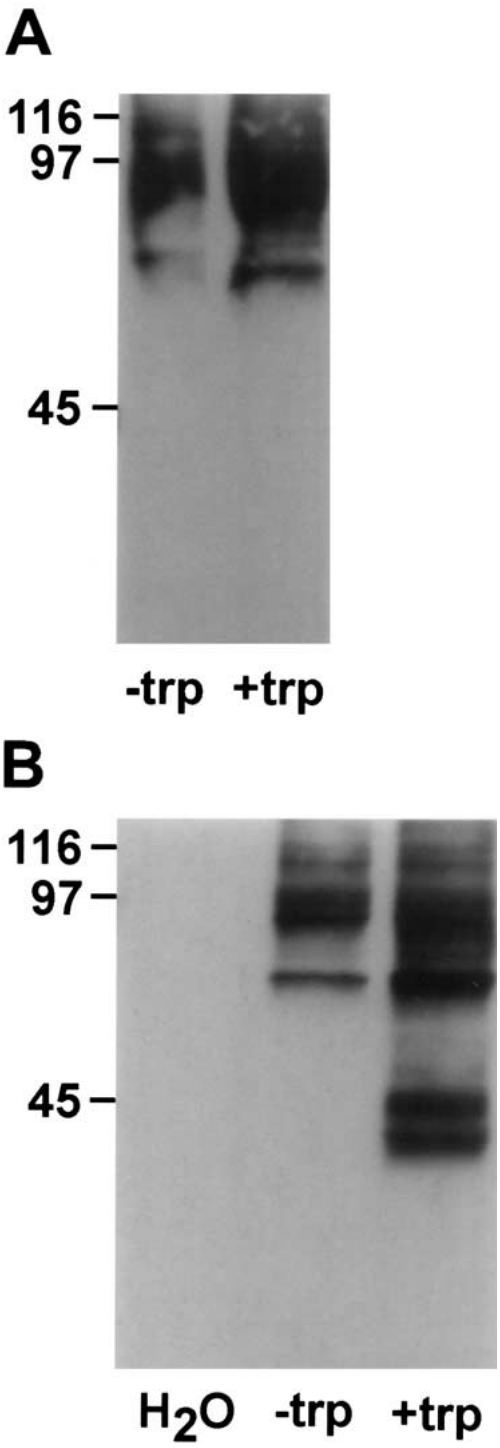

Fig. 7. Western blots of oocytes treated with or without trypsin. Oocytes were injected with WT RNA. After lysis, the yolk-free homogenates of a pool of three oocytes were solubilized in sample buffer with (*A*) or without (*B*) 1,4-Dithiothreitol (DTT). Immunoblotting was performed with a rabbit polyclonal antibody directed against the N-terminus of NaP_i-IIa.

untreated oocytes. Also, trypsin treatment did not alter basal Na/P_i-transport of control (uninjected) oocytes.

Discussion

Recent studies have revealed that the Na/P_i-cotransporter NaP_i-IIa may span the membrane eight times. According to this model, three (N-terminal) transmembrane segments are separated from five (C-terminal) transmembrane segments by a large extracellular loop (Murer & Biber, 1994). To date, however, nothing is known about the relative arrangement of the transmembrane segments of the NaP_i-IIa protein. Helix packing has been studied biochemically by a variety of methods, such as interhelical, oxidative crosslinking via disulfide bridges (Wu & Kaback, 1996; Loo & Clarke, 1997; Qiu et al., 2001) or using bifunctional crosslinking reagents (Xie, Turk & Wright, 2000; Guan, Weinglass & Kaback, 2001). The aim of the present study was to establish a functional split NaP_i-IIa cotransporter to allow the investigation of the arrangement of the proposed eight transmembrane segments by crosslinking. To generate two halves of the NaP_i-IIa protein, we took advantage of the observation that in purified renal brush border membranes part of the NaP_i-IIa protein appears in a proteolytically cleaved form (Biber et al., 1996; Xiao et al., 1997). This cleavage has been shown to occur in the large extracellular loop between the two N-glycosylation sites (positions 298 and 328) and between two cysteine residues forming a disulfide bridge (positions 306 and 334) (Paquin et al., 1999). Accordingly, RNAs encoding the two fragments p40 and p45 were constructed and the proteins were analyzed after expression (individually or coexpressed) in oocytes of *X. laevis*. The resulting Na/P_i-cotransport activity was compared with the characteristics of the WT-(unsplit) NaP_i-IIa protein.

As demonstrated, neither of the single halves (p40 or p45) showed any Na/P_i-cotransport activity when the corresponding RNAs were injected alone. As evident from Western blot analysis, both fragments were expressed; however, by immunofluorescence, they could not be detected in the plasma membrane. On the other hand, when coinjected, expression of both fragments was observed and it was possible to localize them in the plasma membrane. Since neither fragment was sorted to the plasma membrane on its own, this data suggests that formation of the complete NaP_i-IIa protein occurred prior to membrane insertion. Further, Western blot analysis revealed that the two fragments are linked by a disulfide bridge, which resembles the situation previously documented in purified renal brush border membranes (Biber et al., 1996; Xiao et al., 1997). As demonstrated earlier, intramolecular disulfide bridges in the NaP_i-IIa protein seem to be important for its localization to the plasma membrane. Lambert et al. (2000b), were able to show that extracellular treatment with reducing agents (TCEP) leads to inacti-

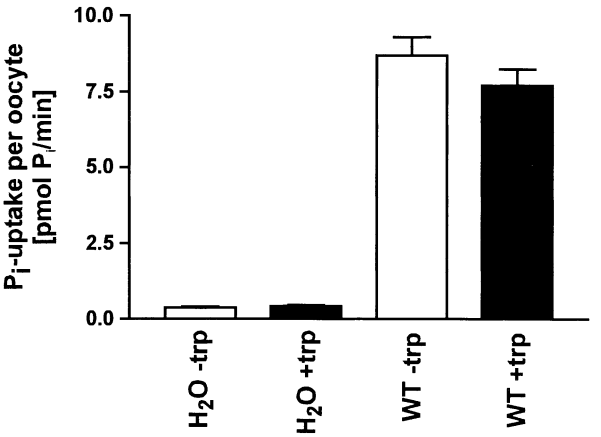

Fig. 8. Na/P_i-uptake in WT NaP_i-IIa-expressing oocytes treated with or without trypsin. Oocytes of the same batch as in Fig. 7 were incubated for 30 min at 25°C in the presence of [$^{32}P_i$]-P_i (1 mM) and Na^+ (100 mM). After several washing steps, $^{32}P_i$ incorporated in single oocytes was determined. The bars represent the mean ± SE ($n \geq 10$).

vation and degradation of the protein. In addition, it has been demonstrated that these cysteine bridges are necessary for the Na^+/P_i-cotransport function of the protein (Xiao et al., 1997; Lambert et al., 2000b).

After coinjection of p40 and p45, Na^+-dependent P_i-cotransport was observed, which in terms of all known characteristics (pH dependency, apparent K_m-values for substrates and electrogenic properties) was indistinguishable from Na/P_i-cotransport mediated by WT NaP_i-IIa. Similar results were previously obtained for other transporters expressed in two complementary parts (Bibi & Kaback, 1990; Groves, Wang & Tanner, 1998; Kohl et al., 1998; Xie et al., 2000; Ottolia et al., 2001).

In summary we have shown that cleavage of the Na/P_i-cotransporter IIa in the extracellular loop ECL-2, introduced either by a proteolytic treatment of the intact transporter or by expression in two fragments, does not affect the formation of the Na/P_i-transport pathway. Therefore, to study the relative arrangement of the proposed transmembrane segments by crosslinking experiments using the split NaP_i-IIa cotransporter appears to be a feasible approach.

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References

Beck, L., Karaplis, A.C., Amizuka, N., Hewson, A.S., Ozawa, H., Tenenhouse, H.S. 1998. Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciuria, and skeletal abnormalities. *Proc. Natl. Acad. Sci.* (*USA*) **95:**5372–5377

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

Bibi, E., Kaback, H.R. 1990. In vivo expression of the lacY gene in two segments leads to functional lac permease. *Proc. Natl. Acad. Sci.* (*USA*) **87:**4325–4329

Custer, M., Lötscher, 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–F774

Forster, I., Hernando, N., Biber, J., Murer, H. 1998. The voltage dependence of a cloned mammalian renal type II Na^+/P_i cotransporter (NaP_i-2). *J. Gen. Physiol.* **112:**1–18

Groves, J.D., Wang, L., Tanner, M.J. 1998. Functional reassembly of the anion transport domain of human red cell band 3 (AE1) from multiple and non-complementary fragments. *FEBS Lett.* **433:**223–227

Guan, L., Weinglass, A.B., Kaback, H.R. 2001. Helix packing in the lactose permease of *Escherichia coli*: localization of helix VI. *J. Mol. Biol.* **312:**69–77

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

Kaback, H.R., Voss, J., Wu, J. 1997. Helix packing in polytopic membrane proteins: the lactose permease of *Escherichia coli*. *Curr. Opin. Struc. Biol.* **7:**537–542

Kohl, B., Wagner, C.A., Huelseweh, B., Busch, A.E., Werner, A. 1998. The Na^+-phosphate cotransport system (NaP_i-II) with a cleaved protein backbone: implications on function and membrane insertion. *J. Physiol.* **508:**341–350

Kohler, K., Forster, I.C., Lambert, G., Biber, J., Murer, H. 2000. The functional unit of the renal type IIa Na^+/P_i cotransporter is a monomer. *J. Biol. Chem.* **275:**26113–26120

Kohler, K., Forster, I.C., Stange, G., Biber J., Murer, H. 2002. Identification of functionally important sites in the 1st intracellular loop of the NaP_i-IIa cotransporter. *Am. J. Physiol.* **282:**F687–F696

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

Lambert, G., Traebert, M., Biber, J., Murer, H. 2000b. 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. *J. Membrane Biol.* **176:**143–149

Loo, T.W., Clarke, D.M. 1997. Drug-stimulated ATPase activity of human P-glycoprotein requires movement between transmembrane segments 6 and 12. *J. Biol. Chem.* **272:**20986–20989

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

Murer, H., Biber, J. 1994. Renal sodium-phosphate cotransport. *Curr. Opin. Nephrol. Hy.* **3:**504–510

Murer, H., Hernando, N., Forster, I., Biber, J. 2000. Proximal tubular phosphate reabsorption: molecular mechanisms. *Physiol. Rev.* **80:**1373–1409

Ottolia, M., John, S., Qiu, Z., Philipson, K.D. 2001. Split Na^+-Ca^+ exchangers. Implications for function and expression. *J. Biol. Chem.* **276:**19603–19609

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

Qiu, Z., Nicoll, D.A., Philipson, K.D. 2001. Helix packing of functionally important regions of the cardiac Na^+-Ca^{2+} exchanger. *J. Biol. Chem.* **276:**194–199

Traebert, M., Kohler, K., Lambert, G., Biber, J., Forster, I., Murer, H. 2001. Investigating the surface expression of the renal type IIa Na^+/P_i-cotransporter in *Xenopus laevis* oocytes. *J. Membrane Biol.* **180:**83–90

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

Wu, J., Kaback, H.R. 1996. A general method for determining helix packing in membrane proteins in situ**:** helices I and II are close to helix VII in the lactose permease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **93:**14498–14502

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

Xie, Z., Turk, E., Wright, E.M. 2000. Characterization of the *Vibrio parahaemolyticus* Na^+/Glucose cotransporter. A bacterial member of the sodium/glucose transporter (SGLT) family. *J. Biol. Chem.* **275:**25959–25964