An apical expression signal of the renal type IIc \( \text{Na}^+ \)-dependent phosphate cotransporter in renal epithelial cells

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Ito M, Sakurai A, Hayashi K, Ohi A, Kangawa N, Nishiyama T, Sugino S, Uehata Y, Kamahara A, Sakata M, Tatsuni S, Kuwahata M, Taketani Y, Segawa H, Miyamoto K. An apical expression signal of the renal type IIc \( \text{Na}^+ \)-dependent phosphate cotransporter in renal epithelial cells. Am J Physiol Renal Physiol 299:F243–F254, 2010. First published April 21, 2010; doi:10.1152/ajprenal.00189.2009.—The type IIc \( \text{Na}^+ \)-dependent phosphate cotransporter (NaPi-IIc) is specifically targeted to, and expressed on, the apical membrane of renal proximal tubular cells and mediates phosphate transport. In the present study, we investigated the signals that determine apical expression of NaPi-IIc with a focus on the role of the N- and the C-terminal tails of mouse NaPi-IIc in renal epithelial cells [opossum kidney (OK) and Madin-Darby canine kidney cells]. Wild-type NaPi-IIc, the cotransporter NaPi-IIa, as well as several IIa-IIc chimeras and deletion mutants, were fused to enhanced green fluorescent protein (EGFP), and their cellular localization was analyzed in polarized renal epithelial cells by confocal microscopy and by cell-surface biotinylation. Fluorescent EGFP-fused NaPi-IIc transporter proteins are correctly expressed in the apical membrane of OK cells. The apical expression of N-terminal deletion mutants (deletion of N-terminal 25, 50, or 69 amino acids) was not affected by truncation. In contrast, C-terminal deletion mutants (deletion of C-terminal 45, 50, or 62 amino acids) did not have correct apical expression. A more detailed mutational analysis indicated that a domain (amino acids WLHSL) in the cytoplasmic C terminus is required for apical expression of NaPi-IIc in renal epithelial cells. We conclude that targeting of NaPi-IIc to the apical cell surface is regulated by a unique amino acid motif in the cytoplasmic C-terminal domain.

Npt2c; SLC34A3; localization; OK cell; hereditary hypophosphatemic rickets with hypercalciuria

INORGANIC PHOSPHATE (Pi) reabsorption in renal proximal tubules is required for \( \text{P}_\text{i} \) homeostasis in the body. \( \text{Na}^+ \)-dependent \( \text{P}_\text{i} \) transporters (NaPi) in the brush-border membrane (BBM) of proximal tubule cells mediate the rate-limiting step in the overall \( \text{P}_\text{i} \)-reabsorption process (26, 28). Type IIa (NaPi-IIa) and type IIc (NaPi-IIc) NaPi cotransporters are expressed in the apical membrane of proximal tubule cells and mediate \( \text{P}_\text{i} \) transport (27, 38). The extent of \( \text{P}_\text{i} \) reabsorption in the proximal tubule is determined largely by the abundance of the NaPi-IIa cotransporter (11, 28, 29).

Several groups have shown that hereditary hypophosphatemic rickets with hypercalciuria (HHRH), a Mendelian disorder of renal \( \text{P}_\text{i} \) reabsorption (39, 42), results from the lack of a functional NaPi-IIc protein, leading to severe renal wasting with hypophosphatemia (5, 16, 19, 22, 25). These data suggest that the NaPi-IIc cotransporter plays an important role in renal \( \text{P}_\text{i} \) reabsorption and may be a key determinant of plasma \( \text{P}_\text{i} \) concentrations in humans. However, it is not clear why a loss of function of the less abundant and energetically less favorable electroneutral NaPi-IIc transporter causes hypophosphatemia/rickets and osteomalacia in humans, whereas mutations in the more abundant electrogenic NaPi-IIa elicits a mild skeletal phenotype (3).

The plasma membrane of epithelial cells is divided into two separate membrane compartments, the apical and the basolateral domains (36, 37). This polarity is maintained by intracellular machinery that directs newly synthesized material into the correct target membrane. Apical protein sorting and retention require specific signals that depend on the recognition of apical signals within these proteins by cellular machinery. Numerous studies have indicated that expression of a PDZ-binding motif, or O- and N-glycan protein modification, is important for protein apical localization (6, 43).

The sequences of NaPi-IIc and NaPi-IIa are \( \sim 54\% \) homologous and the proteins are likely to have a similar topological structure (44). The major differences between the sequences of the NaPi-IIc and NaPi-IIa cotransporters are in the N- and C-terminal regions. The homology between the NaPi-IIc and NaPi-IIa cotransporters offers the possibility of identification of molecular “domains” within the two transporters that may be associated with isoform-specific localization and transport.

NaPi-IIa also shows a high degree of homology to another transporter, NaPi-Iib, at the level of primary structure (\( \sim 70\% \) similarity), but these transporters show different expression patterns in vivo (9, 15). NaPi-IIa is expressed on the apical membrane of renal proximal tubules, and NaPi-Iib is expressed in the intestine and lung epithelium. When transfected into epithelial cells, these molecules are localized in different subcellular locations. Thus NaPi-IIa is apically expressed in renal proximal cells [opossum kidney (OK)] but is mostly intracellular in human intestinal cancer cells (Caco-2), whereas NaPi-Iib is apically expressed in both cell types. Recent studies have indicated that these two highly conserved transporters contain very different determinants for apical expression within their C-terminal tails (14, 21). Of the known amino acid motifs involved in protein localization, PR- and TRL-based motifs are present in NaPi-IIa, and an L-based motif and cysteine stretch are present in NaPi-Iib. In contrast, the C-terminal tail of NaPi-IIc contains a PR motif, but does not contain a PDZ-binding consensus motif S/T-X-V/L or an L-based motif (30, 38).
Thus the signals that determine apical expression of NaPi-IIc in epithelial cells remain unknown. In the present study, we examined the role of N- and C-terminal domains of the NaPi-IIc protein in determination of its apical localization in OK and Madin-Darby canine kidney (MDCK) cells.

MATERIALS AND METHODS

Materials. Oligonucleotide primers were obtained from Sigma Genosys (Hokkaido, Japan). The PrimeSTAR polymerase for PCR and restriction enzymes were purchased from Takara (Kyoto, Japan). All chemicals were purchased from Sigma (St. Louis, MO).

RT-PCR analysis of NaPi expression. Total RNA was prepared using ISOGEN (Wako Pure Chemical, Tokyo, Japan) as described in the manufacturer’s manual. A First-strand synthesis kit (Invitrogen, Carlsbad, CA) was used to generate full-length cDNAs from 1 μg of total RNA. Opossum NaPi-IIc was amplified with the primers 5'-CAAGGACAATGTTGCTGTC-3' (forward) and 5'-ACTGTCGACGTTGAAATTGCACATTGCTC-3' (reverse). Opossum NaPi-IIa (oNaPi-IIa), termed NaPi-4, was amplified with the primers 5'-TATGCGCTCTTCTAGTACC-3' (forward) and 5'-GTGCGTACACAGATGAGAG-3' (reverse). Canine NaPi-IIc was amplified from MDCK cells with the primers 5'-AAAGGACACAGCTGGTGTGCTC-3' (forward) and 5'-ATGTGACACAGGAGTGGAAGCAGTT-3' (reverse). Canine NaPi-IIa was amplified with the primers 5'-GAGGCCACAAAGCCGAG-3' (forward) and 5'-AAAGGACACAGGAGAAGG-3' (reverse). GAPDH was amplified as an internal control with the primers 5'-CTGTCACCACACACTGCTTAGC-3' (forward) and 5'-GCCTGTTCCACACCCTCTTTG-3' (reverse).

Plasmids for construction of the enhanced green fluorescent protein-NaPi-IIc fusion protein. For construction of the enhanced green fluorescent protein (EGFP)-NaPi-IIc fusion protein, wild-type (WT) mouse NaPi-IIc cotransporter (WT NaPi-IIc) cDNA was fused to the C terminus of EGFP by insertion of the full-length cDNA into the pEGFP-C1 vector (Stratagene, La Jolla, CA). For construction of human NaPi-IIa and mouse intestinal EGFP-NaPi-IIb fusion proteins, WT cDNA, including all coding sequences, was fused to the C terminus of EGFP (17). The N-terminal and C-terminal deletion mutants of NaPi-IIc were generated by PCR amplification using primers flanked by the appropriate restriction enzyme sites. The resulting PCR fragments were digested and used to replace the corresponding region in the WT EGFP-NaPi-IIc constructs.

Chimeric EGFP-NaPi-IIa/IIc constructs were constructed by three rounds of PCR. Chimeras constructed included chimeras in which the C-terminal tail of IIc [amino acids (aa) 540–601] was replaced with the WT-IIa C-terminal tail (aa 564 to 639; IIc/IIa C-term), or with the IIa C-terminal tail in which the TRL sequence was deleted (IIc/IIa C-term [del TRL]); a chimera in which the N-terminal domain of IIa (aa 1–101) was replaced by the N-terminal domain of IIc (aa 1–69; IIc N-term IIa); and chimeras in which the C-terminal tail of IIa was replaced with the WT C-terminal of IIc (IIa/IIc C-term or with the C-terminal tail of IIc that had 45 aa (aa 567–601) deleted (IIa/IIc del C45) or that was deleted in the motif WLHSL (IIa/IIc del C50) (Fig. 1). The PCR fragments were digested with restriction enzymes and then substituted into the appropriate construct. Deletion mutants with deletions of five aa in the C-terminal intracellular domain and single aa mutations in the WLHSL domain were generated by PCR amplification and were subcloned into EGFP-NaPi-IIc WT constructs to replace the corresponding WT domain.

Cell culture. OK cells (clone 3B2), derived from opossum kidney, were kindly provided by Dr. J. Biber (Institute of Physiology, University of Zurich, Zurich, Switzerland) and were maintained in DMEM-F12 (Sigma) (17, 35). The cell lines LLC-PK1, derived from porcine kidney epithelia, Caco-2, derived from a human lung-metastasized intestinal carcinoma, and the MDCK cell line, derived from dog renal distal cells, were obtained from the RIKEN Bioresource Center (Tokyo, Japan). Caco-2 cells were cultured in DMEM with high glucose and were supplemented with 1% nonessential aa (Invitrogen). LLC-PK1 cells were cultured in DMEM with low glucose, and MDCK cells were cultured in DMEM with high glucose. All media contained 10% fetal bovine serum (Invitrogen) and penicillin/streptomycin (Invitrogen). All cells were grown at 37°C in a humidified environment (5% CO2-95% air).

Expression of an inducible NaPi-IIc construct in OK cells. The OK cell line has been extensively used for the study of Pi transport, and mimics the function of renal proximal tubular epithelial cells in a variety of ways, including the formation of tight junctions (24). When cultured on permeable supports, OK cell sheets have been shown to have polarized Pi transport (24). However, OK cell sheets cultured on Transwell supports possess a leaky paracellular pathway resembling that of the proximal tubule epithelium in vivo. Due to these properties, it takes 6–7 days for the cells to form tight junctions after plating.

On a second critical point is that the expression of NaPi-IIc is toxic to the cell. Therefore, our attempts to establish an NaPi-IIc stable cell line using conventional methods failed as all of these cells died (data not shown). Thus long-term overexpression of NaPi-IIc may be toxic for both OK and MDCK cells. For these reasons, we established an inducible cell line in which NaPi-IIc is stably expressed only following induction with tetracycline/doxycycline (Dox). In an additional attempt to reduce cell damage in these experiments, we also reduced the size of the expressed NaPi-IIc protein by tagging with FLAG (1 kDa) instead of with EGFP (27 kDa). A commercial “Tet-on” (Clontech, Palo Alto, CA) kit was used to express mouse NaPi-IIc plasmids under the control of a Dox-inducible promoter in OK cells. The OKP tet-on cell line was developed from WT OK cells by transfection with the regulator pTet-on plasmid using LipofectAMINE2000, according to the manufacturer’s instructions. Colonies were grown and isolated in the presence of 100 μg/ml G418. G418-resistant clones were expanded and screened by transient transfection using pTRE2-2EGFP and pTRE-Hyg-luc reporter plasmids. To construct the pTRE/Mouse NaPi-IIc plasmids, WT NaPi-IIc, del C40, del C45, del C50, del C62, del AWPLP, or del WLHSL with a FLAG tag at the N terminus were cloned into a pTRE2-Hyg plasmid. The plasmid was then transfected into the OKP tet-on cell line. Colonies were isolated, expanded in the presence of 100 μg/ml G418 and 200 μg/ml hygromycin, and tested for NaPi-IIc expression in the presence and absence of Dox (1 μg/ml) by Western blotting and immunofluorescence with an anti-FLAG antibody, as described below. A “double-stable” resistant clone (designated as an NaPi-IIc stable cell line), which exhibited low background expression of NaPi-IIc without Dox and a high level of Dox-induced NaPi-IIc expression, was identified and used for further analysis.

Transfection and confocal imaging. Before transfection, the cells were plated in 35-mm glass bottom dishes (Mattek, Ashland, MA). Subconfluent cultures were transfected with 3 μL LipofectAMINE2000 (Invitrogen) and 2 μg plasmids according to the manufacturer’s instruc-
tions. All subsequent experiments were carried out with confluent cells 24 h after transfection. Living cells, expressing green fluorescent NaPi-IIc or mutant cotransporter fusion proteins, were examined and photographed with a Leica (Wetzlar, Germany) TCS-SL confocal laser-scanning microscope equipped with a ×63 oil-immersion objective.

Immunoassayning of OK cells. A peptide identical to the carboxy-terminal 12-aa sequence of oNaPi-IIa (LGVLSQHNLATRL), with the addition of a cysteine at the N terminus, was generated, conjugated to keyhole limpet hemocyanin (KLH), and used to generate rabbit antibodies (Sigma Genosys). Affinity purification of anti-oNaPi-IIa antibodies was carried out by elution of the antibodies from a column to which the antigen had been coupled. A polyclonal anti-opossum NaPi-IIc antibody was generated against a peptide identical to the NaPi-IIc-carboxy-terminal 13-aa sequence (CFENPVVLASQRL) by Medical and Biological Laboratories (Aichi, Japan).

OK cells, grown to subconfluence on glass coverslips or in Transwells (Corning, Corning, NY) were transfected with constructs encoding the NaPi-IIc cotransporter fused with EGFP. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.05% saponin in PBS.

Fig. 2. Expression of wild-type (WT) cotransporter in opossum kidney (OK) cells. A: endogenous NaPi was assayed by RT-PCR in the presence (+) or absence (−) of template. GAPDH was amplified as an internal control. The expression of endogenous NaPi-IIc in OK cells (e; 20 μg/lane) was detected by immunoblotting using an antibody raised against the C-terminal 13 amino acids of opossum NaPi-IIc. The total lysate of cells transiently transfected with WT cotransporter (B) or del C40 cotransporter (C) was analyzed by Western blotting using anti-NaPi-IIc or EGFP antibodies. Samples were separated by 8% SDS-PAGE and transferred to Hybond-P membranes (Amersham Biosciences, Piscataway, NJ). EGFP-NaPi-IIc was analyzed with an anti-EGFP antibody (Clontech). Secondary antibodies, conjugated to horseradish peroxidase, were purchased from Jackson ImmunoResearch (West Grove, PA). Protein bands were visualized with Immobilon Western (Millipore, Billerica, MA). The resulting bands on the filters were analyzed using NIH image software and are graphically presented. The ratio of apically expressed NaPi-IIc to the total surface expressed (apical plus basolateral) NaPi-IIc was then calculated.

Fig. 2A. Western blot analysis of NaPi-IIc. OK cells were grown to subconfluence on glass coverslips or in Transwells. OK cells were transfected with the EGFP-fused N-terminal domain of the WT cotransporters NaPi-IIc or NaPi-IIa and were processed for confocal microscopy equipped with a ×63 oil-immersion objective.

Selective biotinylation of apical and basolateral cell surface proteins was performed as previously described (8). Sulfo-NHS-S-biotin (Pierce, Rockford, IL) was used to label cell surface proteins. Stably transfected OK cells (transfected with WT, del C40, del C45, del 50, del C62, del AWLPL, or del WLHSL) were grown on Transwell filters, before being washed three times with PBS (+) (PBS with 0.1 mM CaCl₂ and 1 mM MgCl₂) and once with biotin buffer (120 mM NaCl, 20 mM NaHCO₃, 1 mM CaCl₂, pH 8.5) at 4°C for 15 min. Sulfo-NHS-biotin (0.5 mg/ml in DMSO) labeling of basolateral or apical aspects of the cells was performed for 20 min at 4°C. Afterward, the cells were washed three times with PBS (+) for 5 min each time at 4°C. The labeled cells were lysed with RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, and 1% SDS). Biotinylated membrane protein was precipitated with avidin beads (Promega, Madison, WI). The NaPi-IIc protein in the total lysate was immunoprecipitated with an excess amount of anti-NaPi-IIc antibody-immobilized beads. Samples were analyzed by Western blotting using anti-NaPi-IIc or EGFP antibodies. Samples were separated by 8% SDS-PAGE and transferred to Hybond-P membranes (Amersham Biosciences, Piscataway, NJ). EGFP-NaPi-IIc was analyzed with an anti-EGFP antibody (Clontech). Secondary antibodies, conjugated to horseradish peroxidase, were purchased from Jackson ImmunoResearch (West Grove, PA). Protein bands were visualized with Immobilon Western (Millipore, Billerica, MA). The resulting bands on the filters were analyzed using NIH image software and are graphically presented. The ratio of apically expressed NaPi-IIc to the total surface expressed (apical plus basolateral) NaPi-IIc was then calculated.

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Fig. 2B. Expression of wild-type (WT) cotransporter in opossum kidney (OK) cells. One representative experiment of four performed is shown. A: endogenous NaPi was assayed by RT-PCR in the presence (+) or absence (−) of template. GAPDH was amplified as an internal control. The expression of endogenous NaPi-IIc in OK cells (e; 20 μg/lane) was detected by immunoblotting using an antibody raised against the C-terminal 13 amino acids of opossum NaPi-IIc. The total lysate of cells transiently transfected with enhanced green fluorescent protein (EGFP)-fused NaPi-IIc in OK-cells (t; 1 μg/lane) was used as a control. B: OK cells were transfected with the EGFP-fused N- or C-terminal domains of NaPi-IIc and were processed for confocal microscopy after reaching confluence. The xz cross section is indicated in the panel below. C: cells were transfected with the EGFP-fused N-terminal domain of the WT cotransporters NaPi-IIc or NaPi-IIa and were processed for confocal microscopy after reaching confluence (left). On the right, costaining of the EGFP-fused cotransporters (green) with actin (red) is shown. The xz cross section is indicated at the bottom. D, top: confluent OK cells were costained with affinity-purified anti-oNaPi-IIa antibody and actin. Staining of oNaPi-IIa is shown in red, actin staining is in green, and merged confocal sections, including the xz cross section, are on the right. Bottom: cells transfected with EGFP-fused WT NaPi-IIc, were stained with affinity-purified anti-oNaPi-IIa antibody. Transfected NaPi-IIc is shown in red, endogenous NaPi-IIa is in green, and merged images are on the right.
Transport measurements. P\textsubscript{i} transport was studied in monolayers of OK and MDCK cell lines grown in Transwells. The cells were exposed to FBS-free assay medium containing \textsuperscript{14}C mannitol in the apical or the basolateral compartments and to the FBS-free assay medium containing 0.25 mM unlabeled mannitol in the opposite compartment (2). P\textsubscript{i} transport was measured using a previously described procedure (18) and was calculated as nanomoles of \textsuperscript{32}P per milligram of protein taken up by the cells over 6 min. The experiments were performed in triplicate. Significant differences (P < 0.05) between the means were determined with paired or unpaired \textit{t}-tests.

RESULTS

Expression of NaPi-II\textsubscript{c} transporter in OK cells. Initially, we analyzed the expression of endogenous and EGFP-fused NaPi-II\textsubscript{c} and II\textsubscript{a} transporter proteins in OK cells, which retain their proximal tubule characteristics. Endogenous NaPi-II\textsubscript{c} and NaPi-II\textsubscript{a} were detected by RT-PCR in these cells (Fig. 2 A). In addition, the endogenous NaPi-II\textsubscript{c} protein was detected by Western blotting using an antibody raised against a peptide corresponding to its C-terminal 13 aa. Endogenous NaPi-II\textsubscript{c} migrated at a molecular weight of 75–100 kDa. In addition, exogenous (transfected) NaPi-II\textsubscript{c} migrated as a broad band of 100–150 kDa (included 27 kDa of EFGP) that may represent NaPi-II\textsubscript{c} modification by glycosylation (Fig. 2 A, e and t). However, this antibody was not suitable for immunohistochemistry as it only recognizes an antigen that is revealed in the denatured protein (data not shown).

When EGFP was fused to the N or C terminus of NaPi-II\textsubscript{c}, the protein was expressed in the apical membrane (Fig. 2 B).

The NaPi-II\textsubscript{c}-EGFP protein did localize at the proper apical location as judged by the appearance of fluorescence patches in the microvilli of OK cells. This localization pattern is similar to that previously reported for NaPi-II\textsubscript{a} (32) and is similar to that for N-terminal EGFP-fused NaPi-II\textsubscript{a} (Fig. 2 C).

We next generated an antibody directed against peptides corresponding to the C terminus of endogenous NaPi-II\textsubscript{a} (oNaPi-II\textsubscript{a}) in OK cells and used this antibody to analyze the colocalization of oNaPi-II\textsubscript{a} with NaPi-II\textsubscript{c} by immunofluorescence analysis. As shown by confocal microscopy, EGFP-fused NaPi-II\textsubscript{c} colocalized with oNaPi-II\textsubscript{a} in apical patches (Fig. 2 D) and no difference in the expression pattern of NaPi-II\textsubscript{a} and NaPi-II\textsubscript{c} in the apical membrane was observed.

The NaPi-II\textsubscript{c} transporter was not observed in intracellular compartments in OK cells.

Comparison of cell-specific apical expression of NaPi-II\textsubscript{c} with that of other NaPi transporters. To analyze whether cellular localization of the transfected transporters might be cell type specific, the expression of EGFP-fused WT NaPi-II\textsubscript{c} was compared with that of NaPi-II\textsubscript{a}, and NaPi-II\textsubscript{b} constructs in LLC-PK\textsubscript{1} cells derived from porcine renal proximal cells, MDCK cells derived from canine renal distal tubules cells, and Caco-2 cells derived from a human intestinal carcinoma cell line (Fig. 3). NaPi-II\textsubscript{c} expression was detected in the apical membrane in LLC-PK\textsubscript{1} and MDCK cells, similar to its expression in OK cells. NaPi-II\textsubscript{a} was detected at the apical region in OK cells, but it had nonpolarized distribution in other cells. NaPi-II\textsubscript{b} was detected in the apical membrane in all of the

Fig. 3. Expression of WT NaPi cotransporters in OK, LLC-PK\textsubscript{1}, Madin-Darby canine kidney (MDCK), and Caco-2 cells. Cells were transfected with the indicated EGFP-fused WT cotransporter (NaPi-II\textsubscript{c}, NaPi-II\textsubscript{a}, or NaPi-II\textsubscript{b}) and processed for confocal microscopy after reaching confluence. The \textit{xz} cross section is indicated at the bottom.
cells, consistent with previous reports (14, 33). In Caco-2 cells, NaPi-IIc and NaPi-IIa were detected in the cytoplasm, but NaPi-IIb was detected in the apical membrane. These results indicate that the cellular localization signals of NaPi-IIc are unique and may be different from those of NaPi-IIa and NaPi-IIb. In addition, the apical localization of NaPi-IIa, -IIb, and -Iic in OK cells compared with all other cell lines shown is very different. Piester et al. (32) reported that visualization of the apical surface of OK cells (clone 3B2) using scanning electron microscopy indicated the expression of microvilli at the apical surface which formed distinct large clusters. The reported diameter of those clusters corresponds well with the diameter of the clusters observed by double immunofluorescent staining for actin and the NaPi-IIa transporter (23, 32). Therefore, the data shown in Figs. 2 and 3 indicate that both endogenous and transfected NaPi-II transporters are predominantly expressed at the apical cell surface (most likely within microvilli).

**Role of N-terminal intracellular domains in apical expression of NaPi-IIc in OK cells.** The domains of integral membrane proteins that are located in the cytoplasm can interact with intracellular molecules. To determine the role of the cytoplasmic domains of NaPi-IIc in its apical expression in OK cells, the cellular localization of the N-terminal intracellular domain of WT, chimeric, or truncated NaPi-IIc proteins, fused to EGFP and transiently expressed in OK cells, was examined by confocal imaging (Fig. 4A). Mutants in which the first 25 (del N25), the first 50 (del N50) aa, or the entire N-terminal domain [the first 69 aa (del N69)] were truncated, were expressed mostly at the apical membrane of OK cells, in a similar manner to WT NaPi-IIc. Furthermore, chimeric proteins, in which the N-terminal domains of IIa and Iic were swapped (IIa-N-term/Iic and Iic N-term/IIa), were also expressed on the apical membrane, similar to WT NaPi-IIa (Fig. 4B). These data suggest that the N-terminal region of NaPi-IIc is not involved in its apical expression in renal proximal tubular cells. In addition, the N-terminal region of NaPi-IIc does not disrupt the apical expression of the NaPi-IIa transporter.

**Role of C-terminal intracellular domains in apical expression of NaPi-IIc in OK cells.** We next examined the role of the C-terminal intracellular domains in NaPi-IIc cellular localization. Partial deletion mutants of the C-terminal cytoplasmic tail were created by deletion of from 5 to 62 aa (del C5 to del C62) (Fig. 5A). The del C5, del C10, del C20, and del C40 mutants were expressed mostly at the apical membrane and were detected in large patches in OK cells, similar to WT NaPi-IIc (Fig. 5B). In contrast, the del C45, del C50, del C55, and del C62 mutants were expressed in a nonpolar manner, being

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**Fig. 4. Expression of N-terminally truncated NaPi-IIc in OK cells.** A: N-terminal amino acid sequence of WT NaPi-IIc, from residue 1 to 72, is shown. Amino acids forming part of the putative first transmembrane domain are underlined. The following N-terminal truncation mutants were generated from EGFP-fused WT NaPi-IIc by a PCR-based method: del N25, deletion of the first 25 amino acids; del N50, deletion of the first 50 amino acids, and del N69, deletion of the first 69 amino acids, which results in truncation of the entire N-terminal cytoplasmic tail. Numbers indicate amino acid positions. These mutants were transfected into OK cells, which were processed for confocal microscopy after reaching confluence. B: chimeric transporters, in which the N-terminal intracellular domain of NaPi-IIc and NaPi-IIa were swapped, were expressed in OK cells. The xz cross section is indicated at the bottom.
detected on both the apical and the basolateral surfaces. Similar observations were made in OK cells on a Transwell filter (Fig. 5C). Following the selective biotinylation of apical and basolateral cell surface proteins of stably transfected OK cells, the apical membrane expression of the del C45, del C50, and del C62 mutant proteins was clearly decreased compared with that of the WT and the del C40 proteins (% of WT: del C45; 27.0/3.8%, del C50; 6.6/3.6%, del C62; 6.0/4.5%) (Fig. 5D). In contrast, increasing deletion of C-terminal aa enhanced the basolateral membrane localization of the mutants. The NaPi cotransporting activity was measured in the Transwell filter assay, as shown in Fig. 5E. The Pi uptake activity of cells expressing del C45 or del C62 was significantly decreased to 73.1 or 54.8%, respectively, compared with that in cells expressing WT and del C40 constructs. In addition, it appears to be the reduction in the apical expression levels of the del C45 and del C62 mutant proteins that is the cause of the low level of Pi uptake activity in these cells (Fig. 5, D and E). These results indicated that signals for NaPi-IIc apical expression exist in the C-terminal domain within the five-aa sequence (WLHSL) that is present in del C40 but deleted in del C45.
Expression of NaPi in MDCK cells derived from distal tubules. To further analyze NaPi-IIc expression, we used MDCK cells, a well-characterized cell line derived from canine kidney distal tubule cells (Fig. 6). In MDCK cells, the cellular expression of transfected NaPi-IIc is different from that of transfected NaPi-IIa (Fig. 3). In addition, although endogenous NaPi-IIc mRNA expression is only weakly detected in mouse or rat kidney distal tubules (38), endogenous NaPi-IIc mRNA can be detected by RT-PCR in MDCK cells (Fig. 6A). In contrast, mRNA for endogenous NaPi-IIa was not detected in MDCK cells. Transporters that were transfected into MDCK cells, which are derived from renal distal cells and lack endogenous NaPi-IIa, had a nonpolarized distribution (Fig. 3) (14). In contrast to NaPi-IIa, NaPi-IIc is detected only apically in both cell types (Figs. 3 and 6B). Similar to the results in OK cells, deletion of the N-terminal intracellular domain (del N69) did not affect the apical expression of NaPi-IIc. However, deletion of the entire C-terminal intracellular domain (del C62)
of NaPi-IIc in MDCK cells led to a nonpolarized expression of NaPi-IIc (Fig. 6C). When deletion mutants of the C-terminal intracellular domain (from del C40 to del C62) were expressed in MDCK cells, apical expression of NaPi-IIc was disrupted in mutants that had >45 aa deleted, similar to the situation observed in OK cells. Since the expression of NaPi-IIc is mostly apical in MDCK cells, it is clear that the cellular expression signals recognized in these cells must be present on the C-terminal tail of NaPi-IIc.

An NaPi-IIa expression signal is not important for apical expression of NaPi-IIc. We next determined whether motifs that have been previously proposed to modulate NaPi-IIa cellular localization are involved in NaPi-IIc localization in OK and MDCK cells. Karim-Jimenez et al. (20) have previously demonstrated that a PR motif in the C-terminal region of NaPi-IIa is essential for its apical localization in OK cells (20). In the C-terminal tail of NaPi-IIa, both internal PR residues and terminal TRL residues may function as apical sorting/targeting signals or as an anchoring motif for apical expression in OK cells (14). We therefore tested the effect of mutation of the PL motif of NaPi-IIc, which corresponds to the PR and PL motifs of NaPi-IIa and IIb respectively (Fig. 7A), on the cellular localization of EGFP-fused NaPi-IIc. Therefore, the C-terminal region of EGFP-NaPi-IIc was mutated either by mutation of S55P of the PL motif to A (S55PL to AL) or by mutation of the L residues surrounding the PL motif to A (S54PL to APA). These mutants were transfected into OK and MDCK cells, and the cellular localization of the proteins was investigated by EGFP fluorescence and confocal microscopy (Fig. 7B). However, all of these mutants retained an apical expression pattern in both OK and MDCK cells, suggesting that the PL motif does not play a role in apical expression of NaPi-IIc.

Determination of apical expression signals in NaPi-IIc C-terminal domain. To elucidate the apical expression signals in the NaPi-IIc C-terminal region, we analyzed the effect of five aa-deletion mutants of the C-terminal domain on EGF-NaPi-IIc cellular localization. The deletions analyzed and their corresponding amino acid sequence were: del C40–44 (del LHS), or del C42–45 (del HSL) were deleted. However, all mutants were correctly expressed at the apical membrane-associated transporter. We further investigated whether single alanine substitutions affected transporter function. However, there was no significant difference in transporter function among these proteins.

Furthermore, we analyzed the localization of deletion mutants that had three aa deletions, in which C40–43 (del WLH), C41–44 (del LHS), or C42–45 (del HSL) were deleted. However, clear apical expression of these mutants could be ob-

Fig. 8. Determination of apical expression signals in the NaPi-IIc C-terminal domain. A: position of the amino acids deleted from the NaPi-IIc C-terminal domain is indicated by asterisks. Deletion of these amino acids generated the EGF-NaPi-IIc deletion mutants del WLHSL, del AWLPL, and del CLQSW, corresponding to del C40–44, del C45–49, and del C50–54, respectively. B: OK and MDCK cells were transfected with the indicated mutated constructs and processed for confocal microscopy after reaching confluence. The xz cross section is indicated at the bottom. C: selective biotinylation of apical and basolateral cell surface proteins. The expression of the del AWLPL or del WLHSL proteins in stable cell lines was analyzed by Western blotting of cell surface (m) or total cell lysate proteins to determine total NaPi-IIc expression (% of total surface) to the total surface expressed (apical plus basolateral) NaPi-IIc was then calculated. The white bars indicate apical membrane-associated transporter, and the gray bars represent basolateral membrane-associated transporter. D: apical expression of alanine mutants of individual amino acids in the WLHSL motif of EGF-NaPi-IIc was similarly tested in OK cells. The xz cross sections are shown (left). The constructs were transiently transfected into OK cells, and the cells were analyzed using a Puptake assay (right).
served in either OK or MDCK cells (data not shown). These results suggest that mutation/deletion of these residues is not sufficient to disrupt apical targeting. The combined data suggest that a single, specific amino acid in the WLHSL sequence is sufficient for determination of apical expression of NaPi-IIc and that at least a five-aa deletion in the C terminus of NaPi-IIc may be required as a signal for apical expression in renal epithelial cells.

Effect of NaPi-IIc C-terminal domain on expression of NaPi-IIa. To confirm the role of the C-terminal intracellular domain of NaPi-IIc in apical expression, we assayed the localization of chimeric mutants in which various regions of NaPi-IIa and NaPi-IIc were swapped (Fig. 9). A previous study had demonstrated that the three aa TRL in the C-terminal tail of NaPi-IIa, which constitute a PDZ-binding domain, are important for its apical expression and that a mutant in which the TRL sequence was deleted showed a nonpolarized localization in OK cells (13). However, even in the absence of the TRL motif, the PR motif in the C-terminal domain of NaPi-IIa can still function as an apical sorting signal as previously described (13). We therefore examined the cellular localization of an IIc/IIa C-term chimera in which the C-terminal domain of NaPi-IIc was replaced with the WT, or a C-terminal TRL-sequence deleted domain of NaPi-IIc was substituted for the corresponding domain in NaPi-IIa. B: MDCK cells were transfected with the chimeras IIc N-term/IIa, IIa/IIc C-term, IIa/IIc del C45, or IIa/IIc del WLHSL in which the N-terminal, C-terminal, delC45 C-terminal, or del WLHSL C-terminal domain of IIc were substituted for the corresponding domain of IIa, respectively. Uptake of sodium-dependent Pi, was measured over 6 min in MDCK cells in Transwells that were transiently transfected with the indicated NaPi-IIc constructs.
tested in MDCK cells (Fig. 9B). Transfected NaPi-IIa had a nonpolarized distribution, whereas NaPi-IIc is correctly localized in the apical membrane in MDCK cells. We reasoned that, if the C-terminal region of NaPi-IIc contains important determinants for polarized targeting, then a NaPi-IIa chimera, in which the C-terminal region of NaPi-IIa was replaced with the C-terminal region of NaPi-IIc, should be correctly localized to the apical membrane in MDCK cells.

The chimera IIc N-term/IIa, in which the N-terminal intracellular domain of NaPi-IIa was replaced with the N-terminal tail of NaPi-IIc, was detected on both the apical and basolateral membranes (Fig. 9B). The IIa/IIc C-terminal chimera, in which the C-terminal domain of NaPi-IIc was replaced with the C-terminal domain of NaPi-IIa, was mostly expressed at the apical membrane and was correctly localized at the apical membrane. Importantly, the apical expression of the IIA/IIc C-terminal chimera was markedly disturbed when the C-terminal domain of NaPi-IIa was replaced with a WLHSL deletion mutant or a del C45 mutant C-terminal region of NaPi-IIc (Fig. 9B). In addition, we further investigated whether the localization of each C-terminal chimeric mutant was disturbed in MDCK cells by measurement of the Pi transport activity in the apical membrane using a Transwell Pi transport assay. As shown in Fig. 9B (bottom), the activities of IIA/IIC C-terminal IIA/IIC del C45 and IIA/IIC del WLHSL mutants were significantly decreased to 42–58% compared with those of the WT and the IIA/IIC C-terminal mutants. Taken together, these data also suggest that the C-terminal domain (WLHSL) of NaPi-IIc contains signals for apical expression in MDCK cells.

The importance of the five aa region of the C terminus of NaPi-IIc, which is implicated in the regulation of NaPi-IIc apical localization, is further emphasized by the fact that this region is highly conserved in rat, human, chimpanzee, Macaca muratta, and opossum, as shown in Fig. 10A. We also outline, in Fig. 10B, the location of the WLHSL apical expression signal in NaPi-IIc in relation to the apical expression signals in the C-terminal region of the other members of the NaPi-II family (IIa and IIb).

**DISCUSSION**

In this study, we examined the N- and C-terminal domains of the NaPi-IIc protein and their potential roles in NaPi-IIc apical localization in OK and MDCK cells. Based on the observed cellular localization of EGFP-tagged chimeric proteins, in which the N-terminal sequences of NaPi-IIc and NaPi-IIa were swapped, we conclude that the N terminus of NaPi-IIc does not have an apical membrane targeting function in OK cells.

With regard to the role of the C-terminal domain, both confocal imaging and surface biotinylation assays consistently showed that NaPi-IIc deletion mutants, with deletions within the last 40 aa of the C-terminal tail, are all localized in the apical membrane while mutants with a deletion of 45 aa or more have a nonpolarized distribution. In MDCK cells, NaPi-IIa was detected at a high level in both the apical and basolateral membranes, whereas NaPi-IIc was exclusively apical. Interestingly, when the C-terminal region of NaPi-IIa was replaced with that of NaPi-IIc, the localization of this chimera was exclusively apical in MDCK cells. This result indicates that the C-terminal region of NaPi-IIc plays a role in NaPi apical expression. In addition, the five aa residues, WLHSL, aa 557–561, in the C terminus of NaPi-IIc, are important for apical membrane expression of NaPi-IIc in OK and MDCK cells. A database search did not reveal any other known membrane proteins that contain the motif WLHSL. However, other epithelial sorting motifs that are shared by different proteins have proved difficult to identify solely on the basis of aa sequence. A sequence similar to WLHSL (WMHSL) was observed in the C-terminal tails of NaPi-IIa and NaPi-IIb.

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However, we have not addressed whether these motifs are involved in the apical expression of NaPi-IIa and NaPi-IIb transporters. It is possible that the WMHSL sequence of NaPi-IIa and NaPi-IIb may not play a dominant role in transporter apical expression in renal and intestinal epithelial cells. The mutant del C62, in which the entire C-terminal domain was deleted, tended to show decreased apical localization and Pi uptake compared with the del C45 mutant, although the differences between these two mutants were not significantly significant. It is still unclear whether a second apical/targeting signal exists within NaPi-IIc. So far, such a second signal has not been detected in this region of the molecule.

A second possibility is that an as yet unidentified vesicle adaptor protein may play a role in the apical targeting of NaPi-IIc. It is known that the trafficking and targeting of the

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**Figure 10.** Evolutionary conservation of the WLHSL motif and a model of the proposed motifs that modulate NaPi-IIc cellular expression. A: alignment of NaPi-IIc C-terminal intracellular domain sequences of mouse (NP_543130), rat (NP_6475544), human (NP_543153), chimpanzee (XP_52867), Macaca muratta (XP_00108762), and opossum (XP_001374838). The shaded areas indicate amino acids conserved across all species. The asterisks indicate hydrophobic amino acids in the mouse sequence. B: predicted expression motifs of NaPi are shown. NaPi-IIa expression is dependent on TRL and PR motifs at the C terminus. NaPi-IIb has an L motif in its C terminus. NaPi-IIc expression may be regulated by a large domain in the C terminus that includes the WLHSL motif.
LDL receptor (LDLR) in MDCK vs. LLC-PK₁ cells is due to the absence of the u1B adaptor subunit of AP1B in LLC-PK₁ cells (10, 12). While this adaptor is involved in basolateral targeting, it is possible that similar mechanisms also regulate the targeting of apical proteins in different cell types. Adaptor proteins have also been implicated in the reversed polarity of apical membrane proteins in the retinal pigment epithelium compared with MDCK cells (36, 45).

It has been shown that the interaction of NaPi-IIa with the PDZ domains of the Na/H exchanger-regulatory factor (NHERF) family occurs primarily via a class I PDZ-binding motif (TRL) located at the C terminus of NaPi-IIa and that this TRL motif is both very important, and required, for either apical sorting, or the mediation of selective retention, of NaPi-IIa in OK cells (7, 13). The interaction of the PDZ-binding motif on the C terminus of NaPi-IIa with members of the NHERF family may also contribute to the stabilization of NaPi-IIa at the apical membrane of renal epithelial cells. Thus the localization of many proteins with a PDZ domain-binding motif, such as the CFTR and the epithelial GABA transporter BGT-1, also appears to involve a combination of targeting and retention mechanisms (1, 4, 31, 40). Combined with the fact that the role of the C-terminal region of NaPi-IIC in membrane localization is also not clear based on this study, these data suggest that the exact role of the PDZ domain in protein localization requires further investigation. A recent study demonstrated that the C-terminal domain of NaPi-IIc, like that of NaPi-IIa mentioned above, can also interact intracellularly with NHERF-3 in OK cells (44). However, although NaPi-IIc has been shown to interact with a few PDZ domain proteins such as NHERF-1/3, this interaction is not essential for apical expression. We further examined the interaction of NaPi-IIc with NHERF-1/3 in the mammalian two-hybrid system, but the interaction was very weak (data not shown). In addition, Reining et al. (34) have recently reported that a protein termed GABARAP associates with the C-terminal PDZ-binding motif of a truncated NaPi-IIa in a membrane yeast-two-hybrid system that used to screen a mouse kidney library. The consensus sequence for binding of GABALAP is centered on a highly conserved tryptophan (W) residue (41). We have not addressed whether GABALAP interacts with the WLHSL of NaPi-IIc.

An NaPi-IIa mutant in which the TRL motif is deleted can still be apically expressed in OK cells (13, 20). Murer and coworkers (20) showed that, in this mutant, the PR motif in the NaPi-IIa C-terminus appears to be important for apical expression of the mutant in OK cells. Therefore, depending on the context, both the TRL and PR motifs in the NaPi-IIa C-terminal region contain important information for apical expression of the NaPi-IIa transporter in OK cells. In contrast to NaPi-IIa, NaPi-IIc has no TRL-PDZ-binding motif in the C-terminal region and the data suggest that the NaPi-IIa-TRL del mutant and NaPi-IIc have similar properties with regard to apical expression. In the present study, we addressed whether the PR-related PL motif in the C-terminal region of NaPi-IIc is essential for apical expression in OK cells. However, this does not seem to be the case for a number of reasons. First, the PR-related PL motif of the mouse NaPi-IIc is not conserved across all species. Second, the deletion and point mutation studies showed that the WLHSL motif of the NaPi-IIc C-terminal region might play a role that corresponds to that of the PR motif of NaPi-IIa. Indeed, the five-aa motif WLHSL of NaPi-IIc is evolutionarily conserved across all species. These data suggest that the C-terminal tail of the NaPi-II family contains several determinants important for polarized targeting.

A contribution of two different motifs to transporter cellular targeting has also been reported for NaPi-Ib, in which both the L- and cysteine stretch motifs have been implicated in protein targeting (14, 21). We have shown that NaPi-Ic is localized intracellularly only in Caco-2 cells (Fig. 3), clearly indicating that the machinery involved in NaPi-Ic sorting in Caco-2 cells is different from that in other cell types. In a previous study, Karim-Jime nez et al. (21) demonstrated that a conserved L691 (mouse NaPi-Ib, mIIb)/689 (human NaPi-Ib, hIIb) chimera in the C-terminal region of NaPi-Ib is essential for the apical membrane localization of the transporters in Caco-2 cells. Truncation at the level of the conserved L691/689 prevented the apical membrane expression of both mIIb and hIIb, respectively, and the mutated proteins were located in intracellular compartments (endosomal and lysosomal structures). The ER and Golgi compartments are involved in “quality control,” and misfolded proteins are unable to leave these compartments. The above results suggest that removal of L691/689 yields a cotransporter (NaPi-Ib) that is conformationally competent for leaving the ER and Golgi. This leucine residue could therefore be part of a targeting motif and/or a motif required for membrane stabilization of the NaPi-Ib transporter protein. In addition, the cysteine stretch in the C-terminal region of NaPi-Ib has been reported as being an important motif for apical expression in Caco-2 cells. However, the L- and cysteine stretch motif are not present in the N- or C-terminal region of NaPi-Ic, suggesting that NaPi-Ic is targeted via other signals.

In summary, we examined the N- and C-terminal domains of the NaPi-IIc protein and their potential roles in NaPi-IIc-apical localization in OK and MDCK cells. We conclude that targeting of NaPi-Ic to the apical cell surface is regulated by a unique aa motif in the cytoplasmic C-terminal domain. Further studies are required to identify binding proteins of the WLHSL region and their potential role in protein targeting.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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