Myosin 5b loss of function leads to defects in polarized signaling: implication for microvillus inclusion disease pathogenesis and treatment

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Submitted 16 May 2014; accepted in final form 11 September 2014

Kravtsov D, Mashukova A, Forteza R, Rodriguez MM, Ameen NA, Salas PJ. Myosin 5b loss of function leads to defects in polarized signaling: implication for microvillus inclusion disease pathogenesis and treatment. Am J Physiol Gastrointest Liver Physiol 307: G992–G1001, 2014. First published September 25, 2014; doi:10.1152/ajpgi.00180.2014.—Microvillus inclusion disease (MVID) is an autosomal recessive condition resulting in intractable secretory diarrhea in newborns due to loss-of-function mutations in myosin Vb (Myo5b). Previous work suggested that the apical recycling endosomal (ARE) compartment is the primary location for phosphoinositide-dependent protein kinase 1 (PDK1) signaling. Because the ARE is disrupted in MVID, we tested the hypothesis that polarized signaling is affected by Myo5b dysfunction. Subcellular distribution of PDK1 was analyzed in human enterocytes from MVID/control patients by immunocytochemistry. Using Myo5b knockdown (kd) in Caco-2BBe cells, we studied phosphorylated kinases downstream of PDK1, electrophysiological parameters, and net water flux. PDK1 was aberrantly localized in human MVID enterocytes and Myo5b-deficient Caco-2kd cells. Two PDK1 target kinases were differentially affected: phosphorylated atypical protein kinase C (aPKC) increased fivefold and phosphoprotein kinase B (PPKB) decreased compared with control. PDK1 redistributed to a soluble (cytosolic) fraction and copurified with basolateral endosomes in Myo5b kd. Myo5b kd cells showed a decrease in net water absorption that could be reverted with PDK1 inhibitors. We conclude that, in addition to altered apical expression of ion transporters, depolarization of PDK1 in MVID enterocytes may lead to aberrant activation of downstream kinases such as aPKC. The findings in this work suggest that PDK1-dependent signaling may provide a therapeutic target for treating MVID.

Apical recycling endosome; atypical protein kinase C; endothelial polarity; phosphoinositide-dependent protein kinase 1; small Ras-like guanosine 3′,5′-monophosphate

LOSS-OF-FUNCTION MUTATIONS in the unconventional myosin Vb (Myo5b) result in microvillus inclusion disease (MVID), a condition characterized by severe untreatable diarrhea with an onset in the first months after birth (3, 6, 27, 36). Currently, parenteral hydration and nutrition are the only palliative treatments. Full gut transplantation is the only available treatment. The diagnosis is based on the finding of Periodic acid-Schiff-positive inclusions in enterocytes, along with decreased cluster of differentiation 10 (CD10) and small Ras-like GTPase (Rab) 8. At the ultrastructural level, microvillus atrophy and the presence of characteristic large inclusions containing brush-border-like microvilli (microvillus inclusions, MI) in about one-fifth of the enterocytes complete the diagnosis (1, 43). Several apical membrane proteins are mislocalized in MVID villus enterocytes, including CD10, cystic fibrosis transmembrane conductance regulator (CFTR), sodium-hydrogen exchanger-3 (NHE3), sucrase isomaltase, alkaline phosphatase, villin, and carinoembryonic antigen. There is also a decrease and relocalization of the Ras-related protein small GTPases Rab8 and Rab11 that reflect disruption of the apical endosomal compartment (1, 15, 37, 47, 49). Typically, these proteins realocalize to the MI, or to a subapical compartment, and are depleted in the apical pole of the cells.

While MVID diarrhea is considered to be secretory, transport analyses of intestinal patient mucosae revealed substantial decreases in unidirectional Na+ fluxes. A greater decrease of mucosal-to-serosal Na+ flux (83%) than the decrease in serosal-to-mucosal flux (70%) was observed. This observation implies that an imbalance of secretion over absorption results in a net secretory Na+ flux in MVID patients, as opposed to the normal net absorptive flux (41), but not a net increase in secretion. The molecular mechanisms underlying these functional changes that lead to severe diarrhea (>120 ml·kg−1·day−1) in children with MVID remain unclear.

Because Myo5b regulates vesicular transport from the apical recycling endosomal (ARE) compartment to the apical membrane (55), there is no question now that the pathogenic mechanism of MVID is a defect in membrane traffic (1). However, some features of the MVID phenotype suggest that membrane traffic alone cannot explain the disease. For example, it remains unclear why Myo5b is expressed in many epithelia but the MVID phenotype is unique to the gut (13). Likewise, because of the magnitude of the diarrhea, one would predict a crypt phenotype. Surprisingly, brush-border membrane defects and MIs are classically observed in mature villus enterocytes of the small intestine (1, 3), which are involved in absorption.

Recently, we demonstrated that a master activator of several important signaling cascades, the phosphoinositide-dependent protein kinase 1 (PDK1), is associated with the ARE and the apical membrane in intestinal epithelia (24). This kinase is necessary to activate other kinases that, in turn, play essential roles in epithelial polarization and transport. Pathways activated by PDK1 include protein kinase B (Akt), atypical protein kinase C (aPKC), and serum/glucocorticoid-regulated kinase 1 (SGK). Akt has been directly implicated in NHE3 exocytosis (22). NHE3 is a major transporter involved in Na+ absorption
on the apical membrane of mature enterocytes. SGK is involved in the activation of NHE3 (10, 40). Finally, aPKC (λ, ζ, and η isoforms) determines the localization and organization of tight junctions (TJ) during the onset of polarization (48) and has also been implicated in apical exocytosis (58). Akt2 is localized to the apical domain of villus enterocytes (21), whereas aPKC is apical in the crypt epithelium (54) and then restricted to the TJ region in differentiated villus enterocytes (53). In other words, these pathways are polarized because of the highly restricted localization of specific kinases.

Myo5b associates with ARE in epithelial cells by binding to small GTPase proteins such as Rab11a/b and Rab8 (16). Furthermore, Rab11, an ARE marker, is delocalized in MVID enterocytes (49, 50). Therefore, because ARE is disrupted in MVID-affected cells (12), it was natural to speculate that Rab11a/b and Rab8 (16). Furthermore, Rab11, an ARE marker, is delocalized in MVID enterocytes (49, 50). Therefore, because ARE is disrupted in MVID-affected cells (12), it was natural to speculate that Rab11a/b and Rab8 are involved in the activation of NHE3 (10, 40). Finally, aPKC (λ, ζ, and η isoforms) determines the localization and organization of tight junctions (TJ) during the onset of polarization (48) and has also been implicated in apical exocytosis (58). Akt2 is localized to the apical domain of villus enterocytes (21), whereas aPKC is apical in the crypt epithelium (54) and then restricted to the TJ region in differentiated villus enterocytes (53). In other words, these pathways are polarized because of the highly restricted localization of specific kinases.

Myo5b loss-of-function mutations.

In this work, we undertook to test the hypothesis that Myo5b, which is essential for the maintenance of apical structures, may be involved in the activation of NHE3 (10, 40). Finally, aPKC (λ, ζ, and η isoforms) determines the localization and organization of tight junctions (TJ) during the onset of polarization (48) and has also been implicated in apical exocytosis (58). Akt2 is localized to the apical domain of villus enterocytes (21), whereas aPKC is apical in the crypt epithelium (54) and then restricted to the TJ region in differentiated villus enterocytes (53). In other words, these pathways are polarized because of the highly restricted localization of specific kinases.

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Materials and Methods

Sample preparation. Deidentified paraffin blocks were retrieved from a repository at the Department of Pathology. Accordingly, the protocol received a nonhuman determination under 45 CFR 46 from the Institutional Review Board.

Cells. Caco-2 and Caco-2BBe cells were originally obtained from ATCC and cultured as described before (45). For experiments, the cells were cultured on 3-μm-pore Transwell filters (Corning) at confluency for 12 days (13–15 days after plating). For lentiviral transduction, the cells were infected with high-titer particles and rapidly selected in puromycin. Typically, the cultures were used for no more than 10 passages, and all the experiments include data from multiple transductions.

Antibodies and reagents. The following were used: anti-Myo5b (NB10-87746; Novus Biologicals), PDK1 (A302-1308; Bethyl), Rab11 [3539 (Cell Signaling); 05-853 (Millipore)], pT555 aPKC (GTX25813; GeneTex), phospho (p)-Akt (9275S; Cell Signaling), Rab11 [3539 (Cell Signaling); 05-853 (Millipore)], pT555 aPKC (GTX25813; GeneTex), phospho (p)-Akt (9275S; Cell Signaling), and its effects have been described before (24). Briefly, confluent, differentiated cells were incubated overnight with the standard medium supplemented with 30 μg/ml transferrin (Tfn) to label endosomes. Cells (1.5 × 10⁶) were suspended in 1 ml PBS supplemented with 1 mM EGTA and antiproteases (P8340; Sigma). The cells were homogenized by 30 strokes in a Teflon pestle homogenizer on ice and spun at 3,000 g for 5 min. The supernatant was then loaded on a 10–40% sucrose gradient in PBS and spun for 20 h at 100,000 g. The gradient was collected in 11 fractions. Any pellet at the bottom of the tube was resuspended in PBS and analyzed as the twelfth fraction. PDK1 enzymatic activity was measured in six aliquots of each fraction in triplicate using the Kinase Assay/Inhibitor Screening Kit (CycLex, MBL) according to the manufacturer’s protocol. Background was estimated in three of the aliquots without adding ATP. Only readings from fractions containing any detectable PDK1 in the immunoblot were shown.

Immunofluorescence procedures were standard. For Rab11 and PDK1, the cells were grown on filters and fixed in 3% paraformaldehyde, 0.1% glutaraldehyde, and then permeabilized in 0.4% saponin. The latter was kept in all solutions to incubate or wash antibodies. The use of detergent to permeabilize the cells was avoided. For immunofluorescence using antiphospho epitope antibodies, the cells were fixed in 10% trichloroacetic acid (17).

The fluorescent images were analyzed with a Leica SP5 confocal microscope using a ×63 oil immersion objective. Confocal stacks were obtained at 1,024 × 1,024 resolution and 0.5 μm spacing. For three-dimensional reconstructions (z–x–y view), confocal stacks were exported to Slidebook (3.1).

Transmission electron microscopy (TEM) of cells grown on filters was performed as described before (18) with the following modifications: filter-grown Caco-2 and Caco-2BBe cells were fixed in 2% gluteraldehyde, 0.2% tannic acid, and 20 mM EGTA in 0.1 M sodium phosphate buffer, pH 7.0, for 10 min at room temperature followed by 50 min on ice, washed, and postfixed in 1% osmium tetroxide, pH 6.5, for 1 h, pretrimmed with 1% uranyl acetate overnight at 4°C, dehydrated, and embedded in EMBed 812 resin (Electron Microscopy Sciences, Hatfield, PA). After solidification, blocks were sectioned on the ultramicrotome at 60 nm setting (silver or silver-gold colored section appearance), and grids with sections were stained with 1% uranyl acetate for 20 min and Reynold’s lead citrate for 1 min at room temperature. Grids were examined under a JEOL 1230 TEM (JEOL, Tokyo, Japan) equipped with a Hamamatsu Orca HR camera (Hamamatsu, Hamamatsu City, Japan).

Cell fractionation. Cell fractionation was performed as described before (24). Briefly, confluent, differentiated cells were incubated overnight with the standard medium supplemented with 30 μg/ml transferrin (Tfn) to label endosomes. Cells (1.5 × 10⁶) were suspended in 1 ml PBS supplemented with 1 mM EGTA and antiproteases (P8340; Sigma). The cells were homogenized by 30 strokes in a Teflon pestle homogenizer on ice and spun at 3,000 g for 5 min. The supernatant was then loaded on a 10–40% sucrose gradient in PBS and spun for 20 h at 100,000 g. The gradient was collected in 11 fractions. Any pellet at the bottom of the tube was resuspended in PBS and analyzed as the twelfth fraction. PDK1 enzymatic activity was measured in six aliquots of each fraction in triplicate using the Kinase Assay/Inhibitor Screening Kit (CycLex, MBL) according to the manufacturer’s protocol. Background was estimated in three of the aliquots without adding ATP. Only readings from fractions containing any detectable PDK1 in the immunoblot were shown.

Transepithelial electrical resistance and short-circuit current measurements. For electrophysiological measurements, the cells were grown on Snapwell 0.4-μm pore filters (Corning) as described above. At the time of measurement, the filters were inserted in a Ussing chamber filled with standard Ringer solution supplemented with 5 mM glucose at 37°C. The chambers were continuously agitated by air bubbling. Measurements were performed via two pairs of Ag/AgCl electrodes through 3 KCl-agarose bridges using a VCC MC6 Multichannel voltage-current clamp (Physiologic Instruments) and recorded via AcqKnowledge software.

Water fluxes. Caco-2BBe cells were grown in 6-mm 3-μm-pore Transwell filters as described above. Before the experiment, tightness of the monolayer was determined by filling the upper chamber to the top. Only filter chambers that could maintain the media level imbalance for over 24 h were used. For each experimental condition, filters in sextuplicate were used. At the beginning of the measurement, media was replaced in both chambers with fresh DMEM-F-12 10% FCS, supplemented with 100 μM penicillin, 100 μg/ml of streptomycin, and 0.25 μg/ml amphotericin. Monolayers of cells were covered with 100 μl (apical “filter” chamber) and with 625 μl in the basal chamber, which resulted in leveled solutions in both chambers. The filter chambers were weighted (including the acrylic ring) after drying the external surface with filter paper, using a Metler Toledo precision balance. A parallel set of filters, sealed with paraffin and without cells, was loaded with an identical amount of apical chamber medium to control for evaporation. All filter chambers were incubated
for 18 h in a regular humidified CO₂ incubator. Weights were measured and repeated after that period. Next, the apical medium was replaced by the same volume containing 100 mM sucrose. The filters were then weighed again and 3 h later to determine osmotic fluxes. Typically, raw data were as follows: total weight of the filter chambers and solution was in the range of 600–700 mg. Weight was reproducible for the same filter to a 1-mg precision. Evaporative loss resulted in 5–15 mg weight loss per filter in the same period. Conversely, osmotic fluxes resulted in 5–20 mg weight loss per filter in the same period. Isoosmotic fluxes resulted in 5–20 mg weight loss per filter in the same period. Conversely, osmotic fluxes resulted in 5–15 mg weight gains in 3 h.

**Statistics.** Data are presented as averages ± SD. Significance was analyzed by Student’s t-test. P values are indicated in the legends for Figs. 1–7.

**RESULTS**

**PDK1 is delocalized in MVID enterocytes and in Myo5b knockdown intestinal cells.** First, we wanted to verify if the polarized distribution of PDK1 observed in mouse small intestine is also present in human enterocytes and affected in MVID. To this end we analyzed existing deidentified paraffin blocks and biopsies from five patients with unrelated diseases (control, 3 small intestine, 2 colon) and from five MVID patients (3 small intestine, 2 colon). After antigen retrieval, the sections were stained with an anti-PDK1 antibody or an isotype control at the same dilution. As expected, in the control samples, PDK1 signal appeared in the subapical region (Fig. 1), even in villus enterocytes. This is a difference with the mouse, where such a distribution was only observed in the crypts, but not in villus (24). Conversely, in MVID patient samples, the distribution was diffuse, mostly perinuclear and in the basal pole of the cells, with some level of apical distribution remaining only in a fraction of the cells (Fig. 1). A similar observation was recently reported in one MVID patient by another group (5). In the MVID colon samples, there was also an overall increase in PDK1 signal throughout the cytoplasm compared with controls (data not shown). Because immunocytochemistry is not quantitative, it is not possible to assert whether the MVID images represent an increase in PDK1 signal or a change in its subcellular localization.

To independently confirm this result, we used Caco-2BBe human colon carcinoma cells, which differentiate and become polarized in culture (25). To this end we analyzed existing deidentified paraffin blocks and biopsies from five patients with unrelated diseases (control, 3 small intestine, 2 colon) and from five MVID patients (3 small intestine, 2 colon). After antigen retrieval, the sections were stained with an anti-PDK1 antibody or an isotype control at the same dilution. As expected, in the control samples, PDK1 signal appeared in the subapical region (Fig. 1), even in villus enterocytes. This is a difference with the mouse, where such a distribution was only observed in the crypts, but not in villus (24). Conversely, in MVID patient samples, the distribution was diffuse, mostly perinuclear and in the basal pole of the cells, with some level of apical distribution remaining only in a fraction of the cells (Fig. 1). A similar observation was recently reported in one MVID patient by another group (5). In the MVID colon samples, there was also an overall increase in PDK1 signal throughout the cytoplasm compared with controls (data not shown). Because immunocytochemistry is not quantitative, it is not possible to assert whether the MVID images represent an increase in PDK1 signal or a change in its subcellular localization.

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Figs. 1–7. **Fig. 1.** Redistribution of phosphoinositide-dependent protein kinase 1 (PDK1) signal in microvillus inclusion disease (MVID) patient intestinal samples. Immunocytochemistry of PDK1 in human samples from patients with unrelated disease (control) or MVID (MVID and isotype control). Arrow shows normal apical distribution. Bars, 10 μm.

**Fig. 2.** Immunoblot analysis of Caco-2BBe cells constitutively expressing scrambled (s) or anti-Myo5b short-hairpin RNA (shRNA) [knockdown (kd)]. A: expression of myosin Vb (Myo5b), PDK1, phospho-(p)-protein kinase C (PKC) ε turn domain (T555), and PKCε/γ, B: in a separate set of experiments, expression and phosphorylation of protein kinase B (Akt) was determined by immunoblot. C: quantification of the signal in the kd extracts relative to the signal in scrambled (both adjusted to loading control) in independent experiments like those shown in A and B (n = 4). *P < 0.01.
well polarized in culture (34). They are considered a model of villus enterocytes in culture because they express NHE3 and CFTR (2, 19, 59). We used shRNA transduced by lentiviral particles followed by puromycin selection. A scrambled shRNA was used as a control. Recently, another group showed that parental Caco-2 cells under lentiviral-delivered constitutive expression of Myo5b shRNA, or under RNAi treatment, reproduce many MVID features (42, 50). The Myo5b knock-
down (kd) was very effective (Fig. 2A). In the same cells, there was little or no change in PDK1 levels. However, the phosphorylation status of a PDK1 target (29), the active forms of αPKC (pT555), increased fivefold (Fig. 2C). Phosphorylation of another PDK1 target, Akt, was not significantly altered (Fig. 2, B and C).

The functional effects of Myo5b kd were first assessed by the development of MI. As a brush-border marker, we used pT567-ezrin, which is the activated form of ezrin. In cells expressing scrambled shRNA, ezrin was localized exclusively to the brush border. Less than 1% of the cells showed MIs, which are characteristic of cell lines derived from carcinomas (52). Conversely, stable transduction of anti-Myo5b shRNA rendered a proportion of cells with MIs that matches that of the human MVID (~20%) phenotype. The MIs were located deep in the cytoplasm at or below the level of the nucleus (Fig. 3A). These results were independently confirmed by TEM (Fig. 3B) and fluorescent phalloidin staining (Fig. 3C).

Next, we tested whether Myo5b kd affects the ARE in cultured cells similar to MVID patients. Rab11, an ARE marker, appeared uniformly distributed under the apical domain in control (scrambled shRNA) cells. This Rab11 apical layer became very discontinuous in most Myo5b kd cells. In fact, Rab11 signal coalesced in spherical supranuclear structures in 18% of the cells (Fig. 3D). The distribution of these structures was very different compared with the subcellular distribution of MIs as shown by phosphoezrin (Fig. 3D).

We then determined the localization of PDK1 to test whether or not a subcellular redistribution could be induced by Myo5b kd, similar to that seen in MVID samples. As described before (24), PDK1 was apically localized in scrambled control shRNA cells. In contrast, PDK1 was concentrated in the basal pole of the Myo5b kd cells (Fig. 3D). This is consistent with the delocalization of PDK1 observed in human MVID enterocytes (Fig. 1). Because the immunoblot analysis showed a substantial increase in active αPKC in Myo5b kd cells, we also localized the active form of this kinase (phosphorylated turn domain). In control cells, it was exquisitely localized to the apicalmost part of the lateral domain, in the TJ region (Fig. 3D). Colocalization of aPKC and TJ has been shown before (57). This localization was disrupted in Myo5b kd cells. Whereas some cells retained a localization that resembled control, grossly delocalized aPKC was observed in approximately one out of four cells. The abnormal images included extensive apical aPKC distribution and lateral domain localization as well as punctate localization in the cytoplasm (Fig. 3D). To determine whether mislocalization of aPKC is indeed dependent on PDK1 activity, Myo5b kd cells were incubated in the presence of the PDK1 inhibitor BX-912 (7) or vehicle. Inhibition of PDK1 abolished the cytoplasmic and lateral membrane pT555-PKCI signal (Fig. 3D, bottom).

To further characterize the PDK1 subcellular compartment(s), we fractionated Caco-2BBe postnuclear supernatants on sucrose gradients. This procedure separates apical membrane and ARE (at the top of the gradient) from common and basolateral (lower in the gradient) endosomes (8, 24, 32). In these experiments, the cells were supplemented with an excess of Tfn from the apical side overnight to label all endosomes. In scrambled control shRNA cells, PDK1 comigrated with Rab11, as we described before in Caco-2 cells. It was not present in the Tfn-containing compartment that excludes Rab11, which is generally identified as common and basolateral endosomes (24) (Fig. 4). Conversely, in the Myo5b kd cells, Rab11 was displaced to a denser fraction (Fig. 4A). PDK1 shifted into two different compartments that exclude Rab11: 1) the top fraction of the gradient, which corresponds to cytosolic protein, and, possibly, floating lipid compartments; and 2) fractions in lanes 6 and 7, where PDK1 copurified with Tfn, but not with Rab11 (Fig. 4A). The appearance of PDK1 in the top fraction was also observed before in cells where endocytosis was disrupted by dynamin inhibition (24). Therefore, the PDK1 shift to the top fraction may be characteristic of a disruption of apical endosomes. Copurification of PDK1 with basolateral endosomes, on the other hand, has not been observed in Caco-2 cells before. It is consistent with a redistribution of PDK1 under Myo5b loss-of-function. Overall, both changes are coherent with the morphological redistribution of PDK1 resulting from Myo5b deficiency observed in human MVID intestine (Fig. 1) and Caco-2BBe cells (Fig. 3). Importantly, PKD1 was active in all membrane compartments to which it relocated but not in the supernatant (Fig. 4B), indicating that the enzyme is still activated when "abnormally" associated with basolateral endosomes.

Overexpression of PDK1 does not phenocopy Myo5b kd. The immunohistochemical distribution of PDK1 in MVID intestine opened the possibility that PDK1 might be upregulated in Myo5b loss-of-function. Although there are no substantial changes in the overall expression of PDK1 in Myo5b kd cells (Fig. 2), we speculated that overexpressing the protein would lead to delocalization. Furthermore, it was expected that overexpression of PDK1 may mimic the increase in aPKC phosphorylation (Fig. 2). Caco-2 cells were stably transduced with PDK1 or green fluorescent protein (as control). The expression levels varied with different transductions but were typically two- to fourfold over the endogenous PDK1 levels. The levels of active aPKC varied only threefold (Fig. 5B), which is less than the change compared with that induced by Myo5b kd (Fig. 2). More importantly, the overexpressed PDK1 was not delocalized but rather remained in the same subapical layer as in control cells (Fig. 5B). Together with the lack of change in PDK1 expression in Myo5b kd cells (Fig. 2), this result supports the notion that delocalization of PDK1 in human MVID (Fig. 1) and the steep upregulation of active aPKC reported above are unlikely to be due to overall changes in PDK1 expression.

Inhibition of PDK1 shows different effects on control or Myo5b kd cell short-circuit current. To further characterize ion transport, we analyzed Caco-2BBe cells expressing scrambled or Myo5b shRNA in Ussing chambers. After 15 days in culture as confluent monolayers, transepithelial electrical resistance (TER) was not impaired by Myo5B kd (Fig. 6A), and well within reported values for these cells (23), indicating that lentiviral transductions do not significantly affect TJ permeability. The TER values were not significantly changed by overnight incubation in BX-912.

Likewise, a modest short-circuit current (Isc, −1 to −2 μA/cm²; Fig. 6B) was detected, similar to previous reports (14), and did not change significantly in Myo5b kd cells. In normal Caco-2 cells, this Isc is completely abrogated by ouabain (14), suggesting that the Na⁺-K⁺-ATPase is the electrogenic component. Accordingly, Isc did not change in scrambled cells when Cl− was replaced by gluconate in the Ringer
solution in the same membranes (Fig. 6B). However, the $I_{sc}$ was abolished by replacing Cl– with gluconate in the Myo5b kd cells. This effect was partially rescued by the PDK1 inhibitor BX-912 (Fig. 6B). This result indicates that, although the absolute $I_{sc}$ is similar, there is a shift in the electrogenic mechanism in Myo5b kd cells from a cation- to an anion-based transport. In normal intestinal epithelia, an electrogenic Cl– transport activated by neurotransmitters was recently reported (35). Although this difference likely reflects differential redistribution of ion transporters due to a membrane traffic defect, it highlights the fact that the changes may be also dependent on intracellular signaling pathways regulating those transporters since it can be modified by BX-912.

**Inhibition of PDK1 shows differential effects on isotonic net water transport that is dependent on Myo5b expression.** To measure net water flux, we developed a method modified from Ref. 30 by seeding Caco-2BBe cells on Transwell filters and determining changes in chamber weight as a function of time, after accounting for evaporation. For more precise measurements and stronger statistical power, we used 6-mm inserts in sextuplicate for 18-h periods (Fig. 7A). The same method was applied to measure osmotically induced water fluxes (Fig. 7B).

The absolute values of net water flux in control cells ($2.8 \pm 0.4 \mu l\cdot h^{-1}\cdot cm^{-2}$ mucosal to serosal) in the absence of external osmotic or hydrostatic pressures were almost identical to those reported in the original publication (30), and ~58-fold lower than those reported for basal isosmotic water absorption in the small intestine in vivo (4). This factor is consistent with the folding in the intestinal epithelium (56) when compared with a flat monolayer of epithelial cells in culture. Myo5b kd resulted in a significant reduction in water absorption that was fully rescued by BX-912 (Fig. 7A). The same experiments were independently repeated using a nonphosphorylatable PDK1 decoy peptide that contains the PDK1 binding and target domains. This myristoylated peptide has been shown to inhibit PDK1 (24). Like BX-912, the decoy peptide also rescued water absorption. PDK1 expression, on the other hand, significantly increased the absorptive net water flux. This result also bodes well with the effect of BX-912 on scrambled control cells, which decreased the flux (Fig. 7). In summary, inhibition of PDK1 has opposite effects in the Myo5b kd background compared with the scrambled control. Because stable expression renders PDK1 in its normal location (Fig. 5) while Myo5b kd delocalizes PDK1, these data suggest that depolarization of PDK1 (Figs. 1–4) is responsible for decreased water absorption under Myo5b loss-of-function conditions.

When untreated control (scrambled shRNA) cells were subjected to an apical 100 mosmol/kgH2O gradient, the net water flux was $11 \pm 2.3 \mu l\cdot h^{-1}\cdot cm^{-2}$ serosal to mucosal. This value is slightly below that observed in a previous publication (30) using T84 cells ($8 \mu l\cdot h^{-1}\cdot cm^{-2}$ for the same gradient). Importantly, neither Myo5b kd nor PDK1 expression had any effect on osmotically driven water flows. BX-912 decreased the water flux in control cells (Fig. 7B). This result suggests that the changes induced by PDK1 and Myo5b kd in water absorption may be related to activity or distribution of ion transporters rather than transepithelial water permeability.

**DISCUSSION**

Decreased expression of ion transporters on the enterocyte apical membrane has been demonstrated in MVID. In the current study, we show that a Myo5b loss-of-function mutation also results in changes in polarized signaling. More importantly, inhibition of PDK1 restores normal isotonic net water (mucosal to serosal) flux in Myo5b kd Caco-2 cells, suggesting that modulation of apical signaling pathways could restore intestinal fluid loss in MVID.
Endosomes are broadly recognized as the location for essential intracellular signaling events. Disruption of membrane traffic resulting in modifications of endosomal compartments lead to dramatic changes in the signaling cascades associated to the endosomes (26, 33). Several pathways, including Akt (31), are known to require endosomal scaffolding. However, the functional role of endosomal scaffolds in other pathways that rely on PDK1 activation remains unclear. In this study, we found that PDK1-associated apical endosomes in intestinal epithelia are dramatically relocalized as a consequence of Myo5b loss-of-function, both in human MVID and in Caco-2BBe cells in culture. Furthermore, pharmacological inhibition of PDK1 rescues functional signaling changes associated with the Myo5b loss-of-function, including net water absorption and Na⁺-dependent \( I_{sc} \). Therefore, the change in PDK1 subcellular distribution seems to be an as yet unrecognized factor controlling water transport. Accordingly, we speculate that PDK1 redistribution and the resulting changes in the downstream cascades are contributing factors to diarrhea in MVID.

The lack of an animal model for MVID has required use of a cultured polarized intestinal Caco-2BBe cell model. MIs have been observed in enterocytes from a Rab8 knockout mouse model (47). However, mutations in Rab8 have not been identified in human MVID. These animals mimic the MVID phenotype at the subcellular level because the mutation affects apical protein distribution only and preserves basolateral polarity. The Rab8 knockout mice show intestinal malabsorption but survive 3–4 wk after birth. Whereas the original publication does not show water balance data in the mice, these survival rates are not compatible with severe secretory diarrhea that is observed in human MVID. Re
cently, a conditional cdc42 knockout mouse model revealed MIs and brush-border disorganization. These animals lack diarrhea and survive >50 days. Conversely, they display gross changes in the mucosal structure, uncharacteristic of MVID (25, 44). It is important to highlight that MIs are diagnostic of MVID in the clinical context of the early intractable diarrhea. However, MI-like structures have been observed in epithelial cells under conditions independent of MVID. MI-like structures (also termed VACs) can be observed in epithelial cells prevented from forming normal lateral domains (51), as well as in a variety of carcinomas (“intracellular lumens”) (38, 52). As a matter of fact, Caco-2 cells display MIs/VACs in ~1% of the cells spontaneously. In this study, we showed that the fraction of cells with MIs increases about 20-fold as a result of the Myo5b kd. Therefore, the strength of the Caco-2 cell model of MVID resides not only in the morphological phenotype but also in the abnormal water transport as demonstrated in the Myo5b kd, a feature that has not been observed in available animal models. An additional advantage of the Caco-2 model is that it may allow a simple reproducible method to measure net water flux in medium or high-throughput screens to test small molecules with therapeutic value in MVID. The current study provides proof-of-principle to validate such a method.

Following efficient Myo5b kd, Caco-2BBe cells seem to closely mimic the features of the MVID epithelia in terms of numbers of MIs, PDK1 distribution, and changes in water flux. Several additional similarities between the constitutive Myo5b kd in Caco-2 cells and MVID intestinal epithelia have been recently published, including: 1) loss of apical microvilli; 2) delocalization of Rab11, early endosome antigen, as well as lysosome-associated membrane protein-2; and 3) decreased lipid absorption, which mimics the nutrient malabsorption in MVID (42, 50). Our results differ from Ref. 50 in that we did not find any change in TER, which is consistent with published data from human MVID mucosae (41). Their measurements were performed at days 5 and 10, when the monolayers are not fully differentiated, whereas we measured TER after 2 wk in culture. More importantly, we used a subclone of the Caco-2 cell, the Caco-2BBe, which was originally selected to have higher levels of TER than the parental Caco-2 line. The reason for using this subclone is that, unlike the parental cell line, it expresses CFTR and better mimics the villus enterocytes. Caco-2BBe, therefore, are better suited for water transport studies. Admittedly, even in these cells the Myo5b kd model did not show a net secretory phenotype, only a decrease in absorption. Nonetheless, it allowed us to demonstrate that a mild overexpression of PDK1 or pharmacological inhibition of the same kinase results in significant effects on net water fluxes that change in the Myo5b kd background. It is worth mentioning that a PDK1 hypomorphic mouse model (39) shows decreased amino acid absorption in the intestine and a decrease in NHE3 brush-border activity (46), which is consistent with our BX-912 data. In other words, a decreased apical PDK1 activity (either by partial loss of expression in the hypomorphic mice or by redistribution in Myo5b loss-of-function) is expected to result in reduced NHE-3 activity, possibly through the local decrease in SGK activation (10). These examples highlight the idea that the effects shown in the current study are not necessarily direct effects of PDK1 activity on ion channels or transporters.

The observed immunohistochemical distribution of PDK1 in MVID patient biopsies suggested two possible nonmutually excluding consequences of Myo5b mutations on PDK1: 1) a change in localization; and 2) a possible upregulation. We could not verify the latter in the biopsies because of the minimal amount of material available. Two independent pieces of evidence suggest that increased amounts of PDK1 are not involved in the phenotype: 1) a mild overexpression of PDK1 did not result in mislocalization of the enzyme or phospho-$$\text{Akt}$$ signal (Fig. 5); and 2) increased expression of PDK1 resulted in an increase, rather than decrease, of isotonic water absorption (Fig. 7). Finally, published data from the hypomorphic PDK1 mouse model suggest that there is normally a large excess of the enzyme (20). Therefore, although we cannot fully rule out an increase in PDK1 levels in MVID human patients, we are inclined to consider localization of the enzyme in abnormal compartments (such as basolateral endosomes), a more consistent explanation for the functional data. In this scenario, inhibition of the mislocalized enzyme (either with BX-912 or the decoy peptide) rescues the abnormal water fluxes. Conversely, this model is also consistent with the finding that normally localized PDK1 in control cells causes the opposite effect (Fig. 7).

The mechanism for apical localization of PDK1 remains unclear (24). PDK1 is known to localize to membranes through the COOH-terminal PH domain, which binds phosphatidylinositol 3-phosphate (PIP3) (60). This poses a conundrum, because PIP3 has been found in the basolateral domain in MDCK cells (11) and in common recycling endosomes (9). Conversely, in the intestine, Akt2 (which also displays a PH domain to attach to membranes) and phosphatidylinositol 3-kinase (PI3K) have been shown to be present in brush-border membranes (21). Accordingly, we hypothesize that the most likely mechanism to explain the delocalization of PDK1 would be a redistribution of PtdIns downstream of Myo5b loss-of-function.

The central premise arising from our data, however, has potential implications for MVID. In early studies on MVID patients, researchers speculated that partial resection of the gut would balance the diarrhea. It was concluded, however, that even a small segment of remaining MVID-affected small intestine would lead to dehydration (41). This observation implies an absolute requirement for any gene replacement to be effective in most stem cells. Such a therapy for MVID remains technologically impossible at this time. Therapeutic manipulation of signaling cascades, on the other hand, is commonly employed for cancer and other diseases. Akt and PI3K inhibitors are in clinical trials for a variety of malignancies, and sirolimus (rapamycin) has been tried as a treatment for polycystic kidney disease. While PDK1 inhibitors may be too toxic for an actual therapeutic use in infants, we speculate that detailed studies of the changes in polarized signaling resulting from Myo5b loss-of-function mutations may result in possible clinical therapies for MVID diarrhea. Those studies should include the PDK1 target kinases affected by PDK1 relocalization and the ion channels/transporters that functionally change as a consequence of signaling changes. The results presented here indicate that indeed pharmacological manipulation of
signaling can rescue Myo5b-dependent changes in isotonic water fluxes and $I_{sc}$.

ACKNOWLEDGMENTS

We are thankful to Dr. Robert Warren for critically reading the manuscript and Yolanda Menendez for excellent technical assistance. The water transport method was inspired by the teachings of Dr. Mario N. Parisi.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01-DK-087359 and R01-DK-076652. R. Forteza was a recipient of postdoctoral fellowship F32-DK-095503.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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