The role of an intracellular cysteine stretch in the sorting of the type II Na/phosphate cotransporter

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Abstract

The type II Na/phosphate cotransporters (NaPi-II) are critical for the control of plasma phosphate levels in vertebrates. NaPi-Iib mediates phosphate uptake from the small intestine followed by glomerular filtration and selective reabsorption from the renal proximal tubule by NaPi-Ila and NaPi-Ilc. A C-terminal stretch of cysteine residues represents the hallmark of the NaPi-Iib isoforms. This motif is well conserved among NaPi-Ilb type transporters but not found in other membrane proteins.

To investigate the role of this motif we analyzed NaPi-II constructs in transiently and stably transfected MDCK cells. This cell line targets the NaPi-Ilb isoforms from flounder and mouse to the apical membrane whereas the mouse Ila isoform shows no plasma membrane preference. Different parts of mouse NaPi-Ila and NaPi-Ilb C-termini were fused to GFP-tagged flounder NaPi-II. The constructs showed strong staining of the plasma membrane with NaPi-Ilb related constructs sorted predominantly apically, the Ila constructs localized apically and basolaterally with slight intracellular retention. When the cysteine stretch was inserted into the NaPi-Ila C-terminus, the construct was retained in a cytoplasmic compartment. 2-bromopalmitate, a specific palmitoylation inhibitor, released the transporter to apical and basolateral membranes. The drug also leads to a redistribution of the NaPi-Ilb construct to both plasma membrane compartments. Immunoprecipitation of tagged NaPi-II constructs from [³H]-palmitate labeled MDCK cells indicated that the cysteine stretch is palmitoylated. Our results suggest that the modified cysteine motif prevents the constructs from basolateral sorting. Additional sorting
determinants located downstream of the cysteine stretch may release the cargo to the apical compartment.

**Keywords**

Na/phosphate cotransporter, MDCK cells, intracellular sorting, palmitoylation
**Introduction**

The homeostasis of $P_i$ in mammals is maintained by intestinal uptake followed by tightly controlled renal excretion. Both transport steps are mediated by Na-dependent $P_i$ cotransport systems of the protein family denoted NaPi-II [1, 2]. The expression of NaPi-II is regulated by a number of hormonal and metabolic factors known to control $P_i$ homeostasis (parathyroid hormone, growth factors, $P_i$ availability and pH) [3]. In mammals NaPi-IIa is expressed in the renal proximal tubule, NaPi-Ilb is predominantly confined to intestine and lung [4]. Lower vertebrates express NaPi-Ilb related cotransporters in both kidney and intestine [2]. Remarkably, in flounder the transporter is found in the basolateral membrane of the proximal tubular segment PII and apically in collecting tubule cells, consistent with the observation that fish can secrete and reabsorb $P_i$ in the kidney [5].

The type II Na/$P_i$ cotransporters share a common secondary structure. The model proposes eight transmembrane spanning domains and two hydrophobic hinge regions (figure 1) [6, 7]. The two termini are cytoplasmic [8]. The main differences between the isoforms are found in the N-terminus, the large extracellular loop and the C-terminus. The C-terminus of NaPi-Ilb isoforms contains an extended stretch of cysteine residues. It has recently been shown that the C-termini of NaPi-II transporter harbor a number of sorting motifs that are read differentially in various cellular backgrounds [9-11]. Interestingly, in MDCK cells the NaPi-Ilb isoform is strictly confined to the brush border whereas NaPi-IIa is sorted to both the apical and the basolateral membranes [11]. This prompted us to use the MDCK cells to investigate a putative biological role of the cysteine string.
Material and Methods

**Generation of Constructs:** The N-terminal tagging of flounder NaPi-IIb with a FLAG epitope has been reported earlier [8]. The DNA fragment containing the tag and the entire transporter was excised from pSport with KpnI/XbaI and ligated into pcDNA3 linearized with the same restriction enzymes. For transient transfections a transporter with an N-terminal EGFP tag was generated. EGFP was amplified by PCR and ligated in frame into pcDNA3 containing flounder NaPi-IIb. Then, the restriction sites SacII and Nsil were introduced by PCR into the NaPi-II sequences at the junction of the transmembrane domain with the C-terminus and after the stop codon. This strategy allowed the whole C-terminus to be switched to generate chimeric sequences. The constructs and chimeras were generated by overlapping PCR using flanking primers that contained either a SacII or an Nsil site, respectively. The sequence of the primers is given in the supplementary material. All the constructs were confirmed by sequencing.

**Cell culture:** MDCK cells (strain II, ATCC, Rockville, USA) were maintained in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Boehringer Mannheim/Sigma), 2 mM L-Glutamine, 0.5 mM β-mercaptoethanol, 1 mM Na+-pyruvate, 1 x MEM non-essential amino acids, 100 U/ml penicillin, and 100 µg streptomycin (Gibco BRL). The culture conditions were 5% CO₂, 95 % air at 37 °C.

**Transient transfections:** MDCK cells were grown on glass coverslips in 6-well plates to approximately 40% confluence. Cells were transfected with 1 µg endotoxin-free DNA per well with Effectene reagent (Qiagen) according to the manufacturers protocol. Cells
were grown to confluence (48 hours), fixed (3% PFA in PBS) and counterstained with rhodamine conjugated wheat germ agglutinin (Vector Laboratories) to label the apical membrane. 2-bromopalmitate (Sigma) was added for 6 hours or overnight at concentrations of 20 and 50 µM. Cellular localization of the chimeric proteins was evaluated by confocal laser scanning microscopy. A total of at least 30 cells from three different transfection experiments were evaluated for apical, basolateral and intracellular staining. To estimate the distribution of fluorescence each compartment was scored from 0 to 5. The sum of all scores yielded the total fluorescence and the individual compartments were related to this number.

**Stable transfections:** Transfection was performed at a cell density of about 60 % using lipofectamine (Gibco BRL). The cells were serum depleted (30 min) and incubated with the transfection mix for 5 hours at 37 °C. They were allowed to recover in complete medium and grown to confluence. After trypsinisation, the cells were diluted 1:20, grown for 22 hours followed by a change to culture medium containing geneticine sulfate (5 mg/ml). Single clones were recovered after 10 days and tested for Na-dependent Pi uptake [12].

**Metabolic labeling and immunoprecipitation:** The cells were incubated with approximately 50 µCi [³H] palmitic acid (MP Biomedicals)/ 10⁶ cells for 3-4 hours in DMEM. The labeled cells were washed 4 times with ice cold PBS and lysed with 1.3ml of RIPA buffer [150 mM sodium chloride, 1% NP-40, 0.5% deoxy cholate, 0.1% sodium dodecyl sulfate, 50 mM Tris HCl (pH 8.0)] containing a protease inhibitor cocktail (Roche). Anti GFP antiserum (Abcam, ab290) was added according to the supplier’s information
(0.66 µl/35mm dish) and incubated overnight at 4 °C under constant agitation. The lysates were transferred to Eppendorf tubes and spun for 10 minutes at full speed. 1 ml of the supernatant was used for immunoprecipitation with µMACS™ Protein G MicroBeads (Miltenyi Biotech) according to the suppliers protocol. Volumes and radioactivity of all liquid samples were measured (flow through, wash, eluate and column resin). In addition, transfection efficiency was assessed by confocal microscopy in parallel samples.

For the immunoprecipitation from the stably transfected cell line a monoclonal anti flag antibody (M2, Sigma) in RIPA buffer was used. Protein G coupled Sepharose (Sigma) was added to the FLAG- antibody complex and the resin was recovered by centrifugation. Radioactivity was measured in the different liquid samples by scintillation counting.
Results

All NaPi-II cotransporter proteins share a similar predicted membrane topology despite functional differences (figure 1) [13]. Amino acids that are essential for the kinetic fingerprint have been identified and located within the conserved core region of the transporters [4]. The role of the extended cysteine stretch of the NaPi-IIb isoforms has not been investigated thus far. Theoretically, the cysteine motif could have an impact on the functional properties of the transporter as well as its regulation. Functional importance, however, is rather unlikely despite the fact, that the C-terminus containing the cysteine stretch is essential for functional expression of NaPi-II transporters in *Xenopus* oocytes (Werner, unpublished observations). A number of studies characterized the functional properties of chimeric transporters comprising type IIa or IIb transmembrane cores with different C-termini and the kinetics were unaffected by the nature of the C-terminus, IIa or IIb, respectively [14, 15]. Therefore we focused on the NaPi-IIb cysteine string in the context of protein trafficking. The expression of NaPi-II cotransporters in MDCK cells results in differential sorting patterns. Mouse NaPi-IIa is expressed in both apical and basolateral membranes, whereas mouse and flounder NaPi-IIb is sorted apically [11].

In order to investigate the impact of the NaPi-IIb C-terminus on intracellular trafficking we generated a number of chimeras: We fused the C-terminus of both isoforms to core regions of various membrane proteins including mouse NaPi-IIa, mouse NaPi-IIb and flounder NaPi-IIb as well as human CD4 (figure 2). All constructs contained a GFP tag at the N-terminus. This modification has previously been shown not to interfere with apical sorting of various NaPi-II isoforms in OK cells [11]. Schematic representations of the chimeras are shown in the corresponding figures. The EGFP coupled constructs were transiently expressed in MDCK cells and their cellular localization was evaluated by
confocal laser scanning microscopy. The apical membrane of the cells was counterstained with rhodamine conjugated wheat germ agglutinin. The NaPi-IIb isoforms from mouse and flounder were sorted to the apical membrane, mouse IIa was localized to both plasma membrane compartments (figure 2a and [11]. A comparable situation was observed when the flounder core region was combined with the C-termini of mouse IIa (sorted apically and basolaterally) and mouse IIb (sorted apically), respectively. The core from mouse NaPi-IIa was sorted about equally to the apical and the basolateral membrane with both IIa and IIb C-termini. Interestingly, the mouse IIb core combined with the IIa C-terminus was completely trapped intracellularly (figure 2a, bottom). The CD4 constructs failed to reach the membrane altogether regardless of the fused C-termini (figure 2B), indicating that the tail alone does not contain sufficient targeting information to direct the fusion proteins to the plasma membrane.

Based on the finding that both flounder-mouse chimeras reached the plasma membrane but showed different membrane localization we generated a number of constructs containing the flounder core region and chimeric variations of IIa and IIb C-termini (figure 1). The construct containing the cysteine-rich stretch combined with the IIb C-terminal part (abb) showed a strong bias to sorting towards the apical membrane. The other chimeras which either lack the cysteine-rich motif (aab and baa) or lack the IIb C-terminal part (bba) were localised to both apical and basolateral membranes. In a number of cells construct bba also showed considerable intracellular staining (figure 3). Comparison of the results presented in figures 2, 3 and 4 indicate that the most proximal part of the C-terminus (axx and bxx, respectively) does not contribute significantly to the intracellular sorting of the chimeras. On the other hand both distal segments influenced the localization of the chimeric transporters. We observed increased intracellular retention if the cysteine stretch
was not paired with its own C-terminus (bba). The cysteine motif could therefore promote apical sorting by blocking basolateral delivery of the transporter.

To investigate this further we switched the cysteine motif with the corresponding amino acids of the mouse NaPi-IIa C-terminus and vice versa to generate the chimeras aba and bab. The sorting of bab was strongly biased to the apical membrane compartment with some intracellular staining. Interestingly, the cysteine residues failed to induce apical sorting of the construct aba. Most of the exogenous protein was trapped intracellularly and did not reach the membrane (figure 4). Staining of the cells with the lysosomal marker lysotracker red (Molecular Probes) failed to demonstrate co-localisation with the transporter construct (not shown). However, when cells were incubated with 2-bromopalmitate, a specific inhibitor of cysteine palmitoylation, transporters were released from intracellular stores and reached the apical and the basolateral membrane (figure 4). Interestingly, the same procedure also relocated part of the flounder/mouseIIb constructs from the apical to the basolateral compartment. This indicates that the cysteine string of NaPi-IIb may be palmitoylated. The modification seems to prevent basolateral delivery and promote an apical localization of the transporter.

To further investigate the lipid modification of the NaPi-IIb C-terminus we labelled the constructs with [$^3$H] palmitic acid and immunoprecipitated the proteins using two different antibodies. First we generated stably transfected MDCK-II cells with epitope tagged flounder NaPi-Iib. Other constructs proved genetically unstable and transfected cells failed to integrate the transgene. The transfected cell line was extensively tested for transgene expression. As expected, the transporter was confined to the apical membrane and showed reduced transport activity at pH 6.5 (supplementary material 1). We used these cells to label palmitoylated proteins with tritium and immunoprecipitate the
transporter using the anti-flag antibody. In two preliminary experiments 9.3±0.7 % of the incorporated radioactivity was precipitated from transfected cells as compared to 1.2±0.8 % in control samples (without the first antibody). Unfortunately, the transgene proved to be unstable and to disappear after few passages and one freeze/thaw cycle.

In order to corroborate this result we chose an antibody against GFP to precipitate palmitoylated transporter chimeras from transiently transfected cells. We transfected the cells with the constructs bab and aba and included non-transfected cells as controls. Transfections were done in duplicates, one sample was used for labeling and immunoprecipitation, the other one to assess the transfection efficiency by confocal microscopy. Usually there were no expression differences observed between the two chimeric transporter constructs. Cells were labeled with [3H] palmitic acid at 37°C for 3 hours, lysis and GFP antibody binding was performed at 4°C overnight. The GFP-antibody complex was recovered using magnetic microspheres. Every step was precisely monitored in order to detect and compensate for experimental variations. Comparable experiments were performed using constructs containing the entire mouse IIa or IIb C-terminus. In all of the 9 independent experiments constructs containing the Cys-motif (IIb or aba) returned the highest [3H] incorporation and untransfected cells gave the lowest counts. The constructs –IIa or bab as controls for unspecific incorporation always returned counts between the values of untransfected cells and –IIb or aba, respectively. Table 1 shows a summary of all labeling experiments including the transiently and stably transfected cells. Both sets of experiments indicate that the cysteine motif of NaPi-IIb is indeed palmitoylated in MDCK cells.
We propose that the conserved cysteine string in Na\textsubscript{i}/P\textsubscript{i} transporters type IIb has an impact on protein trafficking. Palmitoylation of the motif may prevent the transporter from delivery to the plasma membrane. The modified transporter is then either retained intracellularly or – if the motif is followed by other sorting motifs, possibly positively charged amino acids and a cluster of negatively charged residues, it may be sorted to the apical membrane.
Discussion

The Na/Pi cotransporters type II represent interesting model proteins to study intracellular sorting. In vivo, NaPi-IIb isoforms can be found on the apical and the basolateral membrane of epithelia whereas the NaPi-IIa isoforms are strictly apically localized [1, 2]. It has been suggested that the C-terminus contains important sorting determinants [11]. The C-terminus has previously been shown to locate to the intracellular compartment and not to influence the functional properties of the transporter [7, 8, 14].

The intracellular trafficking of NaPi-IIb from mouse was investigated in MDCK cells as well as in OK and Caco-2 cells by Hernando et al. [11]. In the latter two cell lines a leucine residue (L\textsubscript{691} in mouse NaPi-IIb and L\textsubscript{689} in human) was found to be crucial for apical delivery [16, 17]. Truncations of the C-terminus that included these residues resulted in an intracellular retention of the protein.

The sorting of NaPi-IIa depends crucially on the very C-terminal amino acids -ATRL. This motif binds to the PDZ domains of the adaptor proteins NHE-RF and PDZK1 [18, 19]. NHE-RF knock out mice show impaired renal P\textsubscript{i} reabsorption caused by intracellular NaPi-IIa protein retention [20]. (Targeted disruption of PDZK-1 did not result in missorting of NaPi-IIa [21]). The repertoire of PDZ domain containing adaptor proteins may result in cell specific variations in directing membrane or membrane attached proteins as observed, for example with NaPi-II expressed in various different cell lines [11, 22].

We have focused our study on the stretch of cysteines and positively charged amino acids that are well conserved in all NaPi-IIb isoforms. Interestingly, such accumulation of cysteine residues is very rare in vertebrate proteins and unique in mammalian solute transporters. We have found that the cysteine string indeed influences sorting possibly by
preventing basolateral delivery. This may lead to internal retention or apical localization of the cargo. In a previous study by Hernando et al. it was reported that a deletion of five of the 16 cysteine residues in the cluster had no effect on membrane delivery [16]. The apparent discrepancy between these findings and our results could be a consequence of the different cellular background; i.e. MDCK cells apply different sorting strategies as compared to OK or CaCo-2 cells [11]. Alternatively, the remaining cluster of 11 cysteine residues (\(-RVCCRVCCMVGCKCCRCSKCC-\)) in the \(\Delta 5C\) mutant may be sufficient to guarantee modification and correct sorting. This assumption concurs with a recently reported functional analysis of the cysteine string protein Csp [23]. This protein comprises an extended stretch of cysteine residues but lack a transmembrane anchor [24]. Only selected cysteine residues are essential for correct intracellular targeting and for palmitoylation of Csp. Charged amino acids within or adjacent to the cysteine motif may orient the stretch within the membrane/cytosolic interface and are required for palmitoylation of the protein [23]. The comparison of two Csp isoforms revealed another parallel between the cysteine motifs in NaPi-IIb and Csp's: The palmitoylated Csp\(\alpha\) and the non palmitoylated Csp\(\beta\) show different intracellular sorting in clonal \(\beta\)-cells. Mutation analysis of the cysteine motif to convert the \(\alpha\)-specific motif into the \(\beta\)-specific motif failed to influence trafficking. It was suggested that another C-terminal motif containing an accumulation of glutamic acid residues may be required for palmitoylation of Csp [25]. Interestingly, a similar motif including negatively charged amino acids is well conserved within NaPi-IIb isoforms.

We provide evidence that the cysteine residues of NaPi-IIb constructs are palmitoylated. Our experiments cannot detect transient modifications and do not quantify the extent of acylation. The fact that 2-bromopalmitate causes relocation of both flounder-mouse-IIb
and the aba chimera indicates that both constructs are palmitoylated. The enzymes responsible for palmitoylation, acyl transferases, are membrane associated and expressed in specific compartments [26, 27]. Putative substrates are modified as they pass through the same environment. Differentially expressed acyl transferases represent an intriguing possibility to influence the sorting of NaPi-II isoforms. This phenomenon may contribute to cell specific variations in protein trafficking.

Protein palmitoylation is a frequent posttranslational modification and influences protein trafficking or confers membrane association for soluble proteins [28]. Prominent examples of palmitoylated integral membrane proteins include transporters, 7 transmembrane spanning G-protein-coupled receptors and viral proteins [29]. The modification occurs generally early in the exocytotic pathway [30]. Acylation may promote sequestration of the protein in a specific lipid environment and thus influence protein trafficking and stability.

The palmitoylated cysteine stretch of NaPi-IIb, however, shows exceptional features. In other proteins most palmitoylated cysteine residues are located within 10 residues from the transmembrane spanning domain. The spacer in NaPi-IIb is about 50 amino acids. The chemokine receptor CCR5 shows a situation similar to NaPi-IIb with a spacer of 20 amino acids. If palmitoylation of CCR5 is abrogated by mutation of cysteines to alanines the receptor trafficking and signalling are impaired [31-33]. In addition, palmitoylation sites usually comprise not more than 2 to 3 cysteine residues close to positively charged residues [34]. Such an environment enables the interaction between the protein chain and the negatively charged membrane interface [28]. Whether the unusually high number of cysteine residues in the NaPi-IIb C-terminus and the extended spacer between the membrane spanning core and the cysteine stretch may be related remains to be established.
Our results also confirm that NaPi-II cotransporters contain sorting determinants within the transmembrane region [11]. The NaPi-II core regions from flounder and mouse IIa reached the plasma membrane with both mouse IIa and IIb C-termini. The mouse IIb core depended on its own C-terminus for plasma membrane delivery. Transmembrane domains are known to influence sorting possibly via the association of the protein with distinct lipid compartments such as lipid rafts [35, 36]. Hydrophobic regions could influence the packing of the transmembrane helices and affect the distribution of the protein to different membrane compartments [37]. It can be speculated that the lipid anchor and possibly adjacent motifs are required for the maturation of the NaPi-IIb isoforms. The maturation and intracellular trafficking of CFTR in conjunction with Csp may show interesting parallels to NaPi-IIb processing. Csp expression is inversely correlated with CFTR expression caused by retention of the channel protein in the ER. Csp recruits chaperones (Hsp70) that assist the correct folding of CFTR but also limits the exit of mature protein from the ER [38]. A correlation between Csp palmitoylation and ER retention, however, remains to be established.

We provide insights into the role of the cysteine stretch in NaPi-IIb isoforms. The motif is evolutionary well conserved in NaPi-IIb isoforms but not found in other membrane proteins or isoforms of the functionally related NaPi-IIa and NaPi-IIc transporters. The proposed acylation and the concomitant impact on protein sorting reveal interesting parallels with the family of CSP proteins.
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Bibliography


Fig 1: Structure of the NaPi-II transporters. The topological model shown on the left integrates computer based predictions and experimental findings. The cysteine stretch at the C-terminus is indicated. The right panel shows the amino acid sequence of the C-termini from mouse NaPi-IIa (mIIa), mouse NaPi-IIb (mIIb) and flounder (fIIb). The three parts used to generate the different chimeras shown in figures 3 and 4 are boxed. According to their origin they are abbreviated with a (mouse NaPi-IIa) or b (mouse NaPi-IIb). The gene bank accession numbers are: mouse NaPi-IIa, Q60825; mouse NaPi-IIb, NP_035532; flounder NaPi-IIb, AAB39696.
Fig 2: Transient transfection of mouse NaPi-IIa and IIb C-termini fused to various transmembrane regions. Cells were grown on coverslips and transiently transfected with the EGFP coupled constructs. Cells were fixed 48 hours post transfection and the apical membrane was counterstained with rhodamine-coupled wheat germ agglutinin. (A) The mouse IIa, and IIb C-termini were fused to the core region of flounder NaPi-IIb or mouse NaPi-IIa and -IIb as indicated. (B) Human CD4 constructs were transfected including a truncated C-terminus (·) or fused to mouse NaPi-IIa and -IIb C-termini and assayed as above.
Fig 3: Transient transfection of various mouse NaPi-IIa and IIb C-terminal chimeras fused to the flounder NaPi-IIb transmembrane core region. The constructs are denoted abb, aab, baa and bba according to their origin, mouse NaPi-IIa or mouse NaPi-IIb, respectively. The cells were transfected and stained as indicated; xz sections are shown.
Fig 4: Transient transfection of flounder/mouse NaPi-IIa/IIb chimeras. The cells were transfected with the indicated constructs aba and bab, respectively. Cells transfected with the flounder-mouse IIb construct were included. The cells shown in the two bottom panels were treated with 2-bromopalmitate. Incubation with the drug at 50 µM for 6 hours or 20 µM overnight gave comparable results.
Table 1: Incorporation of $^3$H-palmitate into NaPi-II constructs

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Table 1: Palmitoylation of NaPi-IIB chimeras expressed in MDCK cells. Upper panel: Cells were transiently transfected with the indicated constructs and labelled with $^3$H-palmitate. Immunoprecipitation of GFP or FLAG tagged transporter and purification of the complex was strictly monitored. At all stages radioactivity was related to the original total counts in each sample. The numbers presented are compared to the maximal signal obtained with cells transfected with a C-stretch containing construct (aba or –IIb). Lower panel: The stably transfected cells were incubated overnight with $^3$H-palmitate and the FLAG epitope tagged transporter was immunoprecipitated. In the control samples the primary antibody was omitted.