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Effect of Two Tyrosine Mutations on the Activity and Regulation of the Renal Type II Na/P_i-Cotransporter Expressed in Oocytes

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Abstract. The rat renal type II Na/Pi-cotransporter (NaPi2), which is regulated by mechanisms involving endocytosis and lysosomal degradation, contains two sequences that show high homology with two tyrosine (Y)-based consensus motifs previously reported to be involved in such intracellular trafficking: $GY_{402}FAM$ matching the consensus sequence GYXXZ, and $Y_{509}RWF$ matching the motif YXXO. Mutations of any of these two Y nearly abolished the NaPi2 mediated $^{32}P_i$-uptake after cRNA-injection into oocytes. The mechanisms underlying these defects are however different. Mutation of the Y402 results in a lack of glycosylation and reduced surface expression of the cotransporter, that are specific for the Y402 mutation since substitution of the neighboring F403 did not have any effect. The inhibitory effect of the Y509 mutation is related to a functional inactivation of the protein expressed in the plasma membrane; mutation of the neighboring R510 also led to a decrease in the cotransporter activity. Pharmacological activation of the protein kinase C cascade by DOG induced the retrieval of both wild-type (WT) as well as Y509 cotransporters from the oocyte plasma membrane. These data suggest that the Y402 is important for the surface expression whereas Y509 for the function of the type II Na/P_i-cotransporter expressed in oocytes. Y509 seems not to be involved in the membrane retrieval of the cotransporter.

Key words: Na/P_i-cotransporter — PTH — Endocytosis — Tyrosine-based signals

Introduction

Phosphate reabsorption in the proximal tubule of the mammalian kidney is a sodium-dependent process involving sodium/phosphate (Na/P_i) cotransport at the brush border membrane [25]. In recent years 3 different Na/P_i-cotransporters have been identified: type I, type II and type III [27]. The type II Na/P_i-cotransporter is predominantly expressed in renal proximal tubular brush border and it is involved in the physiological regulation of the renal P_i-reabsorption [1, 25–27]. Type II cotransporters are highly conserved among different species, with the more variable stretches clustered in the N and C terminal domains [17, 21, 35, 39; for review *see* ref 26].

Studies on renal proximal tubules as well as on OK cells have shown that parathyroid hormone (PTH) treatment leads to an inhibition of the brush border membrane Na/P_i-cotransport activity, due to a retrieval from the apical membrane and subsequent lysosomal targeting and degradation of the type II Na/P_i-cotransporter [19, 20, 29, 30]. Removal of PTH is associated with an increased brush border membrane expression of the type II cotransporter [29]. In OK cells the PTH-induced inhibition seems to be mediated by both the protein kinase A (PKA) and protein kinase C (PKC) pathways [31]. In addition, pharmacological activation of the PKC cascade has been shown to have an inhibitory effect on the rat (NaPi-2) and human (NaPi-3) type II cotransporters expressed in *Xenopus laevis* oocytes [15, 36]. Recent data indicate that PKC-induced inhibition of the rat cotransporter expressed in oocytes is also mediated by the retrieval of the cotransporter from the plasma membrane (I. Forster et al., *in preparation*).

In an effort to identify particular amino acid signals involved in the membrane retrieval of the type II Na/P_i-cotransporter we have mutated two tyrosine residues (Y) located at positions 402 (GYFAM) and 509 (YRWF) of the rat cotransporter (NaPi2). Based on hydrophobicity plots these residues are most probably located in the transmembrane domain 5 (Y402) and in the intracellular loop connecting transmembrane segments 6 and 7 (Y509) [21, 26]. Both sequences are conserved in all the

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type II cotransporters cloned so far, and show a high homology with two tyrosine-based consensus motifs previously reported to be involved in intracellular trafficking: GYXXZ(Z = M/L/V/F/I) for Y402, and YXXO (where O is a bulky residue as F/R/C or S) for Y509. Such motifs seem to be important for the internalization of endocytic receptors [5], for the biosynthetic targeting of lysosomal integral proteins [13, 40], for the sorting from early to late endosomes [33] and for the targeting to the basolateral membrane in polarized epithelial cells [18, 22, 23]. Our data indicate that the tyrosine residues Y402 and Y509 play an important role in the expression/function of the type II Na/P_i-cotransporter expressed in oocytes. Thus, mutation of the Y402 prevents the expression of the cotransporter in the oocyte membrane, while mutation of the Y509 interferes with the transport function but not with the membrane targeting nor membrane retrieval of the cotransporter.

MATERIALS AND METHODS

SITE-DIRECTED MUTAGENESIS

Mutations of the tyrosines Y402 and Y509 to both alanine (Y402A and Y509A) or phenylalanine (Y402F and Y509F), as well as the neighboring F403 and R510 to A (F403A and R510A) were done both in the wild-type (WT) as well as in the FLAGed cotransporter (F-WT, *see below*). For this purpose, plasmids containing either the wild-type or the FLAGed cotransporter were used as template for amplification using a site-directed mutagenesis kit (Strategene). Briefly, 10 ng of plasmid were amplified with 2.5 U of Pfu DNA Polymerase in the presence of 250 nM of complementary sense (S) and antisense (AS) primers both of which contained in the middle of its sequence the desired mutated codon (bold codons, *see below*). PCR amplification was performed as 20 cycles of 90°C (30 sec.), 55°C (1 min) and 68°C (12 min). Then, 10 U of DpnI were added directly to the amplification reaction, and the sample was incubated for 1 hr. at 37°C to digest the parental DNA. Finally, XL1-blue supercompetent cells were transformed with an aliquot of the ligation mixture and plated onto LB-ampicillin-methicillin plates. The following primers were used to introduce the mutations:

```
Y402-A-S:    CACTTGGGTCACAGGCGCCTTTGCCATGGTGGTGG
Y402-A-AS:   CCACCACCATGGCAAAGGCGCCTGTGACCCAAGTG
Y402-F-S:    CACTTGGGTCACAGGCTTCTTTGCCATGGTGGTGG
Y402-F-AS:   CCACCACCATGGCAAAGAAGCCTGTGACCCAAGTG
Y509-A-S:    GCAAACGCACTGCCAAGGCCCGCTGGTTTGCCGTCC
Y509-A-AS:   GGACGGCAAACCAGCGGGCCTTGGCAGTGCGTTTGC
Y509-F-S:    GCAAACGCACTGCCAAGTTCCGCTGGTTTGCCGTCC
Y509-F-AS:   GGACGGCAAACCAGCGGAACTTGGCAGTGCGTTTGC
F403-A-S:    CTTGGGTCACAGGCTACGCCGCCATGGTGGTGGGCG
F403-A-AS:   CGCCCACCACCATGGCGGCGTAGCCTGTGACCCAAG
R510-A-S:    CGCACTGCCAAGTACGCCTGGTTTGCCGTCCTC
R510-A-AS:   GAGGACGGCAAACCAGGCGTACTTGGCAGTGCG
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Due to the lack of NaPi2 antibodies directed against extracellular domains of the protein we constructed a FLAGed cotransporter which contains in the second extracellular loop the FLAG octapeptide DYKDDDDK for which monoclonal antibodies are available. For this purpose the 24 bp fragment encoding the FLAG was introduced between residues C306 and Q307 of the NaPi2 cotransporter, using the same site-directed mutagenesis kit described earlier. Since this system only allows up to 12 bp insertions, the FLAG-encoding sequence was split between the sense (FLAG-S) and antisense (FLAG-AS) pair of primers (bold codons). After PCR amplification and DpnI digestion, the amplified strands were phosphorilated for 1 hr. at 37°C in the presence of Polynucleotide Kinase, and ligated over night at 16°C with T4 DNA Ligase. Finally, XL1-blue supercompetent cells were transformed as described before. The following primers were used for the introduction of the FLAG epitope:

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FLAG-S:
GACGATGACAAGCAACCAGAGACAAAAGAGGCTTCCACTTC(316).
FLAG-AS:
GTCCTTGTAGTCACACCAAATCGGAATGAGACTGTGATTCC(297).
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For all the constructs, the correctness of the single amino acid mutation and/or FLAG sequence was confirmed by sequencing.

In vitro Translation

The in vitro translation of the mutated NaPi2 constructs as well as the wild-type clone was done using a rabbit reticulocyte lysate system (Promega). All the cRNAs were translated in the presence of ^{35}S-Met, both with and without pancreatic microsomes (Promega), following supplier's protocol. As a negative control, a reaction was carried out in the absence of cRNA. The in vitro translated products were separated in SDS-PAGE gels. After staining with Comassie Blue and destaining with 10% acetic acid, the gels were soaked in Amplify Reagent (Amersham), dried under vacuum and exposed on X-OMAT AR film (Kodak).

X. laevis Oocyte Expression and Transport Assay

The procedures for oocyte preparation and cRNA injection have been described already in detail [38]. Briefly, in vitro synthesis and capping of cRNAs were done by incubating each of the NaPi2 constructs, previously linearized by Bgl II digestion, in the presence of 40 U of SP6 RNA Polymerase and Cap Analog (Promega). Oocytes were injected with either 50 nl of water or 50 nl of water containing 10 ng of cRNA. $^{32}P_i$-uptakes were performed 2 days after injection in the presence or absence of 100 mM NaCl as previously reported [38]. P_i-induced currents were recorded 2 days after injection using the two electrode voltage-clamp technique [10]. For this purpose oocytes were voltage clamped at –50 mV, and the P_i-induced current at 1 mM P_i was recorded.

IMMUNOBLOT OF OOCYTES HOMOGENATES

Yolk-free homogenates were prepared 2 days after injection. Pools of 2 oocytes were lysed with 20 μl/oocyte of homogenization buffer (1% Elugent (Calbiochem) in 100 mM NaCl, 20 mM Tris.Cl, pH 7.6), by mild vortexing. To pellet the yolk proteins, samples were centrifuged twice at 16,000 × *g* for 3 min, at 22°C. Aliquotes of supernatants equivalent to 1 oocyte were separated in a 9% SDS-PAGE gel, and proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was processed according to standard procedures [34], using an anti-rabbit polyclonal antibody raised against an N-terminal synthetic peptide of the NaPi2 cotransporter [4]. Immunoreactive proteins were detected with an enhanced chemiluminescent (ECL) system (Amersham).

Northernblot of Oocytes Total RNA

Total RNA was extracted from oocytes 2 days after injection, using the TRIzol reagent (Gibco-BRL). Briefly, pools of 10 oocytes were lysed with 500 μl of TRIzol, extracted once with chloroform and precipitated with isopropanol at room temperature (RT). Pellets were washed with 75% ethanol and resuspended in water. Aliquotes containing 10 μg of total RNA were separated in a formaldehyde-containing 0.8% agarose gel. Transfer of RNAs to a nylon membrane (Biodyne) and processing of the membrane were done following standard methods [34]. As probe, we used a full length NaPi2 cDNA, random-priming labelled with ^{32}P.

Binding of ^{125}I-M2 Antibody

The M2 monoclonal antibody (Kodak) which specifically recognizes the FLAG octapeptide was labelled with ^{125}I as previously reported [9]. Binding of the iodinated antibody to intact oocytes was performed 2 days after cRNA-injection. Pools of 10 oocytes were first preincubated for 1 hr. on ice with 500 ml of Barth's solution (1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$), 2.4 mM $NaHCO_3$, 88 mM NaCl and 10 mM Hepes, pH 7.4) containing 10% heat-inactivated calf serum. After replacement of the blocking media with 100 ml of Barth's containing 12 nM iodinated antibody, the oocytes were incubated again on ice for 1 hr. Finally, they were washed extensively with Barth's and transferred individually to Eppendorf tubes. The amount of bound antibody was determined by counting the ^{125}I content.

Immunocytochemistry

The fixation and sectioning of *X. laevis* oocytes has been previously described [14]. For immunostaining, cryosections of 5 mm were blocked in PBS containing 3% milk powder and 0.3% Triton X-100. Then, they were first incubated overnight either with the anti-FLAG M2 monoclonal antibody or with an anti-NaPi2 polyclonal antisera, followed by incubation with a goat anti-mouse or swine anti-rabbit IgG-conjugated fluorescein isothiocyanate (FITC) secondary antibody (Dakopatts). Finally, sections were coverslipped using DAKO Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo(2.2.2) octane (Sigma) as a fading retardant.

Results

We first studied the Na^+-dependent $^{32}P_i$-uptake of oocytes injected with the different cRNAs. As shown in Fig. 1*A,* the uptake of oocytes expressing the tyrosine mutants Y402A and Y509A was greatly reduced compared with that of the oocytes expressing the wild-type-NaPi2 transporter. For both mutants, the inhibition was slightly smaller when the Y was replaced by F instead of A (Y402F and Y509F). The mutation of the neighboring F403 (F403A) did not affect the activity of the cotransporter, while replacement of the R510 (R510A) also resulted in a reduced uptake. For all the constructs the Na^+-independent $^{32}P_i$-uptake remained unchanged (Fig. 1*B*). The ^{32}P-uptake pattern of the FLAGed constructs was similar to that of their unflaged counterparts (*data not shown*). Consistently with the low $^{32}P_i$-uptake exhibited by the mutants, also a very reduced transport activity was detected in electrophysiological measurements. Thus, currents of less than 5 nA were recorded after superfusion with 1 mM P_i in oocytes expressing the Y509 mutation, while under similar conditions the WT transporter induces a current of 50 nA (*data not shown,* ref. 11).

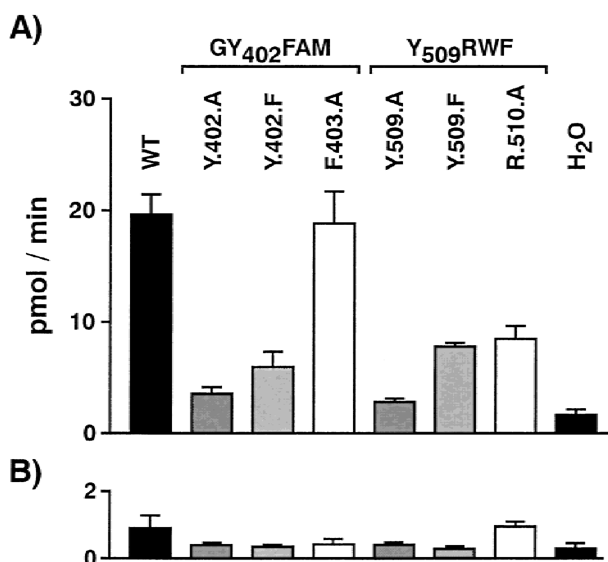

Fig. 1. Na^+ dependent and independent $^{32}P_i$-uptake in oocytes. Oocytes injected with water (most right bars) or with 10 ng of the indicated cRNAs were incubated during 30 min at 25°C, in the presence of $^{32}P_i$ as described in Materials and Methods. The uptake was measured in the presence (*A*) and absence (*B*) of 100 mM NaCl. After extensive washing, single oocytes were transferred to vials and the $^{32}P_i$ measured. The bars represent the mean ± SE of 10 oocytes of a representative experiment.

On Western-blots the wild-type-NaPi2 protein expressed in oocytes appears as a broad band between 70–110 kDa and a lower distinct band at around 64 KDa (Fig. 2). The higher molecular weight smear represents the glycosylated transporters whereas the lower discrete band relates to unglycosylated or core-glycosylated proteins [14]. None of these bands is detected in water-injected oocytes. As shown in Fig. 2, the mutated NaPi2 proteins show a different pattern of expression than the WT-cotransporter. For all the different constructs the lower molecular weight band was detected at similar levels than in the WT. However, while mutation of the Y402 led to a strong reduction in the expression of the glycosylated forms, the level of these proteins remained unchanged in the Y509 mutants. An expression comparable to the WT was also detected in oocytes injected with the F403A mutant, while the level of the glycosylated bands was reduced in the oocytes injected with the R510A cRNA. Thus, although all the cRNAs are translated in oocytes, the mutated proteins behave different in their maturation to fully glycosylated forms.

These differences in protein expression could at

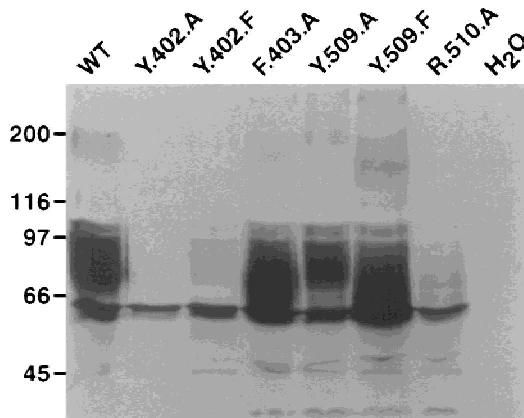

Fig. 2. Western blot of oocytes lysates. Aliquots of oocytes lysates equivalent to 1 oocyte were mixed with SDS-PAGE loading buffer (2% SDS, 20% glycerol 100 mM DTT in 120 mM Tris-HCl, pH 6.8, final concentration) and separated on a 9% SDS-PAGE. After transfer to nitrocellulose the blot was incubated with an antibody against a N-terminal synthetic peptide of the NaPi2 cotransporter. As negative control, lysates from water injected oocytes were used (most right lane). The position of molecular markers is shown on the left.

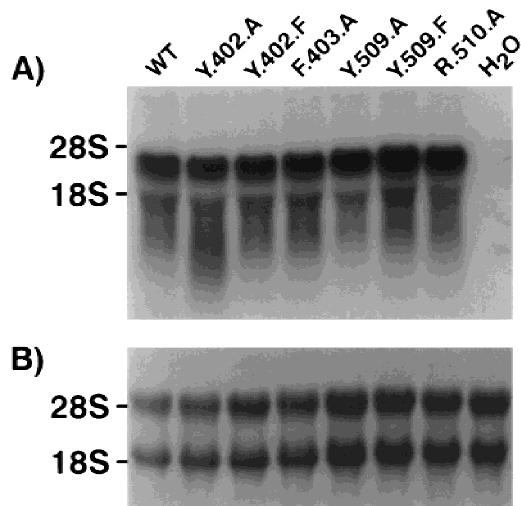

Fig. 3. Northern blot of oocytes total RNA. Aliquots of 10 μg of total RNA were processed as indicated in Material and Methods. Incubation of the blot with a NaPi2 probe reveals the presence of a single band of similar intensity in all the group except in the water injected (*A*). The positions of the 28S and 18S ribosomal RNAs are indicated. As loading control, a picture of the membrane containing the ethidium bromide stained ribosomal RNAs is shown (*B*).

least in part be related to different stabilities of the cRNAs injected into the oocytes. This possibility was ruled out since similar amounts of NaPi2-related cRNAs were detected in all oocytes two days after injection (Fig. 3*A*). As loading control, a picture of the nylon membrane containing the ethidium bromide stained rRNAs is shown in Fig. 3*B*.

We also investigated whether differences in the intrinsic translational efficiencies of the cRNAs were involved in the distinct protein expression. Translation of the different cRNAs in the absence of canine microsomes led to similar amounts of a low molecular weight (unglycosylated) band of about 64 KDa (Fig. 4*A*). The in vitro translation in the presence of pancreatic microsomes led to a similar shift to higher molecular weight forms in all the cRNAs (Fig. 4*B*). This result allows us to exclude differences in the intrinsic translation and glycosylation efficiencies of the cRNAs as being responsible for the different protein expression patterns observed in oocytes (Fig. 2). In addition to the major 64 KDa band, several smaller bands were detected in all the cases, both in the absence and presence of microsomes; these bands could be either products of partially degraded cRNAs, or partially degraded or incompletely translated proteins.

The lack of transport activity detected in the mutants (Fig. 1) could be related either to the lack of surface expression of the mutated cotransporter or to the functional inactivation of the mutant expressed at the surface. We have previously reported that the lack of glycosylation does not *per se* result in an inactive cotransporter, but it rather induces a reduction in the surface expression of the cotransporter [14]. Therefore we next studied the surface expression of the tyrosine mutants by quantifying the binding of the M2 antibody to the FLAGed cotransporters, as well as by immunocytochemical studies. Based on the amount of ^{125}I-M2 antibody bound to intact oocytes, the levels of NaPi2-related protein detected on the plasma membrane of oocytes injected with the F-Y509A mutant were similar to the ones detected in oocytes injected with the F-WT (Fig. 5). On the contrary, no protein was detected in the plasma membrane of oocytes injected with the F-Y402A mutant. In agreement with these data, immunostaining of oocytes' frozen sections with the M2 antibody showed a surface membrane staining in oocytes injected with the F-WT (Fig. 6*B*) and F-Y509A (Fig. 6*D*) cRNAs, but not with the F-Y402A cRNA (Fig. 6*C*) that was indistinguisable from the water injected oocytes (Fig. 6*A*).

Previously we have shown that pharmacological activation of protein kinase C (PKC) in oocytes leads to an inhibition of the NaPi2-related transport function [15, 36]. Recent data indicate that this PKC-induced inhibition is also related to the membrane retrieval of the cotransporter (I. Forster et al., *in preparation*). As reported above, mutation of Y509 yields a protein that although inactive it is expressed at the plasma membrane. Therefore we have studied whether this mutation would interfere with the internalization of the cotransporter after activation of PKC with 5 mM DOG for 1 hr. As shown in Fig. 7 the mutated cotransporter was efficiently removed from the oocyte surface, similar to the WT protein. These data indicate that substitution of the Y509 does not interfere with the PKC-induced internalization

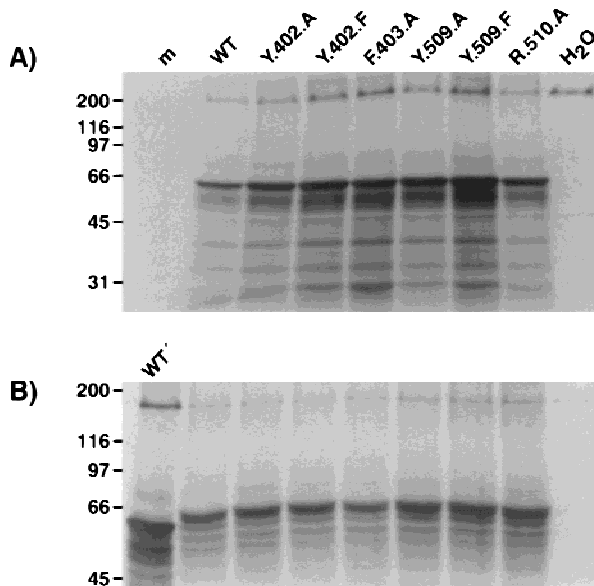

Fig. 4. In vitro translation. Aliquotes of 5 ml of the in vitro translation reactions carried out in the absence of pancreatic microsomes were separated in a 9% SDS-PAGE gel (*A*). A parallel experiment was carried out in the presence of pancreatic microsomes to allow glycosylation, and the products were separated in an 8% gel (*B*). To visualize the shift in the mobility of the protein after glycosylation, an aliquot of the unglycosylated wild type protein was included (*B,* WT′). The position of the molecular markers is shown on the left.

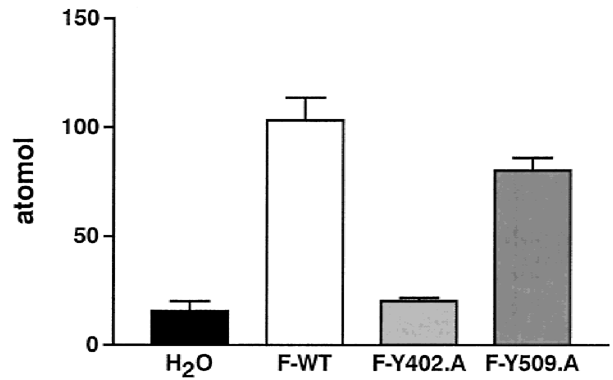

Fig. 5. ^{125}I-M2 binding. Pools of 10 oocytes injected either with water (most left lane) or with 10 ng of the indicated FLAG-cRNA were assayed for binding of the ^{125}I-labeled M2 antibody two days after injection. After extensive washing the oocytes were individually transferred to vials, and ^{125}I was measured. The bars represent the mean ± SE obtained from 10 oocytes of a representative experiment.

of the cotransporter. As the transport activity of the Y509 mutant is rather low it was not possible to support this observation also by functional data.

Discussion

Inhibition of the type II Na/P_i-cotransporter (NaPi2) by PTH involves its internalization from the plasma membrane and ulterior lysosomal targeting and degradation [19, 20, 29, 30]. Inhibition of the NaPi2-mediated P_i-uptake due to the internalization of the cotransporter can be mimicked in oocytes by pharmacological activation of the PKC pathway with phorbol ester, and prevented by staurosporine [15, 36, I. Forster et al., *in preparation*].

Among the best characterized subcellular sorting signals are sequences of four to six amino acids present in the cytoplasmic domain of integral membrane proteins containing a critical tyrosine (Y) residue. They are suggested to be involved in a wide variety of trafficking processes, including internalization of proteins from the plasma membrane and lysosomal targeting [3, 5, 13, 37, 40]. Although the mechanisms by which these signals mediate protein trafficking are not fully understood, it is known that they require the interaction of the critical Y residue with the medium chains of the clathrin-associate AP1, AP2 and AP3 adaptor complexes [7, 16, 28, 32]. Thus in an in vitro binding assay, the rates of AP-1 and AP-2 association with peptides containing the Y-motifs correlated with the rates of internalization [16].

The NaPi2 cotransporter has two tyrosine residues within putative Y-based sorting motifs: $GY_{402}FAM$ and $Y_{509}RWF$. Moreover, a colocalization of the cotransporter with AP2 adaptor proteins can be detected in proximal tubular cells after PTH treatment (M. Traebert et al., *in preparation*). Mutation of any of the two Y residues led to a strong reduction of the transporter activity after injection of the cRNAs in *Xenopus laevis* oocytes. However, the mechanisms by which this inhibition was achieved are different. The decrease in the activity of the Y402 mutants was related to a strong reduction in the amount of plasma membrane-bound NaPi2, associated with an apparent lack of glycosylation of the mutated protein. This effect seems to be specific since mutation of the neighboring F403 did not induce any change in the cotransporter expression and activity. The decrease in the activity of the Y509 mutants, on the other hand, did not involve significant changes either in the total NaPi2 content or in the plasma membrane expression of the transporter. Thus, the primary defect of the Y509 mutations is not protein misrouting but rather functional impairment.

Interestingly, recent studies on different membrane proteins including the aquaporin-2 (AQP2) and the cystic fibrosis membrane conductance regulator (CFTR) have shown that several single point mutations are associated with an impaired routing of the mutant proteins to the plasma membrane of oocytes rather than with a functional impairment [2, 6, 8, 24, 41, 42]. Most of these mutants are retained in the endoplasmic reticulum (ER) and rapidly degraded in a preGolgi nonlysosomal compartment, probably the ER itself [12]. Here we provide some data suggesting that the trafficking of the Y402

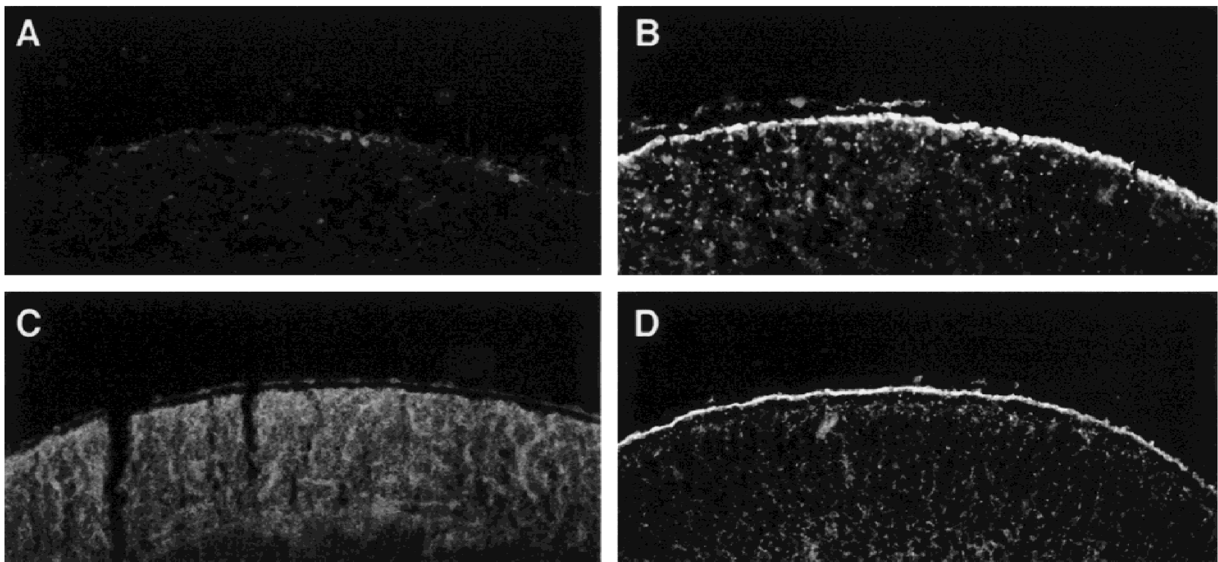

Fig. 6. Immunocytochemical detection of NaPi2 in oocytes. Cryosections of paraformaldehyde-fixed oocytes injected either with water (*A*) or with 10 ng of F-WT (*B*), F-Y402A (*C*) and F-Y509A (*D*), were labeled with the anti-FLAG M2 antibody. Specific immunostaining is detected in F-WT and F-Y509A, but not in F-Y402A and water injected oocytes.

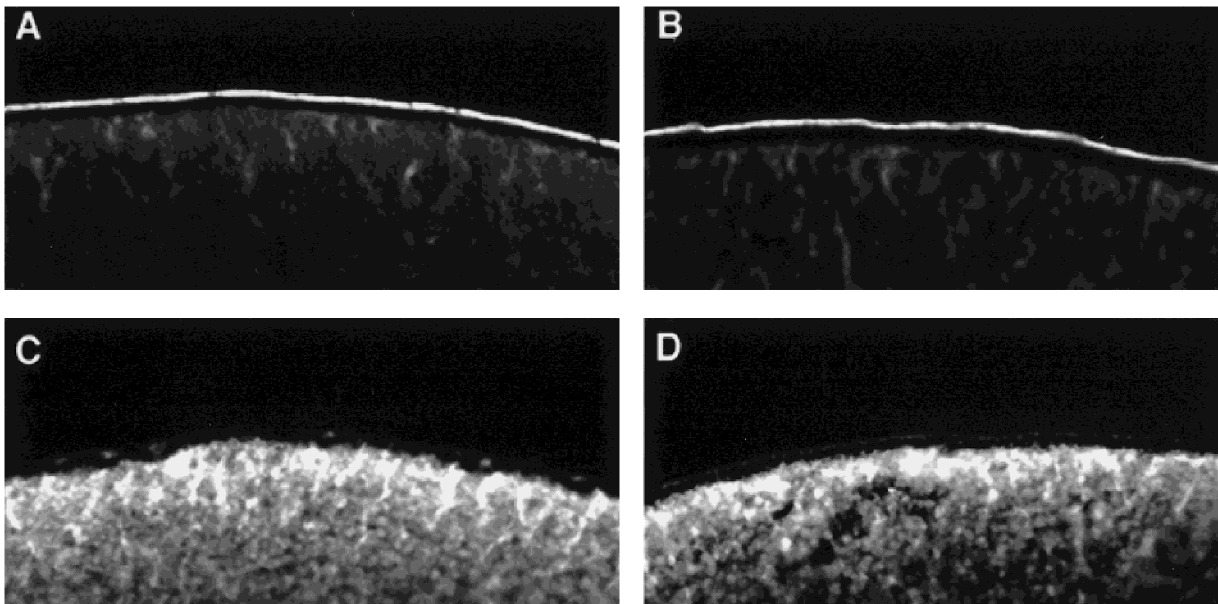

Fig. 7. Immunocytochemical detection of NaPi2 in oocytes treated with DOG. Cryosections of oocytes injected either with the wild type or with the Y509A NaPi2 cRNAs, not treated (*A* and *B*) and treated with DOG (*C* and *D*), were labelled with an antibody against the N-terminus of the cotransporter.

mutants from the ER to the Golgi may also be impaired. Thus, the Y402A and Y402F mutations affected mostly the high molecular weight glycosylated products, while the reduction of the unglycosylated 64 KDa product was negligible, suggesting a defect in the transport of the newly synthesized peptide from the ER to the Golgi. The fact that mutation of the neighboring F403 to A did not have any effect on the cotransporter glycosylation nor on the membrane delivery indicates that the impaired processing of the Y402 mutants is specifically related to the Y residue.

As indicated above, PTH-induced inhibition of the type II Na/P_i-cotransport activity involves both the PKA and PKC pathways [31], and it is mediated by the membrane retrieval of the transporter protein [19, 20, 29, 30].

This inhibition can be mimicked in oocytes by pharmacological activation of the PKC cascade [15, 36]. The inhibition of the cotransporter expressed in oocytes is also mediated by the retrieval of the protein from the plasma membrane (I. Foster et al., *in preparation*). We found that despite its functional impairment the Y509F mutant, which is efficiently targeted to the oocyte membrane, also undergoes internalization after PKC activation with DOG, suggesting that this Y-containing sequence is not involved in the PKC-induced internalization of the type II cotransporter.

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