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FXYD6 IS A NOVEL REGULATOR OF NA,K-ATPASE EXPRESSED IN THE INNER EAR.

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Running Title: FXYD6, a novel regulator of Na,K-ATPase

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The exquisite sensitivity of the cochlea, which mediates the transduction of sound waves into nerve impulses, depends on the endolymph ionic composition and the endocochlear potential. A key protein in the maintenance of the electrochemical composition of the endolymph is the Na,K-ATPase. In this study, we have looked for the presence in the rat inner ear of members of the FXYD protein family, recently identified as tissue-specific modulators of Na,K-ATPase. Only FXYD6 is detected at the protein level. FXYD6 is expressed in various epithelial cells bordering the endolymph space, and in the auditory neurons. FXYD6 co-localizes with Na,K-ATPase in the stria vascularis and can be co-immunoprecipitated with Na,K-ATPase. After expression in *Xenopus* oocytes, FXYD6 associates with Na,K-ATPase α1-β1 and α1-β2 isozymes, which are preferentially expressed in different regions of the inner ear, and also with gastric and non-gastric H,K-ATPases. The apparent K^+ and Na^+ affinities of α1-β1 and α1-β2 isozymes are different. Association of FXYD6 with Na,K-ATPase α1-β1 isozymes slightly decreases their apparent K^+ affinity and significantly decreases their apparent Na^+ affinity. On the other hand, association with α1-β2 isozymes increases their apparent K^+ and Na^+ affinity. The effects of FXYD6 on the apparent Na^+ affinity of Na,K-ATPase and the voltage-dependence of its K^+ effect are distinct from other FXYD proteins. In conclusion, this study defines the last FXYD protein of unknown function as a modulator of Na,K-ATPase. Among FXYD protein, FXYD6 is unique in its expression in the inner ear suggesting a role in endolymph composition.

The mammalian inner ear is composed of two distinct sensory organs, the cochlea, the organ of hearing and the vestibule, the organ of equilibrium. Proper functioning of the sensory hair cells depends on the ionic composition of endolymph and perilymph. Whereas perilymph resembles most other interstitial fluids in containing low K^+ and high Na^+, endolymph has an ionic composition characterized by high K^+ and low Na^+, similar to the intracellular fluid (1-3). K^+ is central to the inner ear physiology, since it is the charge-carrying ion for the sensory transduction. In the cochlea, K^+ is secreted by the marginal cells of the stria vascularis to maintain a very high concentration (150 mM) in the endolymph, the extracellular fluid bathing the stereocilia of the sensory hair cells. This results in a positive potential (+90 mV), which provides a large driving force for K^+ entry into the sensory hair cell. The unique ionic composition of endolymph, along with a highly positive endocochlear potential, are thus essential to mechanoelectric transduction by hair cells and to normal hearing (4-6).

Maintenance of both the ionic composition of endolymph and the endocochlear potential depends on the activity of Na,K-ATPase (5, 6). The Na,K-ATPase is an ubiquitous enzyme consisting of an α and a β subunit, which is responsible for the creation and maintenance of the Na^+ and K^+ gradients across the cell membrane by transporting three Na^+ out of and two K^+ into the cell. This function is crucial for cell survival and body homeostasis because the Na^+ gradient is used as an energy source to transport ions or solutes, and is at the origin of the vectorial Na^+ reabsorption in the kidney and of action potentials in excitable tissues. Regulation of the activity and expression of Na,K-ATPase is tight and governed by a variety of mechanisms. Short-term regulation involves protein kinases and results in modulation of the cell surface expression of the Na,K-ATPase, whereas long-term regulation, mediated by mineralocorticoid

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or thyroid hormones, leads to a change in the total number of Na,K-ATPase units (for review, see (7, 8)). Moreover, the existence of multiple α and β isoforms permits the production of isozymes with different transport properties (9). Finally, recent experimental evidence shows that members of the FXYD protein family specifically associate with and modulate the transport properties of Na,K-ATPase (for review, see (10, 11)).

The mammalian FXYD family contains seven members that share a common signature sequence encompassing the transmembrane and adjacent regions (12). Most characterized FXYD proteins exhibit a similar structure with a single transmembrane domain and a type I orientation that is achieved, in some, but not all cases, by the cleavage of an N-terminal signal peptide (for references see (10)). So far, six out of the seven members of the FXYD protein family have been shown to regulate Na,K-ATPase in a tissue- and isozyme-specific way. Four FXYD proteins (FXYD1, FXYD3, FXYD4 and FXYD7) affect in a distinct way the apparent affinity for extracellular K^+ of the Na,K-ATPase, which in the case of the brain-specific FXYD7 may be physiologically relevant in neuronal excitability (13). In addition, FXYD1 (phospholemman) (14), FXYD2 (γ subunit) (15-17), FXYD3 (18) and FXYD4 (19, 20) affect the apparent affinity for internal Na^+ of Na,K-ATPase in a way that is consistent with the physiological demands of the tissues in which they are expressed. These results suggest that at least one common function of all FXYD proteins could be the regulation of Na,K-ATPase transport properties.

Given the key role of the Na,K-ATPase in the maintenance of the electrochemical composition of the endolymph and the modulatory role of FXYD proteins on its transport properties, we have looked in this study for the expression of several FXYD proteins in the inner ear. The only FXYD protein that we were able to detect by Western blot was FXYD6. We, therefore, characterized FXYD6, the last member of the FXYD family of unknown function. FXYD6, also known as phosphohippolin, was previously identified as a phospholemman-like protein expressed at high levels in brain and at a lesser level in lung, testis and colon (21).

In this study, we investigated the association of FXYD6 with Na,K- and H,K-ATPases, the modulation of Na,K-ATPase function, and its cellular distribution in the inner ear and in PC12 cells. Our results show that FXYD6 has functional characteristics that are distinct from other FXYD proteins and that the protein may have a crucial role in endolymph homeostasis.

EXPERIMENTAL PROCEDURES

cDNAs. Mouse FXYD6 cDNA, subcloned between the 5' and 3' domains of *Xenopus* β-globin, was kindly provided by H. Garty (Weizmann Institute of Science, Israel). Cloning of rat Na,K-ATPase α1, α2, α3, β1 and β2 cDNAs has been described (9). cDNAs for the rabbit, gastric H,K-ATPase α and β subunits were kindly provided by G. Sachs (UCLA, USA) and cDNA for rat, colonic H,K-ATPase α subunits by Fréderic Jaisser (INSERM, U478, Paris, France). All cDNAs were introduced into the pSD5 vector and cRNAs were prepared by *in vitro* translation (22).

Protein expression in *Xenopus* laevis oocytes. Stage V–VI oocytes were obtained from *X. laevis* as described (23). cRNAs coding for mouse FXYD6, rat Na,K-ATPase α1, α2, α3, β1 and β2 subunits or rabbit gastric or rat colonic H,K-ATPase α and gastric H,K-ATPase β subunits were injected into oocytes in different combinations as described in the figure legends. To study protein expression and association, oocytes were incubated in modified Barth's solution (MBS), in the presence of 0.7-1 mCi/ml ^{35}S-methionine (Easy Tag Express [^{35}S] Protein Labelling Kit, PerkinElmer). Oocytes were subjected to a 6 h pulse and to 24–48 h chase periods in MBS containing 10 mM cold methionine. After the pulse and chase periods, microsomes were prepared as described (23) and subjected to immunoprecipitations with a FXYD6 antibody under non-denaturing conditions (see below).

Preparation of extracts of rat inner ear. Rat cochleae were isolated and ground in a mortar. The tissue was homogenized manually in a phosphate buffered saline (pH 7.4) containing antiproteases (Roche). The homogenate was diluted 1:1 with a lysis buffer containing 200 mM NaCl, 2% digitonin, 10 mM PMSF, 10 mM EDTA, 40 mM Tris-HCl, pH 7.4, for 1 h at 4°C and centrifuged at 10,000 x g for 5 min. The supernatants were used for immuoprecipitation and Western blotting.

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Antibodies, immunoprecipitation and Western blotting. A polyclonal FXYD6-antibody directed against a C-terminal GST fusion peptide of mouse FXYD6 (62-CSFNQKPRAPGDEEAQVENLITTNAAEPQK AEN) was produced by Pascal Béguin. The corresponding cDNA region was cloned in frame into the pGEX-4T1 vector. After purification using glutathione beads, and dialysis against phosphate-buffered saline (PBS), the fusion protein was used for immunization of rabbits (Cocalico Biologicals). This antibody was used for co-immunoprecipitation experiments of metabolically labeled Na,K- and H,K-ATPase expressed in *Xenopus* oocytes under non-denaturing conditions (24). Immunoprecipitates were resolved on 5–13% SDS-polyacrylamide gels or SDS-Tricine gels and revealed by fluorography.

FXYD1 (14), FXYD2 (16), FXYD3 (18), FXYD4 (19), FXYD6 and FXYD7 (13) antibodies were used in Western blots to test the expression of FXYD proteins in the inner ear.

To demonstrate association of FXYD6 with Na,K-ATPase in rat cochlea, 50 µg protein of cochlear extracts were incubated with a Na,K-ATPase α1 subunit antibody (Bioreagents) overnight at 4°C. Immunoprecipitates were migrated on a 5–13% SDS-polyacrylamide gel and transferred onto nitrocellulose. FXYD6 was revealed with the FXYD6 antibody and chemiluminescence detection (ECL, Amersham Pharmacia).

Electrophysiology. Electrophysiological measurements were performed 3 days after oocyte injection with rat Na,K-ATPase α1–β1 or α1–β2 cRNAs alone or together with FXYD6 cRNA by using the two-electrode voltage clamp technique. Measurements of the apparent external K^+ affinity were carried out as described (16) in the presence of 1 µM ouabain, which inhibits the endogenous, oocyte Na,K-pumps, but not the expressed ouabain-resistant rat Na,K-ATPase isozymes. The maximal Na,K-pump current and the apparent K^+ affinity ($K_{1/2}$ K^+) measured in the presence of 90 mM external Na^+ were obtained by fitting the Hill equation to the data with a Hill coefficient of 1.6 (25). Measurements of the apparent Na^+ affinity of Na,K-ATPase in intact cells were performed as described (26) by co-expressing rat α1 with rat β1 or β2 cRNAs together with rat epithelial Na^+ channel (ENaC) α-, β-, and γ-subunit cRNAs in the presence or absence of FXYD6 cRNA. The maximal current (Imax) and the apparent Na^+ affinity ($K_{1/2}$ Na^+) were fitted by using the Hill equation and a Hill coefficient of 3 (26). Statistical analysis was performed by Student's t-test.

Immunocytochemistry of FXYD6. The rats were deeply anesthetized and then fixed intraaortically with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Cochleae were dissected, the oval and round windows were opened, and a hole was drilled at the apex before an overnight postfixation at 4 °C. Cochleae were then transferred to a phosphate buffer containing 20% sucrose for cryoprotection and frozen. Fourteen µm - thick sections were cut with a Reichert-Jung 2800 cryostat microtome and stored at -20 °C until use. The immunohistochemical procedures were similar to those described (27). The sections were rinsed (3 - 5 min) in phosphate-buffered saline (PBS), preincubated 1 h in 30% normal goat serum with 0.3% Triton X-100 and incubated overnight at 4 °C with FXYD6 (1:500), Na,K-ATPase α1 subunit (1:100) and parvalbumin (Swants, 1:750) antibodies diluted in PBS with 1% normal goat serum. They were then rinsed in PBS (3 - 8 min) and incubated for 2 h with goat anti-rabbit IgGs conjugated to Alexa 488 (Molecular Probes) diluted 1:2000 in PBS, anti-mouse conjugated CY3 (Molecular Probes) and anti-goat conjugated CY5 (Molecular Probes). The sections were then rinsed in PBS (3 - 8 min) and mounted in Mowiol. For co-localization experiments of FXYD6 and barttin in the stria vascualris, a chicken barttin antibody (28) (kindly provided by Shinishi Uchida) was used at a dilution of 1:100. The sections were observed with a ZEISS LSM510 Meta laser scanning confocal microscope. No immunostaining was observed when the primary antibody was omitted in the first incubation step, or when preimmune sera were used.

PC12 Cell Culture. Rat pheochromocytoma (PC12) cells (kindly provided by Bernard Thorens, CIG, Lausanne), a cell model commonly used to study neurite formation, were maintained in 100 mm^2 culture dishes in culture medium DMEM (Invitrogen) supplemented with 6% horse serum, 6% fetal bovine serum, 20 mM Hepes, 2 mM glutamine, 1 mM Na-pyruvate and 100 µg/ml penicillin/streptomycin at 37°C, 5% CO2. To induce differentiation, culture medium was supplemented with nerve growth factor (10 µg/ml) for one week. For immunocytochemistry, cells were plated at 4 x 10^5 cells/well on 6-well

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glass slides. All samples were washed in PBS, fixed in 4% paraformaldehyde for 10 minutes and rinsed with PBS. Cells were permeabilized using 0.3 % Triton X-100, rinsed with PBS and blocked with 3% BSA in PBS for 1 hour. Cells were then co-incubated with a FXYD6 antibody and Na,K-ATPase α1 subunit monoclonal antibody in 1% BSA for 1 h at room temperature, washed with PBS and incubated with fluorescence-labeled secondary antibodies (Alexa 488-conjugated goat anti-rabbit IgG, 1:100, and Cy3-conjugated goat anti-mouse, 1:150) for 1 hour. Samples were rinsed with PBS and cover slipped with Fluorsave (Calbiochem). Immunolabeling was examined using a Zeiss LSM 510 Meta laser scanning confocal microscope. Cellular extracts of PC12 cells were prepared in a lysis buffer containing 100 mM NaCl, 1% digitonin, 5 mM PMSF, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4 and used for co-immunoprecipitation assays.

RESULTS

Location of FXYD6 in the cochlea and interaction with Na,K-ATPase *in situ*. Previously, expression of several FXYD RNAs has been reported in the inner ear of mice (29, 30). We performed Western blot analysis, which revealed that, out of 6 FXYD proteins tested, only FXYD6 could be detected in extracts of rat inner ear (Fig.1A). FXYD6 expression was low at developmental stage P0 but increased at P12 and P30 (Fig.1A, lanes 17-19, Fig.1B). FXYD6 was revealed as a doublet with a major band at about 20 kDa corresponding to the molecular mass of FXYD6 expressed in brain (21) and a minor band at about 18 kDa of unknown origin.

The cellular localization of the FXYD6 protein in the cochlea was investigated by immunohistochemistry. In the adult rat cochlea, FXYD6 was expressed in various epithelia bordering the endolymph (the interdental cell, the outer sulcus cells and their root process and the stria vascularis) (Fig.1C, green). Moreover, the protein was expressed in somas and dendrites of the spiral ganglion neurons. FXYD6 co-localized with Na,K-ATPase (Fig.1C, red and merged) in several regions including the stria vascularis. A Na,K-ATPase α subunit antibody was able to co-immunoprecipitate FXYD6 in the inner ear at developmental stage P0 and P12 (Fig.1D) indicating that FXYD6 is associated with Na,K-ATPase.

Location of FXYD6 in the stria vascularis. FXYD6 is expressed in the stria vascularis where it co-localizes with Na,K-ATPase (Fig. 2A). A strong signal was diffusely detected in the middle part of the stria vascularis where basolateral membranes of marginal cells and apical membranes of intermediate cells are localized (Fig.6). The resolution of confocal microscopic analysis was not sufficient to precisely determine the localization of FXYD6, because the basolateral membrane of marginal cells and the apical membrane of intermediate cells are associated in close vicinity of 150~200 Å and prominently invaginated (31, 32). Barttin is a β subunit of chloride channels specifically expressed in the basolateral membrane of marginal cells (33) (Fig.6). Co-immunolabeling of FXYD6 and barttin showed co-localization of the two proteins (Fig.2B) suggesting the presence of FXYD6 in the basolateral membrane of marginal cells but not excluding expression in intermediate cells.

Subcellular localization of FXYD6 in PC12 cells. In addition to brain, Kadowaki et al. (21) have shown that FXYD6 is expressed in non-differentiated pheochromocytoma (PC12) cells. To verify whether FXYD6 co-localizes with Na,K-ATPase in these cells, we performed co-immunostaining with FXYD6 and Na,K-ATPase antibodies, which revealed co-localization of FXYD6 and Na,K-ATPase at the plasma membrane of non-differentiated PC12 cells (Fig.3A, upper panels). Moreover, in differentiated PC12 cells of neuronal phenotype, FXYD6 co-localized with Na,K-ATPase in dendrites and the cell body (Fig.3A, lower panels). Both in non-differentiated (Fig.3B, left panel) and differentiated (Fig.3B, right panel) PC12 cells, FXYD6 was associated with Na,K-ATPase since it could be co-immunoprecipitated with a Na,K-ATPase α subunit antibody.

Association of FXYD6 with Na,K-ATPase and H,K-ATPase in *Xenopus* oocytes. The association of FXYD6 with Na,K-ATPase was investigated after co-expression of FXYD6 in *Xenopus* oocytes together with Na,K-ATPase α1–β1, α2–β1, α3–β1 or α1-β2 isozymes. After metabolic labeling and various pulse–chase periods, microsomes were prepared and subjected to immunoprecipitations under nondenaturing conditions with a FXYD6 antibody. The FXYD6 antibody immunoprecipitated the 20 kDa form of FXYD6 (Fig.4A). The intensity of the FXYD6 band was intrinsically low because FXYD6 contains only 1

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methionine, which can be metabolically labeled. Western blot analysis confirmed the efficient expression of FXYD6 and also revealed the 18 kDa form of FXYD6 observed in the inner ear (data not shown). Na,K-ATPase α and β subunits of all α-β1 isozymes could be co-immunoprecipitated with FXYD6 after the pulse and prolonged chase periods (Fig.4A, lanes 1–21). Moreover, FXYD6 antibodies co-immunoprecipitated Na,K-ATPase α1-β2 isozymes (Fig.1B, lanes 1-6) which is the prominent Na,K-ATPase isozyme in the stria vascularis (34). Similar experiments were performed with gastric and non-gastric H,K-ATPase and they showed that both H,K-ATPases, co-expressed with FXYD6, could be co-immunoprecipitated with the FXYD6 antibody over prolonged chase periods (Fig.4C).

Functional effects of FXYD6 on Na,K-ATPase transport properties. The functional effects of FXYD6 on Na,K-ATPase transport properties were investigated by electrophysiological measurements in *Xenopus* oocytes expressing rat Na,K-ATPase α1–β1 or α1-β2 isozymes with or without FXYD6.

As shown in Fig.5A, Na,K-ATPase α1-β2 isozymes expressed without FXYD6 had a significantly lower apparent K^+ affinity than α1-β1 isozymes and the voltage-dependence of the $K_{1/2}$ K^+ values was more pronounced at negative membrane potentials. FXYD6 produced a small but significant decrease in the apparent K^+ affinity of the Na,K-ATPase α1-β1 isozyme at slightly negative and positive membrane potentials. In contrast, FXYD6 produced a significant increase in the apparent K^+ affinity of Na,K-ATPase α1-β2 isozymes at negative membrane potentials (Fig.5A). FXYD6 also had significant and distinct effects on the apparent affinity for internal Na^+ of the two Na,K-ATPase isozymes, and produced a 2-fold decrease in the apparent Na^+ affinity of the α1–β1 isozyme without significantly affecting maximal Na,K-pump currents, and a slight increase in the apparent Na^+ affinity of the α1-β2 isozyme (Fig.5B).

DISCUSSION

In the present paper, we provide evidence that FXYD6, the last uncharacterized FXYD protein, may also be a tissue-specific regulator of Na,K-ATPase, similar to other members of the FXYD protein family. Indeed, our results show that FXYD6 is associated with Na,K-ATPase in the inner ear and that it modifies the transport properties of different Na,K-ATPase isozymes after co-expression in *Xenopus* oocytes.

Expression of FXYD6 in the inner ear

RNA expression of several members of the FXYD family in the inner ear has been reported (29, 30). In this study, we show that, out of 6 FXYD proteins, only FXYD6 was detected in this organ at the protein level. Thus, the other FXYD proteins are either not expressed or at much lower levels than FXYD6.

Interaction of FXYD6 with P-Type ATPases.

A common feature of all FXYD proteins so far characterized is their interaction with Na,K-ATPase both in expression systems and *in situ* (for review, see (10, 11)). FXYD6 shares this property but shows a broader specificity than other FXYD proteins. Similar to mouse FXYD3 (18), FXYD6 stably interacts also with both gastric and non-gastric H,K-ATPases after expression in *Xenopus* oocytes. In the cochlea, FXYD6 is localized in neurons and interestingly, in the stria vascularis, which co-expresses Na,K-ATPase and gastric H,K-ATPase at the basolateral side of marginal cells (28) (Fig.6). Further experiments are needed to confirm the potential association of FXYD6 and H,K-ATPase in this organ.

Putative physiological relevance of the regulation of Na,K-ATPase by FXYD6 in the maintenance of the endolymph electrochemical composition.

Compared to other FXYD proteins studied, association of FXYD6 modulates the transport properties of Na,K-ATPase in a distinct way. In view of the cellular localization of FXYD6 in the cochlea, we could predict at least two sites of action, which preferentially express either Na,K-ATPase α1-β1 or α1-β2 isozymes. Interestingly, our study shows that these two rat Na,K-ATPase isozymes have different functional properties (Fig.5A). These results confirm our previous observation that β isoforms can differentially modulate the transport properties of Na,K-ATPase (9, 25).

The first site of action of FXYD6 might be the auditory neurons, mainly expressing Na,K-ATPase α1-β1 isozymes. Intracellular Na^+ concentrations in dendrites increase significantly during neuronal activity passing from 11 mM to 50 mM (35). The nearly 2-fold decrease in the apparent Na^+ affinity, produced by association of FXYD6 with Na,K-ATPase α1-β1 isozymes,

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may be necessary for the efficient extrusion of Na^+ during neuronal activity. Upon a rise in intracellular Na^+ to 50 mM, Na,K-ATPase α1-β1 isozymes with a $K_{1/2}$ Na^+ of about 10 mM (Fig.5B) would transport at maximal rates. On the other hand, Na,K-ATPase with a $K_{1/2}$ Na^+ of 20 mM, due to association with FXYD6, would have the capacity to increase its transport rate at increased intracellular Na^+ concentrations. A similar decrease in the apparent Na^+ affinity of α1-β1 isozymes is produced by FXYD1 (phospholemman) expressed in skeletal and heart muscle and has been related to efficient extrusion of increased intracellular Na^+ concentrations during action potentials in contractile tissues (14). Of course, at present, we cannot exclude that, alternatively or in addition to the observed Na^+ effect on α1–β1 Na,K-ATPase isozyme, FXYD6 may be involved in other regulatory mechanisms of the expression or function of Na,K-ATPase isozymes in neuronal cells.

The second site of action of FXYD6 might be the stria vascularis. The stria vascularis is made up of three layers of cells, epithelial marginal cells, intermediate cells and epithelial basal cells (Fig.6). Marginal cells interdigitate with intermediate cells and are separated by the intrastrial space. K^+ coming from the spiral ligament enter basal cells and intermediate cells via a dense network of gap junctions. Then it is secreted into the intrastrial space by potassium channels and taken up by marginal cells via Na,K-ATPase and other K^+ transporters e.g. H,K-ATPase. Finally, K^+ is secreted into the scala media to form the endolymph. FXYD6 is strongly expressed in the marginal cells and possibly in the intermediate cells which mainly express α1-β2 Na,K-ATPase isozymes (34). α1-β2 Na,K-ATPase complexes associated with FXYD6, with a high apparent affinity for Na^+ might be necessary to maintain the low intracellular Na^+ concentrations in marginal cells that have been reported by Ikeda et al. (36).

The intrastrial space is critical for the generation of the endocochlear potential. Indeed, the endocochlear potential is essentially a K^+ equilibrium potential that is generated by the conjunction of two K^+ channels, *Kcnj10* (Kir 4.1) and *MERG1a* located in the intermediate cells of the stria vascularis in conjunction with the very low K^+ concentration in the intrastrial fluid and a normally high K^+ concentration in the cytosol of intermediate cells (37-39). Intermediate cells might act as glial cells in siphoning the extracellular K^+ (40). Na,K-ATPase and, as suggested by Nie et al. (39), *Kcnj10* may be implicated in this process, which would permit the secretion of K^+ by the *MERG1a* channel. At 1 mM K^+ in the intrastrial space (37), the α1–β2 isozyme with a $K_{1/2}$ K^+ of about 1.7 mM (Fig.5) would not efficiently transport K^+ at a membrane potential of –110 mV characteristic of the intermediate cells (37). However, an FXYD6-associated α1-β2 complex, with a higher apparent affinity for K^+, would be able to transport at a more efficient rate, and keep a low K^+ concentration in the intrastrial space.

In conclusion, in this study, we have characterized the last FXYD protein of unknown function. Our results show that FXYD6 associates with Na,K-ATPase as other FXYD proteins and has a distinct functional effect on Na,K-ATPase indicating that all mammalian FXYD proteins associate with and modulate the transport properties of Na,K-ATPase in a tissue-specific way. The localization of FXYD6 in the inner ear suggests an important role in endolymph production.

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FIGURE LEGENDS

Fig. 1: FXYD6 is associated with Na,K-ATPase in the inner ear. (A) Expression of FXYD6 in the inner ear. Five μg protein of inner ear extracts from developmental stage P0 (lanes 1, 5, 9, 13, 17, 21), P12 (lanes 2, 6, 10, 14, 18, 22) or P30 (lanes 3, 7, 11, 15, 19, 23) or 10 μg protein of oocyte microsomes (lanes 4, 8, 12, 16, 20, 24) were tested by Western blotting for the expression of FXYD proteins with specific antibodies to FXYD1, FXYD2, FXYD3, FXYD4, FXYD6 and FXYD7. (B) Aliquots (5 μg of protein) from inner ear extracts at different postnatal stages (P0, P12, P30) were used in Western blots and probed with a FXYD6 antibody. (C) Rat inner ear slices (14 μm) were used for triple immunostaining with a FXYD6 antibody (green), a polyclonal parvalbumin antibody (blue) to specifically immunolabel inner hair cells and radial dendrites (41), and a monoclonal Na,K-ATPase α1 subunit antibody (red). After merge, co-localization is indicated in yellow. Magnification 10 x. Bar = 10 μm. SG = spiral ganglion, IC = interdental cells, D = dendrites, OSC = outer sulcus cells, RP = root process, SV = stria vascularis. (D) Aliquots (10 μg of protein) from extracts of inner ear at P0 were directly loaded on a Tricine-gel (input). Other samples of inner ear at P0 or P12 were first immunoprecipitated (50-100 μg of protein) with an antibody against Na,K-ATPase α subunit (IP α) or a preimmune serum (IP PI) (lower panel) under nondenaturating conditions. Proteins were transferred onto nitrocellulose membranes, and Western blot analysis was performed using an FXYD6 antibody.

Fig. 2: FXYD6 is co-localized with Na,K-ATPase in the stria vascularis. (A) Rat inner ear slices were used for double immunostaining with a FXYD6 antibody (green) plus a monoclonal Na,K-ATPase α1 antibody (red). After merge, co-localization is indicated in yellow. Magnification 63 x. (B) Rat inner ear slices were used for double immunostaining with a FXYD6 antibody (red) and a chicken barttin antibody (green). After merge, co-localization is indicated in yellow. Magnification 40 x.

Fig. 3: Subcellular localization of FXYD6 in PC12 cells. (A) Confocal microscope analysis of non-differentiated (upper panel) and differentiated (lower panel) PC12 cells using double immunostaining with a FXYD6 antibody (green) and a monoclonal Na,K-ATPase α1 antibody (red). After merge, colocalization is indicated in yellow. (B) Extracts from non-differentiated (ND, left panel) or differentiated (D, right panel) PC12 cells were directly loaded on a SDS polyacrylamide gel (10 μg of protein, input) or first immunoprecipitated (100 μg of protein) with a Na,K-ATPase α1 subunit antibody (IP α) or with preimmune serum (IP PI) under nondenaturating conditions. Proteins were transferred onto nitrocellulose membranes, and Western blot analysis was performed using a FXYD6 antibody.

Fig. 4: Association of FXYD6 with P-type ATPases in *Xenopus oocytes*. Oocytes were injected with rat Na,K-ATPase α1, α2, or α3 and β1 (A) or α1 and β2 (B) subunit cRNAs (10 ng of α and 1 ng of β cRNAs) in the absence or presence of 8 ng of FXYD6 cRNA. After cRNA injection, oocytes were labeled with [^{35}S]-methionine for 6 h followed by a 24 h and 48 h chase period, and digitonin extracts were prepared. Nondenaturing immunoprecipitations were performed with a FXYD6 antibody, and the immunoprecipitates were resolved by SDS-PAGE and revealed by fluorography. (C) Oocytes were injected with rat colonic H,K-ATPase (c HKA) α subunit (10 ng) and rabbit, gastric H,K-ATPase β subunit (1 ng) cRNAs in the absence or presence of 8 ng of FXYD6 cRNA. Nondenaturing immunoprecipitations with a FXYD6 antibody were performed on digitonin extracts of metabolically labeled oocytes.

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Fig. 5: Effects of FXYD6 on the transport properties of Na,K-ATPase. (A) Three days after injection of rat Na,K-ATPase α1 (10 ng) and β1 (1 ng) or α1 (10 ng) and β2 (1 ng) subunit cRNAs in the absence or presence of FXYD6 cRNA (8 ng), K^+-activation constants ($K_{1/2}$ K^+) of the Na,K-ATPase were determined in the presence of 90 mM external Na^+. Closed triangles, α1 + β1 alone; open triangles, α1 + β1 plus FXYD6; closed circles, α1 + β2 alone, open circles, α1 + β2 plus FXYD6. Shown are means ± SE of 20 oocytes from two different batches. * refers to statistical significance between α1 + β1 alone and α1 + β1 plus FXYD6 ($p<0.05$) or α1 + β2 alone and α1 + β2 plus FXYD6 ($p<0.01$). (B) Two days after injection of rat α1 and β1 or α1 and β2 cRNAs in the absence or presence of FXYD6 cRNA, epithelial Na^+ channel subunit cRNAs (α, β, and γ, 0.3 ng each) were injected. After overnight incubation, Na^+ activation constants ($K_{1/2}$ Na^+) were determined at –50 mV. Shown are means ± SE from 9-10 oocytes from five different batches. ***$p<0.001$. Maximal Na,K-pump currents, Na,K-ATPase α1 + β1 alone: 286±27 mV, Na,K-ATPase α1 + β1 plus FXYD6: 274±18 mV. Maximal Na,K-pump currents, Na,K-ATPase α1 + β2 alone: 196±27 mV, Na,K-ATPase α1 + β2 plus FXYD6: 140±18 mV. For all electrophysiological measurements, endogenous, oocyte Na,K-ATPase was inhibited by the presence of 1 μM ouabain.

Fig.6: Schematic model of the cochlear stria vascularis, K^+ secretion into endolymph and distribution of Na,K-ATPase and FXYD6. For detailed description see text.

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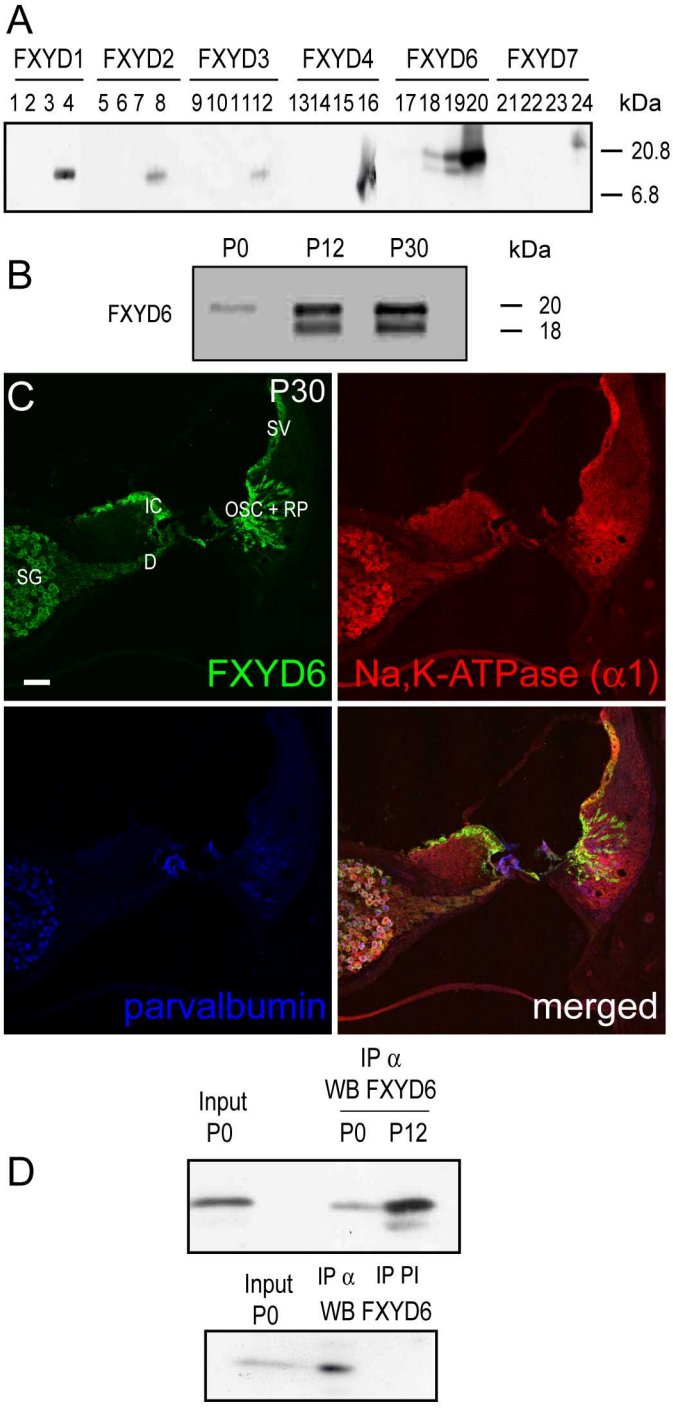


Fig.1

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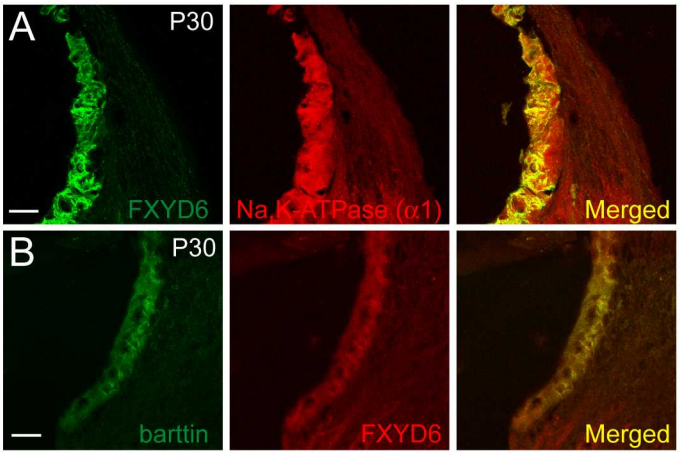


Fig.2

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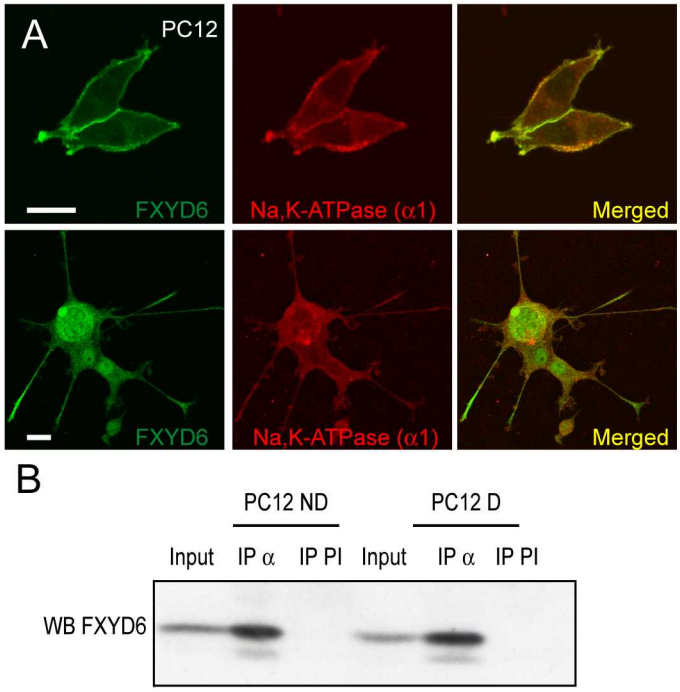


Fig.3

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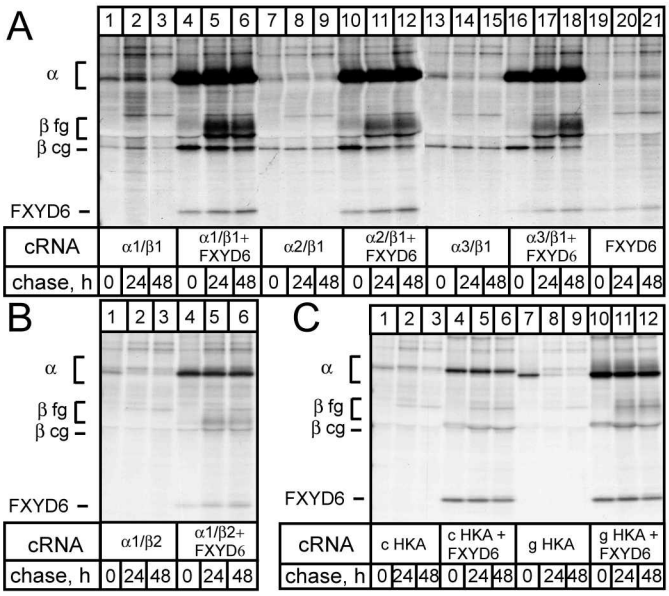


Fig.4

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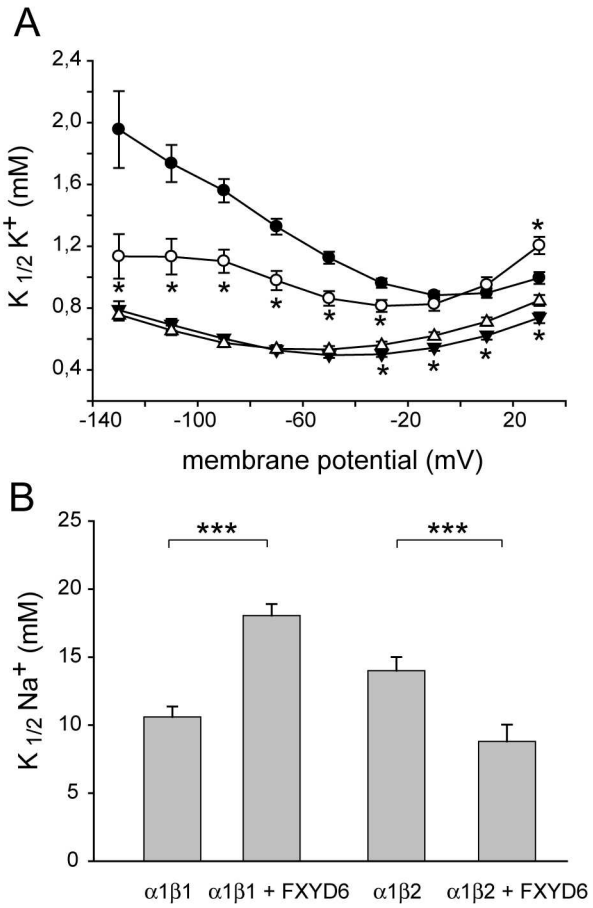


Fig.5

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