Structural and Functional Properties of Two Human FXYD3 (Mat-8) Isoforms*

Received for publication, May 31, 2006, and in revised form, October 26, 2006 Published, JBC Papers in Press, October 31, 2006, DOI 10.1074/jbc.M605221200

Stéphanie Bibert, Sophie Roy, Danièle Schaer, Emanuela Felley-Bosco, and Kathi Geering

From the Department of Pharmacology and Toxicology, University of Lausanne, rue du Bugnon 27, 1005 Lausanne, Switzerland

Six of 7 FXYD proteins have been shown to be tissue-specific modulators of Na,K-ATPase. In this study, we have identified two splice variants of human FXYD3, or Mat-8, in CaCo-2 cells. Short human FXYD3 has 72% sequence identity with mouse FXYD3, whereas long human FXYD3 is identical to short human FXYD3 but has a 26-amino acid insertion after the transmembrane domain. Short and long human FXYD3 RNAs and proteins are differentially expressed during differentiation of CaCo-2 cells. Long human FXYD3 is mainly expressed in non-differentiated cells and short human FXYD3 in differentiated cells, and both FXYD3 variants can be co-immunoprecipitated with a Na,K-ATPase antibody. In contrast to mouse FXYD3, which has two transmembrane domains for lack of cleavage of the signal peptide, human FXYD3 has a cleavable signal peptide and adopts a type I topology. After co-expression in Xenopus oocytes, both human FXYD3 variants associate stably only with Na,K-ATPase isozymes but not with H,K-ATPase or Ca-ATPase. Similar to mouse FXYD3, short human FXYD3 decreases the apparent K+ and Na+ affinity of Na,K-ATPase over a large range of membrane potentials. On the other hand, long human FXYD3 decreases the apparent K+ affinity only at slightly negative and positive membrane potentials and increases the apparent Na+ affinity of Na,K-ATPase. Finally, both short and long human FXYD3 induce a hyperpolarization activated current, similar to that induced by mouse FXYD3. Thus, we have characterized two human FXYD3 isoforms that are differentially expressed in differentiated and non-differentiated cells and show different functional properties.

Na,K-ATPase is an integral membrane protein that belongs to the P-type ATPase family and is responsible for active cation transport. During each reaction cycle, Na,K-ATPase transports 3 Na+ ions out and 2 K+ ions into the cell by using the energy of the hydrolysis of ATP. Na,K-ATPase maintains the electrochemical gradients of Na+ and K+ ions that are necessary to maintain the cell volume and the membrane potential, affect the activity of secondary transport systems, and permit neuronal excitability and muscle contraction. The minimal functional unit of Na,K-ATPase is a heterodimer composed of two subunits, α and β. The α subunit contains 10 transmembrane segments and an ATP-hydrolyzing domain and is responsible for ion transport. The β subunit that is a type II, glycosylated protein, plays a chaperone role by allowing the structural and functional maturation of Na,K-ATPase (1). Four α and 3 β isoforms have been identified. Na,K-ATPase isozymes are expressed in a tissue- and development-specific way, and show different transport properties indicating that each isoform could play specific roles adapted to specific physiological processes (2, 3).

Numerous mechanisms are known to modulate the expression and/or activity of Na,K-ATPase. The Na,K-ATPase activity is tightly coupled to the intracellular concentration of Na+. The number of active Na,K-ATPase units at the plasma membrane is controlled by phosphorylation of the catalytic α subunit by protein kinases, stimulated by peptide hormones and neurotransmitters (4). Finally, mineralocorticoid and thyroid hormones mediate long-term regulation of Na,K-ATPase by affecting α and β gene transcription and leading to an increased number of Na,K-ATPase units at the cell surface (5). Recently, a family of small proteins called FXYD proteins was defined (6). In mammals, the FXYD family contains seven members including phospholemman (FXYD1) (7), the Na,K-ATPase γ subunit (FXYD2) (8), mammary tumor marker 8 (Mat-8) (FXYD3) (9), corticosteroid hormone-induced factor, CHIF (FXYD4) (10), related to ion channel, Ric (FXYD5) (11), phosphohippolin (FXYD6) (12), and FXYD7 (13). Family members have a molecular mass between 7.5 and 24 kDa. They share a common signature of 6 amino acid residues, comprising the FXYD motif, from which the family takes its name, 2 glycine residues and a serine residue. Most characterized FXYD proteins are type I membrane proteins with a single transmembrane domain, adopted in some, but not all cases, by cleavage of a N-terminal signal peptide. FXYD proteins are widely distributed in mammalian tissues that perform fluid and solute transport or that are electrically excitable. However, the expression of FXYD proteins is tissue-specific and at least one of their common functions is their association with Na,K-ATPase and the modulation of its transport properties. To date, all FXYD proteins, except FXYD6, have been shown to associate with and to alter the activity of the Na,K-ATPase. Actually, it seems that each of these proteins modifies the transport properties of Na,K-ATPase in a distinct way adapted to the physiological needs of the tissue in which they are expressed (14). Due to the important role of Na,K-ATPase, variations of FXYD protein expression may be the cause of pathophysiological states as in human primary hypomagnesaemia, which is associated with a mutation in a conserved glycine residue in the transmembrane domain of FXYD2 (15).
The FXYD3 protein, or Mat-8, is expressed at high levels in uterus, stomach, and colon, and at low levels in breast, ovary, lung, small intestine, and thymus (9). Mouse FXYD3 was identified as a protein inducing hyperpolarization activated chloride conductance after expression in Xenopus oocytes (9). This could suggest that FXYD3 may be involved in the regulation of transepithelial ion transport in tissues containing absorptive or secretory epithelia. Recently, we have shown that mouse FXYD3 shares features in common with other members of the FXYD family, namely its capacity to interact with Na,K-ATPase and to modify its transport properties (16). On the other hand, mouse FXYD3 exhibits structural and functional features that differ from the other FXYD proteins. First, the lack of cleavage of the predicted signal peptide indicates that mouse FXYD3, in contrast to other FXYD proteins, may have two transmembrane domains. The second feature that distinguishes mouse FXYD3 from other FXYD proteins is its ability to interact, not only with Na,K-ATPase, but also with gastric and non-gastric H,K-ATPase. So far, the physiological relevance of the functional properties of FXYD3 remains unclear. Interestingly, FXYD3 was shown to be overexpressed in human and mouse breast tumors initiated by the neu and ras oncopgenes, suggesting a correlation between FXYD3 and mammary tumorigenesis (17). Moreover, Grzmil et al. (18) showed that small interfering RNA inhibition of FXYD3 expression promotes a decrease in cell proliferation of prostate cancer cells. On the other hand, it has been shown that FXYD3 mRNA expression is increased after treatment of MCF-7 breast cancer cells with the fluoropyrimidine drug, 5-fluorouracil, and that this activation is dependent on p53 expression (19). It remains to be shown whether there is a correlation between the regulation of the functional properties of Na,K-ATPase by FXYD3 and the possible role of FXYD3 in cell proliferation.

Human adenocarcinoma cells, CaCo-2 cells, spontaneously convert from undifferentiated cells into polarized cells. By investigating the impact of differentiation on gene expression, Anderle et al. (20) have shown that expression of FXYD3 mRNA is increased during differentiation of CaCo-2 cells. In this study, we have identified two human FXYD3 isoforms in CaCo-2 cells that are differentially expressed during cell differentiation and are also expressed in the MCF-7 breast cancer cell line. Both human FXYD3 isoforms are able to stably associate with Na,K-ATPase and play different functional roles in the regulation of Na,K-ATPase activity.

**MATERIALS AND METHODS**

**Cloning and cDNAs**—The human FXYD3 cDNAs (human FXYD3 short form, hFXYD3-sf; human FXYD3 long form, hFXYD3-Lf) were amplified from total RNA of CaCo-2 cells by means of RT-PCR. The PCR was realized with a sense primer paired with an EcoRI site and a different antisense primer paired with a NotI restriction site (5'-CGGCGCGGCTCAGCTTGGGCTGAGC-3') (restriction enzyme sites are underlined). The human FXYD3Δ22 cDNA, lacking 22 N-terminal amino acids, was amplified from human FXYD3 cDNAs by PCR using a sense primer paired with an EcoRI site and containing the start codon ATG at the position of the Asp22 codon (5'-GCGAATTCATGCGAATGTTGATGGTTGATGGCTGTTTGAGCTGGA-3') and the antisense primer described above. The human FXYD3His cDNA containing a C-terminal histidine epitope was amplified from FXYD3 cDNAs by using the sense primer used to amplify human FXYD3 cDNAs and an antisense primer paired with a NotI restriction site and the His tag (5'-TGGCGCAGGCTCAGTGAAGGTTGATGGCTGTTTGAGCTGGA-3'). To produce the FXYD1 and FXYD2 chimeras containing the 26 additional amino acids of human FXYD3-Lf, we first PCR amplified the sequence coding for the N-terminal domain of dog FXYD1 or human FXYD2 by using a sense primer paired with an EcoRI site (5'-GCGAATTCTCATGCACTCCTCACCACATC-3'/5'-GGGATTATCATGCGAATGTTGATGGCTGTTTGAGCTGGA-3') and an antisense primer paired with 15 base pairs coding for the first 26 amino acids of human FXYD3-Lf (5'-CCCCGAGCTCCTCACCACATGCGAATGTTGATGGCTGTTTGAGCTGGA-3'). In parallel, the sequence coding for the additional 26 amino acids of FXYD3-Lf was PCR amplified by using a sense primer paired with 15 base pairs coding for the last amino acids of the N-terminal part of FXYD1/FXYD2 (5'-CTCATGCTCAGCGGCTGAGGAGGGTGGGG-3'/5'-CTCATGCTCCTCACCACATC-3'). Both PCR products were mixed and used for a third PCR by using the sense primer paired with an EcoRI site and the antisense primer paired with the 15 bases pairs coding for the first amino acids of the C-terminal part of FXYD1/FXYD2 (5'-CTCATGCTCAGCGGCTGAGGAGGGTGGGG-3'). The last two PCR products were mixed and used for a final PCR by using the sense primer paired with an EcoRI site and the antisense primer paired with a NotI site (5'-CAGCAGGCGCGCTACCACCGGCTGAGGAGGGTGGGG-3'/5'-CACGCAGGCGCTACCACCGGCTGAGGAGGGTGGGG-3'). The last two PCR products were mixed and used for a final PCR by using the sense primer paired with an EcoRI site and the antisense primer paired with a NotI site. All final products were digested by EcoRI and NotI and cloned into a pSD5 vector. All constructs were sequenced. cDNAs of rat Na,K-ATPase α1 and β1 subunits (kindly provided by J. Lingrel, University of Cincinnati, Cincinnati, OH), human Na,A,K-ATPase α1, α2, α3, and β1 subunits (3), rabbit gastric H,K-ATPase α, β subunits (kindly provided by G. Sachs, UCLA, Los Angeles, CA), rat colonic H,K-ATPase α subunit (kindly provided by F. Jaisser, INSERM U478, Paris, France), human SERCA2a (kindly provided by D. H. MacLennan, University of Toronto, Toronto, Ontario, Canada), and dog FXYD1 (21), human FXYD2 (22), and mouse FXYD3 (16) were subcloned.
Structural and Functional Properties of Human FXYD3 Isoforms

into a pSD5 vector. cRNAs were prepared by in vitro translation (23).

Antibodies—For the preparation of human FXYD3 antibodies, cDNA encoding a C-terminal region (His<sup>1</sup>–Ser<sup>87</sup>) was amplified by PCR and subcloned into the pGEX-4T1 vector (GE Healthcare). Glutathione S-transferase-FXYD3 fusion proteins were produced and purified as indicated by the manufacturer’s instructions. Eluted proteins were dialyzed overnight against phosphate-buffered saline to eliminate reduced glutathione. Purified proteins were used for immunization of rabbits (Cocalico Biologicals, Inc.). These antibodies recognized the short and long forms of human FXYD3.

Protein Expression in Xenopus Oocytes—Stage V–VI Xenopus oocytes were obtained as described (24). To study protein expression and FXYD3 association, cRNA-injected oocytes were incubated in modified Barth’s solution containing 0.7–1 mCi/ml [<sup>35</sup>S]methionine for 6 h and subjected to 24- and 48-h chase periods in modified Barth’s solution containing 10 mM unlabeled methionine. After the various pulse-chase periods, microsomes were prepared and subjected to non-denaturing immunoprecipitations as described (24) with antibodies against the Na<sub>K</sub>-ATPase α subunit (25), the gastric H,K-ATPase β subunit (26), the SERCA2a (Affinity Bioreagents), human FXYD3, mouse FXYD3 (21), dog FXYD1 (kindly provided by H. Garty), or human FXYD2 (22), and resolved either by SDS-PAGE (5–13% polyacrylamide) or on SDS-Tricine (15% polyacrylamide) gels (prepared according to the manufacturer’s instructions).

In Vitro Translation—In vitro translation in reticulocyte lysate of human wild type FXYD3 isoform or mutant FXYD3Δ22 cRNAs, in the presence or absence of canine pancreatic microsomal membranes was performed according to the manufacturer’s instructions (Promega).

Cell Culture—CaCo-2 cells were passaged every 6 days by treatment with trypsin/EDTA and used between passages 35 and 50. The cells were seeded at 4 × 10<sup>5</sup> cells in 25-cm<sup>2</sup> Costar flasks in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 100 units/ml penicillin and 100 µg/ml streptomycin and incubated at 37 °C at 9% CO<sub>2</sub>. The medium was changed every 2 days. For non-differentiated cells, cells were collected at about 80% of confluence. Differentiated cells were changed every 2 days. For non-differentiated cells, cells were harvested 3 weeks after they have reached confluency. MCF-7 breast cancer cells (kindly provided by D. Longley, University of Belfast) were maintained in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 µg/ml penicillin/streptomycin and incubated at 37 °C at 5% CO<sub>2</sub>.

RT-PCR—The extraction and purification of total RNAs from CaCo-2 cells or from human intestinal carcinoma cell lines DLD-1, HT29, and T84 were carried out according to the manufacturer’s specifications (RNeasy Mini Kit, Qiagen). The yield of each RNA was determined and was about 25 µg/culture dish. Total isolated RNA (0.5 µg) was then reverse transcribed using the Titan One Tube RT-PCR System (Roche). Briefly, PCR amplification was performed using 40 cycles with denaturation, annealing, and extension conditions, respectively, at 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min. Specific primers were prepared for the human gene FXYD3 (GenBank accession number NM_005971) (forward, 5‘-ATGCAGAAGGTGACCCCTGGGCTG-3‘, reverse, 5‘-TCAGCTTTGGCTGAGCCTGGG-3‘). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was amplified as a control. PCR products were electrophoresed in a 2% agarose gel, visualized by ethidium bromide, and photographed under UV light.

Cell Lysis and Western Blotting—Cellular extracts for immunoblotting analysis were prepared as follows. Cells grown on Petri dishes were washed 2 times with phosphate-buffered saline and harvested with trypsin/EDTA. The cell suspension was diluted in culture medium and centrifuged at 110 × g for 5 min at 4 °C. Cells were washed 3 times with phosphate-buffered saline before being frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Cells were suspended on ice, in 20 mM Tris-HCl, pH 8, containing 100 mM NaCl, 0.5% digitonin, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 1 µg/ml leupeptin, antipain, and pepstatin A). After 30 min at 4 °C with gentle agitation, resuspended cells were sonicated and centrifuged at 10,600 × g for 4 min at 5 °C. Supernatants were collected and the protein content was determined by the method of Lowry. Proteins (50 µg) were directly subjected to SDS-PAGE or to immunoprecipitations under non-denaturing conditions and then electroblotted onto nitrocellulose membranes, overnight at 40 V. Membranes were blocked with 10% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibody. The following primary antibodies were used: hFXYD3 (1/500), Na,K-ATPase α subunit (1/10,000), villin (Serotec Ltd., United Kingdom) (1/500), and actin (Sigma) (1/5,000). Primary antibody binding was detected using the following secondary antibodies: anti-rabbit IgG and anti-mouse IgG antibodies conjugated to horseradish peroxidase (1/10,000, Amersham Biosciences). Detection was achieved with the ECL chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s protocol.

N-terminal Sequencing—Five ng of His-tagged hFXYD3-Lf cRNA was injected in Xenopus oocytes. After 48 h, microsomes were prepared, and the His-tagged fusion protein was purified by nickel-affinity chromatography (nickel-nitrilotriacetic acid, Qiagen). The column-bound proteins were eluted with 50 mM Tris, 150 mM NaCl, 0.5% digitonin containing 200 mM imidazole. The purified protein was subjected to SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane using 10 mM CAPS buffer, pH 11, containing 10% methanol, overnight at 40 V. The polyvinylidene difluoride membrane was then washed 5 min in distilled water and 5 min in methanol. The protein band was then visualized by staining with 0.1% Coomassie Blue R-250 in 50% methanol, overnight at 4 °C for 5 min. Supernatants were collected and the protein content was determined by the method of Lowry. Proteins (50 µg) were directly subjected to SDS-PAGE or to immunoprecipitations under non-denaturing conditions and then electroblotted onto nitrocellulose membranes, overnight at 40 V. Membranes were blocked with 10% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with primary antibody. The following primary antibodies were used: hFXYD3 (1/500), Na,K-ATPase α subunit (1/10,000), villin (Serotec Ltd., United Kingdom) (1/500), and actin (Sigma) (1/5,000). Primary antibody binding was detected using the following secondary antibodies: anti-rabbit IgG and anti-mouse IgG antibodies conjugated to horseradish peroxidase (1/10,000, Amersham Biosciences). Detection was achieved with the ECL chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s protocol.
FXYD2 cRNA by using the two-electrode voltage clamp technique. Measurements of the apparent external K\(^+\) affinity were carried out as described previously (22) in the presence of 1 \(\mu M\) ouabain that inhibits the endogenous Na,K-pump but not the expressed ouabain-resistant rat Na,K-ATPase. The maximal pump current and the apparent K\(^+\) affinity \((K_{Vmax} / K_{1/2} K^+)\) measured in the presence or absence of external Na\(^+\) \((100\ \text{mm})\) were obtained by fitting the Hill equation to the data using a Hill coefficient of 1.6 or 1, respectively (27). Measurements of the apparent Na\(^+\) affinity of Na,K-ATPase were performed as described previously (28) by co-expressing rat \(\alpha 1\) and rat \(\beta 1\) cRNAs together with rat epithelial Na\(^+\) channel \(\alpha, \beta, \) and \(\gamma\) subunit cRNAs in the presence or absence of FXYD cRNA. The maximal current \((I_{Vmax})\) and the apparent Na\(^+\) affinity \((K_{Vmax} / K_{1/2} \text{Na}^+\) \(\) were fitted by using the Hill equation and a Hill coefficient of 3.

For the determination of FXYD3-induced current, electrophysiological measurements were performed 3 days after oocyte injections with mouse FXYD3, FXYD3-sf, or FXYD3-lf cRNA by using the two-electrode voltage clamp technique. Briefly, oocytes were placed in a recording chamber in a solution containing 150 mm NaCl, 5 mm KCl, 1 mm MgCl\(_2\), 2 mm CaCl\(_2\), 10 mm dextrose, 10 mm HEPES, pH 7.4. Hyperpolarizing and depolarizing voltage steps were done from potentials at \(-10\ \text{mV}\) as described by Morrission et al. (9).

**RESULTS**

Expression Pattern of FXYD3 in Human Colon Adenocarcinoma Cells—Human colon adenocarcinoma cells, CaCo-2 cells, exhibit a typical enterocytic differentiation, spontaneously after confluence. FXYD3 mRNA expression was assessed by using RT-PCR during the differentiation process. Total RNA was amplified using primers that span all exons of human FXYD3. As shown in Fig. 1A, two FXYD3 mRNA species could be revealed, a lower molecular mass species (FXYD3-sf) present all along during differentiation and a higher molecular mass species (FXYD3-lf) present only in non-differentiated CaCo-2 cells. Together with FXYD3-sf, FXYD3-lf was also slightly expressed in three other human intestinal carcinoma cell lines (Fig. 1B, lanes 2–4).

To verify translation and visualize whether alterations of FXYD3 transcript expression during differentiation results in alterations of FXYD3 protein levels, we performed Western blot analysis. Villin, which plays a major role in the initiation of brush-border assembly (29), was used as a marker for enterocytic differentiation of CaCo-2 cells (30) (Fig. 1D, lower panel). Western blot analysis showed 2 FXYD3 translation products, the expression of which differed during differentiation of CaCo-2 cells (Fig. 1, C and D, upper panel). The FXYD3-lf protein was the major form in non-differentiated cells and its expression slightly decreased during differentiation, whereas the FXYD3-sf protein was the major form in differentiated cells and its expression increased during differentiation (Fig. 1E). Moreover, Western blot analysis showed the expression of FXYD3-lf transcripts not only in CaCo-2 cells but also in MCF-7 breast cancer cell line (Fig. 1F), indicating that the expression of FXYD3-lf is not restricted to CaCo-2 cells.
Biosynthesis and processing of human FXYD3 isoforms. A, alignment of amino acid sequences of mouse FXYD3 (mFXYD3-sf), short and long human FXYD3 isoforms (hFXYD3-sf and hFXYD3-Ilf), and truncated human mutant FXYD3 isoforms lacking 22 N-terminal amino acids. The putative transmembrane domain (TM) is indicated by the thick line. The additional 26 amino acids located in the juxtamembrane domain of hFXYD3-Ilf are in bold and boxed. Predicted signal peptides are underlined and arrows indicate the position of predicted cleavage. B, wild type hFXYD3-Ilf (lanes 1 and 2) and truncated hFXYD3-IlfΔ22 (lanes 3 and 4) were translated in a reticulocyte lysate, in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of canine pancreatic microsomes, and labeled with [35S]methionine. Wild type hFXYD3-Ilf (lanes 5 and 6) and truncated hFXYD3-IlfΔ22 (lanes 7 and 8) cRNAs (5 ng/oocyte) were injected into Xenopus oocytes. Oocytes were pulse labeled with [35S]methionine for 6 h (lanes 5 and 7), followed by a 24-h chase period (lanes 6 and 8) before preparation of microsomes. Labeled proteins were then separated by SDS-Tricine gel electrophoresis and revealed by fluorography. C, His-tagged hFXYD3-Ilf and hFXYD3-Ilf cRNAs were injected into Xenopus oocytes. Oocytes were pulse labeled with [35S]methionine for 6 h (lanes 1, 4, and 7) followed by 24- and 48-h chase periods (lanes 2, 5, and 8, and lanes 3, 6, and 9, respectively). Oocyte microsomes were subjected to immunoprecipitation under non-denaturing conditions using a hFXYD3 antibody (lanes 1–6) or a His antibody (lanes 7–9). Samples were then separated by SDS-Tricine gel electrophoresis and revealed by fluorography. D, N-terminal sequencing of purified hFXYD3-Ilf His expressed in Xenopus oocytes gave a unique signal that corresponds to the indicated sequence.

Human FXYD3 Isoforms Associate with Different Na,K-ATPase Isozymes but Not with Other P-type ATPases—The ability of FXYD3 proteins to interact with different Na,K-ATPase isoforms (human α1, α2, α3, and B1) was tested after co-expression in Xenopus oocytes. Xenopus oocytes were metabolically labeled, subjected to various chase periods, and microsomes of Xenopus oocytes were immunoprecipitated under non-denaturing conditions with Na,K-ATPase α subunit or FXYD3 antibodies. As shown in Fig. 3A, the Na,K-ATPase α subunit antibody, recognizing α1, α2, and α3 isoforms, co-immunoprecipitated the Na,K-ATPase β subunit, and mouse and human FXYD3 isoforms after the chase periods, and to a lesser extent, after the pulse period (lanes 2–25). Likewise, a FXYD3 protein, migrated at the same molecular mass in the absence or presence of microsomes and at a higher molecular mass than the N-terminal truncated FXYD3-Ilf mutant (Δ22) (Fig. 2B, lanes 1–4), indicating lack of signal peptide cleavage. On the other hand, human FXYD3-Ilf expressed in Xenopus oocytes exhibits a different pattern after the pulse or a 24-h chase period (lanes 5 and 6). After the pulse period, human FXYD3-Ilf had the same molecular mass as when expressed in vitro (lanes 1 and 5), whereas after a 24-h chase period, a band of lower molecular mass appeared (lane 6) with a similar molecular mass as that of the FXYD3Δ22 mutant (lanes 7 and 8). Similar results were obtained for FXYD3-sf (data not shown). These results seem to indicate that, contrary to mouse FXYD3, the signal peptide of human FXYD3 is cleaved. To verify signal peptide cleavage, we performed N-terminal sequencing of purified, C-terminal His-tagged FXYD3-Ilf expressed in Xenopus oocytes. As shown in Fig. 2C, human FXYD3-Ilf and human His-tagged FXYD3-Ilf exhibited a similar migration pattern during the pulse and chase periods (Fig. 2C, lanes 1–6) indicating that the presence of the His tag did not disturb the processing of the protein. A band of lower molecular mass of His-tagged FXYD3 was revealed with a FXYD3 antibody during the chase periods (lanes 5 and 6). Immunoprecipitation with a His tag antibody revealed only the two higher molecular mass species (compare lanes 5 and 6 to lanes 8 and 9), indicating that the lower molecular mass band could correspond to a partial C-terminal cleavage of the His tag in FXYD3-Ilf. After affinity purification of His-tagged FXYD3-Ilf, N-terminal sequencing provided a NDLEDKN N-terminal amino acid sequence of the mature protein confirming the cleavage of the signal peptide. Altogether, the results indicate that, contrary to mouse FXYD3, the signal peptide of human FXYD3 isoforms is cleaved between Ala20–Asn21. We can conclude that human FXYD3 isoforms are type I proteins like the majority of the other members of the FXYD family.
antibody co-immunoprecipitated α1–β1 isoforms (Fig. 3B). In oocytes expressing Na,K-ATPase with human FXYD3 isoforms, the electrophoretic migration of the associated fully glycosylated β subunit is slightly changed compared with Na,K-ATPase expressed alone (Fig. 3A, compare lanes 2 – 4 with lanes 8 – 25). This indicates that human FXYD3 isoforms influence the glycosylation processing of the β subunit and/or the intracellular sorting of Na,K-ATPase similar to mouse FXYD3 (16) (Fig. 3A, lanes 5 – 7).

Thus, in Xenopus oocytes, human FXYD3 isoforms are able to associate stably with all α1–β1 Na,K-ATPase isoforms. These results indicate that the presence of the additional 26 amino acids in the juxtamembrane domain of human FXYD3-If does not impede the structural association with Na,K-ATPase. The higher association efficiency of human FXYD3 isoforms after the chase period than after the pulse period (compare the intensity of co-immunoprecipitated FXYD3 between the pulse and the chase periods in Fig. 3A) indicates that FXYD3 associates post-translationally with Na,K-ATPase.

The specific association of human FXYD3 with Na,K-ATPase was analyzed by coexpressing human FXYD3 isoforms with gastric or colonic H,K-ATPase or SERCA2a in Xenopus oocytes. Mouse FXYD3 was previously shown (16) to associate stably with gastric (Fig. 4, lanes 1 – 3) and to a lesser extent with colonic (lanes 10 – 12) H,K-ATPase. Human FXYD3 isoforms could be co-immunoprecipitated with H,K-ATPases during the pulse but not during the chase periods, indicating that human FXYD3 did not stably associate with the two H,K-ATPases (lanes 4 – 9 and lanes 13 – 18). The lower association efficiency of human FXYD3 isoforms with H,K-ATPases compared with mouse FXYD3 was also reflected by the fact that human FXYD3 did not influence the glycosylation processing of the gastric H,K-ATPase β subunit, as previously observed with mouse FXYD3 (16) (Fig. 4, compare lanes 1 – 3 to lanes 4 – 9 and lanes 10 – 12 to lanes 13 – 18). Similar to mouse FXYD3, human FXYD3 isoforms did not stably associate with SERCA2a because co-immunoprecipitation could only be observed during the pulse period (lanes 19 – 27). Altogether, these results indicate that human FXYD3 isoforms expressed in Xenopus oocytes associate with several P-type ATPases but stably only with Na,K-ATPase.

**Functional Effects of Human FXYD3 Isoforms**—The functional effects of FXYD3 proteins on the transport properties of Na,K-ATPase were tested by electrophysiological means in Xenopus oocytes expressing rat Na,K-ATPase α1–β1 isoforms either alone or together with mouse or human FXYD3 isoforms. Mouse FXYD3 and human FXYD3-If produced a similar, significant decrease of the apparent affinity for extracellular K⁺ of Na,K-ATPase over a wide range of membrane potentials in the presence of external Na⁺ (Fig. 5A). The effect was also observed in the absence of external Na⁺ (Fig. 5B) suggesting a modification of the intrinsic affinity of the external K⁺ binding site. On the other hand, human FXYD3-If had no effect on the apparent K⁺ affinity of Na,K-ATPase at very negative membrane potentials and an effect on the apparent K⁺ affinity at slightly negative and positive membrane potentials (Fig. 5A), which was nearly abolished in the absence of external Na⁺ (Fig. 5B). Similar to mouse FXYD3, human FXYD3-If produced a significant decrease in the apparent affinity for internal Na⁺, whereas human FXYD3-If produced a slight increase in the apparent Na⁺ affinity of Na,K-ATPase (Fig. 5C).

In addition to the effects of human FXYD3s on the transport properties of Na,K-ATPase, we tested whether they might...
induce ion conductance after expression in oocytes as described for mouse FXYD3 (9). *Xenopus* oocytes expressing human FXYD3 isoforms exhibited a hyperpolarization activated current (Fig. 6A) similar to that produced by mouse FXYD3 (9). During hyperpolarizing pulses, oocytes injected with human FXYD3-lf cRNA had large inward currents (Fig. 6A), whereas H2O-injected oocytes showed no active currents (Fig. 6B). Similar results were obtained with the human FXYD3-sf variant (data not shown). The current-voltage relationship of oocytes expressing human FXYD3 isoforms is similar to currents induced by mouse FXYD3 (Fig. 6C) or FXYD1 (31).

**The Functional Role of the Additional 26 Amino Acids in the Juxtamembrane Domain of Human FXYD3-lf**—To determine the role of the additional 26 amino acids in the effect of FXYD3 on the transport properties of Na,K-ATPase, we introduced the 26 extra amino acids into FXYD1 and FXYD2. We have chosen these FXYD proteins because both FXYD1 (21) and FXYD2 (22) modulate the apparent K⁺ affinity and decrease the apparent Na⁺ affinity of Na,K-ATPase similar to FXYD3-sf. Both chimeras were well expressed in *Xenopus* oocytes and when co-expressed with Na,K-ATPase could be co-immunoprecipitated with a Na,K-ATPase α subunit antibody similar to the wild type FXYD proteins (data not shown).

Results of electrophysiological measurements show that (i) the 26-residue insert of FXYD3-lf counteracts the increase in the $K_{1/2}$ Na⁺ value effected by FXYD3-sf, FXYD1, and FXYD2 (22); (ii) in the case of $K_{1/2}$ K⁺ values, at membrane potential values ranging from −140 mV to approximately −50 mV, the insert
Structural and Functional Properties of Human FXYD3 Isoforms

In this study, we have identified two splice variants of human FXYD3 that are differentially expressed during differentiation of CaCo-2 cells. Both isoforms are able to associate with Na,K-ATPase but each isoform exhibits different functional roles in Na,K-ATPase regulation.

FXYD3 Splice Variants and Cell Differentiation—By analyzing more than 1000 related expressed sequence tags, Sweadner and Rael (6) defined the small membrane protein family FXYD. From consensus nucleotide sequences, they deduced the amino acid sequences of the seven mammalian members of the family. They also identified two splice variants of FXYD2, γα and γβ, which were confirmed by Kuster et al. (32). Moreover, Jones et al. (33) described a third variant γc in mice and humans, although the human FXYD2 γc mRNA may not encode a functional protein because of the absence of an initiator methionine. Sweadner and Rael (6) also identified several splice variants of FXYD1 that were, however, in the 5'-untranslated region and consequently did not change the coding sequence, and, in fact, noted that all members of the family possess occasional expressed sequence tags with deviations in the sequence that could correspond to either splice variants or splicing artifacts. These results indicate that several FXYD proteins may exist in multiple forms that considerably increase the complexity of their possible functions. In this study, we have identified an uncommon form of human FXYD3 in CaCo-2 cells. Searches in GenBank revealed that, in contrast to the three FXYD2 variants, which differ in their N-terminal sequence, the long human FXYD3 variant contains 26 additional amino acids in the C-terminal juxtamembrane domain compared with the common, short human FXYD3 form. Interestingly, in mice, only the short form of FXYD3 could be detected at the mRNA level (9). As shown in our study, the long human FXYD3 protein is the major FXYD3 form in undifferentiated CaCo-2 cells, whereas it becomes the minor form in well differentiated epithelial cells (Fig. 1, C–E). Finally, long FXYD3 is not only present in undifferentiated CaCo-2 cells, but also in several colon adenocarcinoma cell lines (Fig. 1B) and in MCF-7 breast cancer cell lines (Fig. 1F). Together with the observation that mRNA expression of FXYD3 increases during differentiation of CaCo-2 cells (20), these results raise the question of the role of FXYD3 in cell differentiation. Our study indicates that cell differentiation is accompanied by a change in FXYD3 isoform expression. The precise role of the two FXYD3 isoforms in the differentiation process remains to be determined.

FXYD3 Variants Are Type I Membrane Proteins—In all FXYD proteins, the C terminus contains more positive charges compared with the N terminus, indicating, according to the inside-positive rule, a membrane topology with the C terminus exposed to the cytoplasmic side (34). FXYD1 (7), FXYD2, FXYD4 (22), and FXYD7 (13) are indeed type I membrane proteins. Except for FXYD2 and FXYD7, FXYD proteins contain a hydrophobic domain at the N terminus encoding a signal peptide that is predicted to be cleaved during biosynthesis. Interestingly, despite the prediction, the signal peptide of mouse FXYD3 is not cleaved producing a protein with two transmembrane domains (16). Our results show that, in contrast to mouse FXYD3, but similar to the other members of the FXYD family, human FXYD3 variants are type I membrane proteins with the N terminus exposed to the extracytoplasmic side. This membrane orientation is achieved by the cleavage of the signal pep-
stitute. The difference in signal peptide cleavage between mouse and human FXYD3 proteins is surprising in view of the more than 70% sequence homology of the two signal peptide sequences. Similar to FXYD4 (22), the signal peptides of the human FXYD3 variants are cleaved after expression in Xenopus oocytes, but not during in vitro synthesis. Cleavage of the signal peptide of human FXYD3 or FXYD4, expressed in oocytes, is a slow process that may explain the lack of signal peptide cleavage in the in vitro translation system where the time and location for processing are limited. It is not known whether signal peptide cleavage of human FXYD3 occurs during synthesis by a conventional signal peptidase, or rather post-translationally in the endoplasmic reticulum or another cellular compartment by a so far unknown cleavage enzyme. Delayed cleavage of the signal peptide of the human cytomegalovirus US11 polypeptide has been reported (35). Future experiments should reveal whether similar to US11, part of the signal peptide and the transmembrane region affect signal peptide cleavage.

FXYD3 Variants Associate with Na,K-ATPase—Up to now, six of the seven FXYD proteins have been shown to interact with Na,K-ATPase and to regulate its function (14, 36). FXYD proteins are considered to be tissue-specific auxiliary subunits of Na,K-ATPase although they may have other additional functions. Our results show that, similarly to other studied FXYD variants, human FXYD3 variants specifically associate with Na,K-ATPase after co-expression in Xenopus oocytes. In particular, FXYD3 variants are able to interact with α1-β1, α2-β1, α3-β1 Na,K-ATPase isoforms after expression in Xenopus oocytes. Our results show that more FXYD3 forms can be co-immunoprecipitated with Na,K-ATPase during the chase period than during the pulse period (Fig. 3A). During the pulse period, the β subunit associated with the α subunit is mainly in its coreglycosylated form indicating the location of the Na,K-ATPase in the endoplasmic reticulum, whereas during the chase periods, the β subunit associated with the α subunit is mainly in its fully glycosylated form, indicating that Na,K-ATPase has passed the Golgi compartment where full glycosylation occurs. From these data, we can conclude that, similar to FXYD1 (21), association of FXYD3 and Na,K-ATPase mainly occurs post-translationally in compartments different from the endoplasmic reticulum. We do, however, not know whether association occurs during the passage of proteins from the Golgi to the plasma membrane or at the plasma membrane. In contrast to mouse FXYD3, human FXYD3 variants do not stably associate with H,K-ATPase. Finally, in CaCo-2 cells, FXYD3 variants associate with Na,K-ATPase all along during differentiation. CaCo-2 cells are derived from a colon carcinoma cell line and express different phenotypes depending on the differentiation status. At present, we do not know whether the two human FXYD3 variants associate with the same or different pools of Na,K-ATPase. Altogether, our results indicate that similar to other FXYD proteins, human FXYD3 splice variants are possible regulators of Na,K-ATPase.

Functional Effects of FXYD3 Variants on Na,K-ATPase—All FXYD proteins characterized so far such as FXYD1, FXYD2, mouse FXYD3, FXYD4, FXYD5, and FXYD7 influence the function of Na,K-ATPase in a distinct way (14, 36). Recently, we have shown that mouse FXYD3 promotes a decrease in both the apparent Na\(^+\) and K\(^+\) affinities of Na,K-ATPase (16) similar to FXYD1 (21) and FXYD2 (22). In this study, we show that each human FXYD3 variant modulates the transport properties of Na,K-ATPase in a distinct way. Short, human FXYD3 decreases both the apparent Na\(^+\) and K\(^+\) affinities of Na,K-ATPase similar to mouse FXYD3. On the other hand, long human FXYD3 has no effect on the apparent K\(^+\) affinities of Na,K-ATPase at negative membrane potentials but decreases the apparent K\(^+\) affinities of Na,K-ATPase at positive membrane potentials. Moreover, in contrast to short human FXYD3, long human FXYD3 slightly increases the apparent Na\(^+\) affinity of Na,K-ATPase. Comparison of the functional effects of wild type FXYD1 or FXYD2 and chimeric proteins, containing the 26 additional amino acids of long human FXYD3, revealed that the 26 amino acids of long human FXYD3 are able to abolish most, but not all, of the functional effects of FXYD1 and FXYD2. These results indicate that the additional 26 amino acids in the cytoplasmic domain of the long human FXYD3, which apparently are not implicated in the structural association with Na,K-ATPase, may play a role in the regulation of Na,K-ATPase. On the other hand, the additional 26 amino acids in human FXYD3-if do not seem to be implicated in the hyperpolarization activated chloride current because oocytes expressing FXYD3-if have large inward currents similar to those of oocytes expressing mouse FXYD3 or FXYD3-sf (Fig. 6C). Nevertheless, the cellular function of these common inward currents remains to be determined.

At present, it is difficult to speculate on the physiological relevance of the different functional effects of FXYD3 variants on Na,K-ATPase during cell differentiation. It is conceivable that differential regulation of Na,K-ATPase during cell differentiation is necessary to assure a tight control on cellular Na\(^+\)/K\(^+\) gradients. In this line, a switch from FXYD3-if to FXYD3-sf expression and a concomitant decrease in the apparent Na\(^+\) affinity of Na,K-ATPase may lead to slightly higher intracellular Na\(^+\) concentrations that may be important for certain differentiation processes. Moreover, Na,K-ATPase is involved in the formation and maintenance of tight junctions (37), and in cell attachment (38), processes that are important in maintaining the differentiated cell type but also to promote differentiation. In conclusion, differential regulation of the functional properties of Na,K-ATPase, which differentially modifies the ionic cellular milieu, may be important for triggering or maintaining cell differentiation and/or proliferation.

Acknowledgment—We thank Jean-Daniel Horisberger for helpful comments on the manuscript.

REFERENCES
1. Geering, K. (2001) J. Bioenerg. Biomembr. 33, 425–438
2. Blanco, G., and Mercer, R. W. (1998) Am. J. Physiol. 275, F633–F650
3. Crambert, G., Hasler, U., Beggah, A. T., Yu, C., Modyanov, N. N., Horisberger, J. D., Lelievre, L., and Geering, K. (2000) J. Biol. Chem. 275, 1976–1986
4. Therien, A. G., and Blostein, R. (2000) Am. J. Physiol. 279, C541–C566
5. Feraille, E., and Doucet, A. (2001) Physiol. Rev. 81, 345–418
6. Sweadner, K. J., and Rael, E. (2000) Genomics 68, 41–56
7. Palmer, C. J., Scott, B. T., and Jones, L. R. (1991) J. Biol. Chem. 266, 11126–11130
