FXYD5 (dysadherin) regulates the paracellular permeability in cultured kidney collecting duct cells

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Submitted 10 March 2011; accepted in final form 1 September 2011

Lubarski I, Asher C, Garty H. FXYD5 (dysadherin) regulates the paracellular permeability in cultured kidney collecting duct cells. Am J Physiol Renal Physiol 301: F1270–F1280, 2011. First published September 7, 2011; doi:10.1152/ajprenal.00142.2011.—FXYD5 (dysadherin or RIC) is a member of the FXYD family of single-span transmembrane proteins associated with the Na\(^{+}\)-K\(^{+}\)-ATPase. Several studies have demonstrated enhanced expression of FXYD5 during metastasis and effects on cell adhesion and motility. The current study examines effects of FXYD5 on the paracellular permeability in the mouse kidney collecting duct cell line M1. Expressing FXYD5 in these cells leads to a large decrease in amiloride-insensitive transepithelial electrical resistance as well as increased permeability to 4-kDa dextran. Impairment of cell-cell contact was also demonstrated by staining cells for the tight and adherence junction markers zonula occludens-1 and \(\beta\)-catenin, respectively. This is further supported by large expansions of the interstitial spaces, visualized in electron microscope images. Expressing FXYD5 in M1 cells resulted in a decrease in N-glycosylation of \(\beta\)1 Na\(^{+}\)-K\(^{+}\)-ATPase, while silencing it in H1299 cells had an opposite effect. This may provide a mechanism for the above effects, since normal glycosylation of \(\beta\)1 plays an important role in cell-cell contact formation (Vagin O, Tokhtaeva E, Sachs G. J Biol Chem 281: 39573–39587, 2006).

FXYD proteins; RIC; Na\(^{+}\)-K\(^{+}\)-ATPase; tight junction; adherence junction

THE Na\(^{+}\)-K\(^{+}\)-ATPase (the Na\(^{+}\) pump) uses ATP to actively pump three Na\(^{+}\) ions out of the cell in exchange for two K\(^{+}\) ions flowing into the cell. The pump is composed of a catalytic \(\alpha\)- and regulatory \(\beta\)-subunit. There are several isoforms of both \(\alpha\) (\(\alpha\)1–4) and \(\beta\) (\(\beta\)1–3), which are expressed in a tissue- and development-dependent fashion (5). Many studies have provided evidence that in addition to this “classic” role, the Na\(^{+}\)-K\(^{+}\)-ATPase has both structural and signaling functions (26, 28, 41). One such mechanism involves the role of \(\beta\) Na\(^{+}\)-K\(^{+}\)-ATPase in cell adhesion and cell-cell contact (11, 34, 40). It has been demonstrated that interactions between \(\beta\)1-subunits in neighboring Madin-Darby canine kidney (MDCK) cells participate in cell-cell contacts and affect paracellular permeability (40, 41). This interaction is mediated by the carbohydrate moieties on \(\beta\)1 and is largely impaired by inhibiting its N-glycosylation.

In addition to its \(\alpha\)- and \(\beta\)-subunit, the Na\(^{+}\)-K\(^{+}\)-ATPase complex often contains a third subunit, which is a member of the FXYD family. FXYD proteins are short (<100 amino acids with the exception of FXYD5) single-span transmembrane proteins characterized by the invariant motif Phe-XXX-Tyr-Asp in their extracellular domain. All members of this group were found to specifically associate with the Na\(^{+}\)-K\(^{+}\)-ATPase and modulate its kinetic properties (9, 10, 37). They are therefore thought to act as tissue-specific regulators or auxiliary subunits of the pump, whose role is to adjust its kinetic properties to specific requirements of the cell type or the physiological state under which they are expressed, without affecting it elsewhere (9).

FXYD5 (also termed dysadherin, or RIC) is a rather unique member of the FXYD family. Like other FXYD proteins, it specifically interacts with the pump and was shown to increase its \(V_{\text{max}}\) (18, 19, 22). However, other studies also reported effects on cell adhesion, E-cadherin abundance, cell motility, and actin organization (13, 21, 22, 31, 38). FXYD5 is overexpressed in various tumors, and its expression level correlates with high metastasis and poor prognosis (for a review, see Ref. 24). A role for FXYD5 in cell adhesion and cell-cell contact is also supported by the fact that unlike all other FXYD proteins, FXYD5 has a relatively long extracellular domain (i.e., 145 vs. ~30 amino acids in other FXYD proteins), and it was suggested to be heavily glycosylated. In native rodent tissues, FXYD5 runs as a ~20-kDa polypeptide compatible with its calculated molecular weight (18, 19). In tumors and tumor-derived human cell lines, an ~50- to 55-kDa polypeptide was reported, and the difference was suggested to reflect excessive O-glycosylation of the extracellular domain (13, 38).

The current study examines effects of FXYD5 on the rodent kidney collecting duct cell line M1. We found that expressing FXYD5 in these cells results in a large increase in the paracellular permeability measured as amiloride-insensitive transepithelial resistance (TER) and permeation of 4-kDa dextran. Expression of FXYD5 in M1 cells alters the cellular distribution of the tight and adherence markers zonula occludens-1 (ZO-1) and \(\beta\)-catenin and causes large dilations of the interstitial spaces. The expression of FXYD5 was also associated with a decrease in the glycosylation of \(\beta\)1, suggesting a possible mechanism for its effects on cell-cell contacts.

MATERIALS AND METHODS

Expression and silencing of FXYD5 in cultured cells. M1 cells were purchased from the American Type Culture Collection and cultured in a 1:1 mixture of DMEM and F12 media supplemented with 5% fetal calf serum, 5 \(\mu\)M dexamethasone, and penicillin and streptomycin. Cells were transfected with mouse FXYD5 cDNA in which amino acids 103–106 were replaced by a hemagglutinin A (HA) epitope, subcloned into the pIRE5-EGFP vector. This protein region is not conserved among species and therefore assumed to have no essential function. Transfection was done using jetPEI reagent (PolyPlus Transfection) according to the manufacturer’s instructions. Positive clones were isolated by FACS and assayed for expression of FXYD5 using an anti-HA antibody. To silence the transfected cDNA, an FXYD5-expressing cell clone was further transfected with MISSION short...
pipetting and vortexing. Cell debris was removed by centrifugation off the plate, transferred to microtubes, and dispersed by repetitive EGTA, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 20 mg/ml stopped by two washings plus a 20-min incubation with 100 mM Confluent monolayers were surface biotinylated by 10-min incubation, the beads were precipitated and washed three times in the C12E10 buffer. Immunoprecipitated proteins were eluted with 1% Triton X-100. This detergent dissociates the FXYD5-Na⁺/K⁺-ATPase complex but not the association of the antibody with protein A (19). Hence contamination of the immunopellet with the antibody light chain which runs very close to FXYD5 is prevented. Coimmunoprecipitation of α-L-Na⁺/K⁺-ATPase by anti-FXYD5 was done using anti-HA antibody attached to agarse beads (HA Tag IP/Co-IP Kit 26180, Pierce). Eight hundred microliters of detergent-solubilized proteins were mixed with 40 μl anti-HA-agarose in a plugged spin column and rotated for 16 h at 4°C. The spin column was unplugged and centrifuged at 16,000 g for 10 s, and the eluant was removed. The agarose pellet was washed six times with 0.5 ml TBS and the agarose pellet was suspended and washed six times with 0.5 ml TBS+0.05% Tween 20 (TBS-T). The spin column was repurged, and the agarose pellet was suspended in 60 μl 2X nonreducing 1% SDS sample buffer. This was heated on a heat block at 95°C for 10 min, and then the column was centrifuged at 16,000 g for 10 s to elute the immunoprecipitated proteins. The eluted proteins were mixed with 1/10 volume 1 M DTT and were resolved on polyacrylamide Tris tricine gels together with a sample of denatured whole cell lysate. Gel-resolved proteins were transferred to polyvinylidene difluoride membranes using CAPS buffer plus 10% methanol (15 V for 120 min). The blots were blocked with 5% milk (1 h, room temperature) and cut into low- and high-molecular weight segments. These were incubated overnight at 4°C with various antibodies, washed, and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-coupled goat anti-rabbit or goat anti-mouse IgG (1:10,000). ECL substrate (1.25 mM luminol, 0.198 mM cumaric acid, and 0.0094% hydrogen peroxide) was added, and luminescence was visualized by X-ray film and quantified using an ImageQuant LAS 4000 mini chemiluminescent reader (General Electric). All luminescence values to be compared were within the linear concentration range.

Fluorescent and electron microscopy. For visualization of cell junction markers, M1 cells were cultured on coverslips and fixed with 3% paraformaldehyde+0.5% Triton X-100. They were incubated for 1 h at room temperature with polyclonal antibodies to either β-catenin or ZO-1 (1:500) followed by 3 ×5-min washing in PBS and a 30-min incubation at room temperature with Cy3-coupled goat anti-rabbit/mouse antibody (1:500, Jackson Laboratories). Samples were washed three times in PBS, covered with mounting medium (Immuno-mount, Thermo Scientific), and visualized by confocal microscopy (Olympus).

For electron microscopy, confluent monolayers cultivated on porous supports were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed in the same buffer, and postfixed with 1% osmium tetroxide. After en bloc staining with 2% uranylacetate in water for 1 h at room temperature, cells were dehydrated in graded ethanol solutions and embedded in Epon 812. Ultrathin sections (70- to 90-nm thickness) were prepared using an Ultramicrotome Leica UCT (Leica). They were analyzed with a 120-kV SPIRIT Transmission Electron Microscope (FEI, Eindhoven) and digitized with an EAGLE (FEI) CCD camera using TIA (FEI) software.

Transepithelial permeability measurements. Approximately 500,000 cells were seeded in 12-mm 0.4-μm PCF filter bottom cups (Millicell, Millipore). Transepithelial electrical potential and resistance were measured daily using an EVOMX voltohmeter and chopstick STX2 electrodes (World Precision Instruments). Transepithelial contributions to the current and resistance were determined by the addition of 10
μM amiloride to the apical compartment. To assess for ion selectivity via the paracellular pathway, TER was measured in the presence of amiloride in media containing 140 mM NaCl or Na-glucuronate or N-methyl-D-glucamine (NMDG)-Cl.

Macromolecule permeation was measured in confluent monolayers manifesting FXYD5-dependent differences in resistance. At time 0, mixtures of rhodamine B (1 mg/ml) and either 4- or 70-kDa FITC-dextran (1 mg/ml) were added to the upper compartment. Aliquots (100 μl) were removed from the lower compartment at 30-min intervals and replaced by an equal volume of fresh medium. The samples’ fluorescence was measured (rhodamine B: 520-nm excitation, 590-nm emission; FITC: 485-nm excitation, 544-nm emission) and corrected for dilutions in the lower compartments. Data are expressed as percentage of the equilibrium value determined in parallel using filters with no cells.

**Antibodies.** The following antibodies have been used for Western blotting at the indicated dilutions: a monoclonal antibody to the N terminus of α1-Na+−K+−ATPase (6H, 1:2,000, kindly provided by Dr. M. J. Caplan, Yale University School of Medicine); a polyclonal anti-β1-Na+−K+−ATPase (32) (1:10,000); a monoclonal anti-HA (1:2,000, Santa Cruz Biotechnology); a monoclonal 36E-cadherin antibody (1:5,000, BD Biosciences); a monoclonal anti-β-tubulin (1:40,000, Sigma-Aldrich); a polyclonal anti-β-catenin (1:500, Sigma-Aldrich); a monoclonal anti occludin (1:1,000, Invitrogen 33–1500); and a monoclonal anti-FXYD5 (1:500) (19).

**Additional materials.** 2-Acetamido-2-deoxy-α-D-galactopyranoside, FITC-dextran, rhodamine B, and amiloride HCl were purchased from Sigma-Aldrich.

**Statistics.** Data are expressed as means ± SE or means ± SD as indicated in the figure legends. Statistical significance was determined by an unpaired Student’s t-test.

**Fig. 1.** Expression of FXYD5 and its effect on β-Na+−K+−ATPase glycosylation. A: Western blot of microsomes from wild-type (WT) and two hemagglutinin (HA)-FXYD5-transfected M1 clones (4C5 and E12) with anti-HA and anti-α1-Na+−K+−ATPase. B: C12E10-solubilized M1 proteins from WT and HA-FXYD5-transfected (F5) M1 cells were immunoprecipitated with either anti-α1 (top) or anti-HA (middle and bottom) as described in MATERIALS AND METHODS. Five percent of the total detergent-solubilized proteins and the whole immunopellets were blotted with anti-HA (top), anti-α1 (middle), and anti-E-cadherin (bottom). C: Western blot of microsomes from WT and HA-FXYD5-transfected M1 cells with antibodies to α1-Na+−K+−ATPase, β1-Na+−K+−ATPase, and HA. D: top: Western blot of microsomes from WT and FXYD5 silenced (shF5; sh, short hairpin) H1299 cells with anti-α1 Na+−K+−ATPase and anti-FXYD5. Bottom: RT-PCR of total RNA from these cells with FXYD5-specific primers. E: Western blot of microsomes from WT and FXYD5-silenced H1299 cells with anti-β1-Na+−K+−ATPase.
migrated as an ~40-kDa polypeptide, much heavier than the 24-kDa band obtained in transfected M1 cells. Possible reasons for this difference and the discrepancy with the previously reported 50- to 55-kDa polypeptide are discussed in the DISCUSSION.

The data in Fig. 1 seem to indicate that the expression of FXYD5 also causes a decrease in the abundance of α1-Na\(^+-\)K\(^+-\)ATPase (cf. differences between wild-type and clone 4C5 in Fig. 1A and the difference between wild-type and shF5 clone in Fig. 1D). To further explore this issue, confluent cell monolayers from matched cultures that do or do not express FXYD5 were surface biotinylated and total and streptavidin-bound α1- and β1-Na\(^+-\)K\(^+-\)ATPase were quantified. To quantify β1, cell lysates were deglycosylated by incubation with PNGase. Indeed, a substantial downregulation of α1-Na\(^+-\)K\(^+-\)ATPase in FXYD5-transfected M1 cells was apparent, and the expression of FXYD5 lowered the total abundance of this protein by >40% (Fig. 2, Table 1). Surface expression of α1, however, was much less affected by the transfection, and the difference in the streptavidin pulled down fraction of α was hardly significant. For β, neither total nor plasma membrane protein was significantly affected by the expression of FXYD5.

Previously, it was reported that expression of FXYD5 is associated with downregulation of E-cadherin in some, but not all cell lines and tumors (3, 13, 30). We have therefore also examined the effect of FXYD5 on E-cadherin in M1 cells. No effect of FXYD5 on total or plasma membrane abundance of E-cadherin was detected (Fig. 2). Also, no physical association between FXYD5 and E-cadherin was noted (Fig. 1B, bottom). Thus in these cells the effect of FXYD5 on cell adhesion is not secondary to the downregulation of E-cadherin.

M1 cells are known to form polarized high-resistant epithelium expressing the amiloride-blockable Na\(^+\) channel ENaC in the apical membrane and Na\(^+-\)K\(^+-\)ATPase in the basolateral pole (36). Possible effects of FXYD5 on the formation of tight junctions (TJ) were measured by recording the time-dependent increase in TER in cells grown on permeable supports. Wild-type cultures formed monolayers with TER of >1 kΩ·cm\(^2\), typical of tight epithelium (Fig. 3A). These cells were also characterized by a considerable transmembrane potential, giving rise to short-circuit currents (I\(_{sc}\)) of ~25 μA/cm\(^2\) (Fig. 3B). Adding 10 μM amiloride to the luminal compartment abolished I\(_{sc}\) and further increased TER to >2.0 kΩ·cm\(^2\). Under these conditions, TER is dominated by paracellular permeability. In the FXYD5-transfected cells, much lower TER values were recorded and no response to amiloride was seen. Thus FXYD5 appears to inhibit formation of TJs. To verify that the

Table 1. Effects of FXYD5 on total and surface expression of Na\(^+\)-K\(^+-\)ATPase.

<table>
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<tr>
<th></th>
<th>Total Abundance, % of WT</th>
<th>Surface Abundance, % of WT</th>
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<tbody>
<tr>
<td>α1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
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</table>

Values are means ± SE of protein abundances in FXYD5-transfected cells from 10 plates in 6 different experiments normalized to the abundance of β-tubulin and expressed as the percentage of the values in matched wild-type (WT) cultures. Total and surface expressions of α1- and β1-Na\(^+-\)K\(^+-\)ATPase were quantified as described in MATERIALS AND METHODS. Statistical significance was calculated using a 2-tailed paired t-test.
low TER values are indeed secondary to the expression of FXYD5, we performed partial silencing of FXYD5 in a cell clone that manifests the reduced TER. The decreased expression of FXYD5 led indeed to a partial reversal of its effect, resulting in a significant increase in TER and $I_{sc}$ compared with the FXYD5-expressing cells (Fig. 3).

Next, we assessed whether the FXYD5-induced decrease in TER reflects the activation of a particular conducting pathway. Accordingly, TER values were also measured following substitution of NaCl by either NMDG-Cl or Na-gluconate (Fig. 4). The ionic substitutions had only minor effects on TER values and clearly did not abolish the FXYD5-dependent decrease in TER. Thus this decrease is not likely to reflect induction of a Na$^+$- or Cl$^-$-conducting transporter.

In principle, the above observation may be secondary to effects of FXYD5 on the rate of cell growth and inability to form confluent monolayers during the 1-wk period monitored. To exclude such a possibility, we have determined the rate of cell proliferation in wild-type and FXYD5-transfected cells. No effect of FXYD5 on the rate of cell proliferation could be detected, arguing against such an option (Fig. 5A). The possibility that the FXYD5-transfected cells fail to form confluent monolayers is further excluded by the restricted permeation of large molecules as described below. Interestingly, the FXYD5-transfected cells detached from the plate by trypsinization much more quickly than the wild-type cells, confirming differences in cell-cell and/or cell-substrate contacts (Fig. 5B). Tsuji et al. (38) reported that inhibiting O-glycosylation by incubating cells with benzyl 2-acetamido-2-deoxy-$\alpha$-D-galactopyranoside (benzyl-$\alpha$-GalNAc) decreases expression of FXYD5 and weakens its effects on cell adhesion and morphology. In our experiments, no effect of benzyl-$\alpha$-GalNAc (4 mM present during the whole cultivation period) on TER in either wild-type or FXYD5-transfected M1 cells was observed. Analysis of proteins from these cultures on acrylamide gels showed a shift in the electrophoretic mobility of a few protein bands in the benzyl-$\alpha$-GalNAc-treated sample, suggesting that the O-glycosylation inhibition does take place.

Previously, we have demonstrated that FXYD5 affects Na$^+$-K$^+$-ATPase kinetics and increases the pump’s $V_{max}$ by about twofold (18). The next set of experiments was aimed at assessing whether the effect on paracellular TER is secondary to a higher pumping rate and presumably lower cell Na$^+$. This
was done by monitoring the paracellular resistance developed in monolayers cultivated in the continuous presence of amiloride. The rationale is that blocking Na\(^+\) entry into the cells should largely reduce cell Na\(^+\) and slow down Na\(^+\) pumping to its minimal rate, \(V_{\text{max}}\). Under these conditions, no difference in the Na\(^+\)-K\(^+\)-ATPase turnover rate is expected between cells that do or do not express FXYD5. As seen in Table 2, cultivating cells in the continuous presence of amiloride did not influence the effect of FXYD5 on TER, indicating that this effect is not secondary to the increased \(V_{\text{max}}\). We have also verified that the presence of amiloride in the culture medium did not lower surface expression of the Na\(^+\)-K\(^+\)-ATPase (data not shown).

The effect of FXYD5 on paracellular permeability was further confirmed by comparing transepithelial fluxes of three cell-impermeable fluorescent compounds: rhodamine B (molecular weight 536), 4-kDa FITC-dextran, and 70-kDa FITC-dextran. All three compounds permeated empty filters equally quickly and reached equilibrium within 2 h. They permeated cell monolayers at size-dependent rates, and their uptake was linear for at least 4 h (Fig. 6A). Comparing fluxes across wild-type and FXYD5-transfected cell monolayers demonstrated substantial but not dramatic differences; i.e., the FXYD5-expressing monolayers are about twofold more permeable to 4- and 70-kDa dextran than wild-type cultures. However, they still provide an effective barrier for large molecules, and have been associated with higher intercellular water and solute permeability (20, 25). In addition, in phase-contrast microscopy, the FXYD5-expressing cells were more spread shaped than the nontransfected cells (Fig. 7).

Finally, we have examined the appearance of cell-cell junctions by electron microscopy. High-magnification images shown in Fig. 10 demonstrate that expressing FXYD5 evokes large expansions of interstitial spaces just under the TJ (asterisks in Fig. 10). Interestingly, similar dilations were reported in small intestine following glucose-induced permeation of TJ and have been associated with higher intercellular water and solute permeability (20, 25). In addition, TJ in FXYD5-expressing cells appeared somewhat wider and had less electron-dense material. Thus both staining junctional markers and electron microscopic images demonstrate the effects of FXYD5 on the structure of cell-cell junctions, which presumably lead to higher paracellular permeability.

**DISCUSSION**

The current study examines the effects of FXYD5 on cell-cell contacts in a tight epithelium cell line. It was found that expressing FXYD5 in M1 cells decreases paracellular electrical resistance and increases its permeability to macromolecules. Cell-cell junctions of the transfected cells are wider, and impairment of TJ and AJ are also manifested by the cellular distribution or abundance of ZO-1, occludin, and β-catenin. The decrease in TER, increased permeability to dextran, and redistribution of ZO-1 demonstrate that TJ are affected, while the electron microscopy data and β-catenin staining suggest impairment of AJ. These observations, however, do not necessarily mean that both structures are independently affected.

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**Table 2. Effects of amiloride on TER**

<table>
<thead>
<tr>
<th>Addition</th>
<th>TER, kΩ cm(^{-2})</th>
<th>WT</th>
<th>FXYD5-transfected</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>1.50 ± 0.09</td>
<td>0.61 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>+ Amiloride. at day 7</td>
<td>2.82 ± 0.43</td>
<td>0.66 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>+ Amiloride at day 1</td>
<td>2.70 ± 0.18</td>
<td>0.65 ± 0.04</td>
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</table>

Values are means ± SE of transepithelial electrical resistance (TER) values at day 7 from 15 filters in 3 different experiments averaged in each group. WT and FXYD5-expressing cells were cultivated on porous supports for 7 days. The cells were divided into 2 groups. One group was grown as usual, and TER was measured at day 7 before and after the addition of 10 μM amiloride. The other received 10 μM amiloride at day 1 and was grown in the continuous presence of the channel blocker.
permeability was apparent at very different pumping rates. A likely mechanism is that impairment of cell-cell contacts is due to FXYD5’s effect on the structure of the Na\(^{+}\)-K\(^{+}\)-ATPase and possibly on the decrease in β glycosylation. Several studies have provided evidence that β-Na\(^{+}\)-K\(^{+}\)-ATPase functions as a cell adhesion molecule (11, 34, 40). Such a role is also supported by the finding that the structure of the β1 ecto domain resembles an immunoglobulin-like fold, typical of cell adhesion molecules (1). It has been demonstrated that interactions between β1-subunits in neighboring MDCK cells participate in cell-cell contacts and affect paracellular permeability (40, 41). This interaction is mediated by the carbohydrate moieties on β1 and is largely impaired by inhibiting N-glycosylation of β. Thus a decrease in β glycosylation may account for the FXYD5-induced impairment of cell-cell contacts characterized in the current study. The decrease in glycosylation may be due to specific interactions between the ecto domains of β and FXYD5, which limit β accessibility to glycosylation in the ER or Golgi. Specific interactions between the FXYD motif of FXYD10 and β1 are apparent from the three-dimensional structure of the shark Na\(^{+}\)-K\(^{+}\)-ATPase and likely to be relevant to other FXYD proteins as well (33). Also, we found that surface biotinylation of FXYD5 with N-hydroxysuccinimide ester derivatives is very inefficient even though the extracellular domain has seven lysine residues. This could be due to a strong interaction of FXYD5 with other proteins and limited accessibility of the extracellular lysines to biotinylation. A rather intriguing observation is the significant downregulation of α1 by the expression of FXYD5 depicted in Fig. 2 and Table 1. A similar observation has been made before in X. laevis oocytes injected with FXYD5 cRNA (18). It may reflect some interference by FXYD5 of the α-β association and thereby α destabilization. However, since surface expression of the Na\(^{+}\)-K\(^{+}\)-ATPase was hardly affected by the decrease in α1 expression, this effect is unlikely to contribute to the decrease in cell-cell contacts.

Other mechanisms by which interactions between FXYD5 and the Na\(^{+}\)-K\(^{+}\)-ATPase affect paracellular permeability through modified cytoskeletal organization are possible as well. Rajasekaran and coworkers (2, 16, 27) have reported that binding of the p85 subunit of phosphatidylinositol 3-kinase (PI3K) to the cytoplasmic C tail of α-Na\(^{+}\)-K\(^{+}\)-ATPase activates PI3K and produces phosphatidylinositol 3,4,5-trisphosphate (PIP3). Annexin II interacts with PIP3 and the pump’s β-subunit to recruit Rac1 and promote lamellipodia formation. Independently, the Na\(^{+}\)-K\(^{+}\)-ATPase was shown to associate with protein phosphatase-2A (PP2A), and its inhibition resulted in hyperphosphorylation of occludin and paracellular permeabilization (29). Thus association of FXYD5 with the Na\(^{+}\)-K\(^{+}\)-ATPase may influence a number of cellular processes, affecting cytoskeletal organization and cell-cell contacts, independently of its enzymatic activity.

FXYD5 has been originally cloned as an mRNA induced in NIH 3T3 cells by the oncoprotein E2a-Pbx1 (7). The human ortholog was identified as a cancer-associated membrane protein whose expression inhibits E-cadherin and promotes metastasis (13). In a number of clinical studies, a correlation was established between its abundance and the progression and survival chances of various human cancers (for a review, see Ref. 24). Weakening of cell-cell contacts is certainly in agreement with promotion of metastasis and the above clinical
correlations. Interestingly, effects on paracellular resistance were also reported for FXYD3 (Mat-8), another family member overexpressed in cancer cells. Silencing endogenous FXYD3 in Caco-2 cells lowered their transepithelial resistance by twofold (4). While this effect is in the opposite direction of those described in the current study, it may reflect a more general role of FXYD proteins in cell-cell contact.

In previously reported cancer-related studies, FXYD5 migrated as a 50- to 55-kDa polypeptide, much heavier than the <20 kDa predicted by its amino acid sequence. Since the ecto domain of FXYD5 is enriched in serines, threonines, and prolines, and protein expression was downregulated by benzyl-α-GalNAc, it was suggested that FXYD5 in tumor cells is highly O-glycosylated (13, 38). In transfected M1 cells, however, FXYD5 migrated as an ~24-kDa polypeptide, close to the calculated molecular weight. Taken together with the lack of effect of benzyl-α-GalNAc on TER, the data suggest that excessive O-glycosylation of FXYD5 may be characteristic of

Fig. 7. Cellular distribution of the adherence junction marker β-catenin. WT and FXYD5-transfected cells were grown on glass slides. Confluent monolayers were fixed, stained for β-catenin, and visualized by confocal microscopy, as described in MATERIALS AND METHODS.

Fig. 8. Cellular distribution of the tight junction marker zonula occludens-1 (ZO-1). WT and FXYD5-transfected cells were grown on glass slides. Confluent monolayers were fixed, stained for ZO-1, and visualized by confocal microscopy.
the metastatic state but is not essential for the weakening of cell-cell contacts. Interestingly, in H1299 cells a much larger polypeptide of \( \sim 40 \) kDa was apparent. A similar difference in size between the human and mouse proteins has also been reported (21). It cannot be accounted for by sequence differences and may reflect a difference in O-glycosylation of the human and mouse protein or a difference between cells derived from normal (M1) vs. tumor (H1299) tissues.

Another difference from previous data is the fact that we were unable to detect FXYD5-dependent downregulation of either total or surface expressed E-cadherin as reported (13). However, a second E-cadherin-independent mechanism by
which FXYD5 may affect invasion and metastasis has been described (23). This mechanism involves upregulation of the chemokine ligand CCL2, which in turn exerts autocrine and paracrine tumor-promoting effects. Interestingly, CCL2 was found to induce disassembly of the TJ complex in the blood-brain barrier by triggering caveolae-dependent internalization of transmembrane TJ proteins (35). Since FXYD5 is expressed not only during metastasis but also in a variety of normal epithelia (18, 19), one may wonder whether the observed effects on TJ and AJ serve a particular physiological role. One possibility is that this is an integral component of “leaky” low-resistance epithelia. Support for this notion is the fact that in the gastrointestinal tract FXYD5 is relatively abundant in the low-resistance small intestine (duodenum, jejunum, and ileum) and much less in tight epithelium like the distal colon (18). However, FXYD5 was also detected in tight epithelia such as the kidney collecting duct and lung. However, in the collecting duct it is expressed only in intercalated cells and not in the Na\(^{+}\)-transporting principal cells, while its cellular distribution along the airway epithelia has not yet been determined (18).

Another option is that FXYD5 participates in a mechanism responsible for a transient permeation of the paracellular pathway. At least two such mechanisms have been described. One is enhancement of paracellular permeability of small intestine to increase nutrient absorption, triggered by high luminal glucose or alanine (39). This process is characterized by large dilations of the interstitial space, very similar to those shown in Fig. 10 (20, 25). A second option is the cytokines induced permeabilization of TJ to allow egression of leukocytes from the interstitial space to the lumen in intestinal, pulmonary, and renal epithelia (6, 39). Finally, FXYD5 may be involved in epithelial remodeling and recovery from injury (15). This option is supported by the fact that its expression also induces the epithelial-mesenchymal transition marker vimentin (21). Obviously, all putative roles will also involve regulation of the expression or activity of FXYD5. Testing these options and elucidating the physiological role of FXYD5 await further studies.

ACKNOWLEDGMENTS

The authors thank Prof. Benjamin Geiger from the Department of Molecular Cell Biology, The Weizmann Institute of Science, for useful discussions and Dr. Vera Shinder from the Moskowitz Center, The Weizmann Institute of Science, for electron microscope images. H. Garty is the incumbent of the Hella and Derrick Kleeman Chair of Biochemistry.

GRANTS

This study was supported by a research grant from the Israel Science Foundation to H. Garty.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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