Pathogenic mechanisms in microvillus inclusion disease
Dhekne, Herschel Shrikant

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5

DEFECTS IN THE BRUSH BORDER FUSION MACHINERY CAUSE MICROVILLUS INCLUSION DISEASE

Herschel S. Dhekne¹, Rodrigo Perez-Ortega¹, Mallik Zibouche², Carsten Posovszky³, Edmond H.M. Rings¹,⁴, Peter van der Sluijs², Sven C. D. VanIJzendoorn²

¹Department of Cell Biology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands, Department of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Ulm, Germany, ²Department of Pediatrics, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

(Manuscript submitted)
Abstract

Mutations in munc18-2 cause the immune disorder Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL5). Some FHL5 patients also suffer from enterocyte abnormalities that are characteristic of microvillus inclusion disease (MVID). The mechanism for this phenotype is not understood. We show that loss of myosin Vb, munc18-2, or syntaxin-3 function in patient enterocytes, and complementary knockdown experiments in intestinal epithelial Caco-2 cells, all inhibit ezrin-dependent development of brush border microvilli. Our data define a essential role of the vesicle fusion machinery in the pathogenesis of MYO5B-associated MVID, and reveal a common molecular pathway that unifies a subset of congenital diarrheal disorders.

Introduction

MVID is a rare and fatal autosomal disease characterized by intractable diarrhea and nutrient malabsorption. Cellular hallmarks of MVID are microvillus atrophy, intracellular retention of apical brush border membrane (BB) proteins, and microvillus inclusions in the enterocytes’ cytoplasm (Cutz et al, 1989; Philips et al, 1992). MVID is caused by mutations in MYO5B which encodes myosin Vb, an actin motor that together with rab11a regulates protein transport from endosomes to the plasma membrane (Muller et al, 2008; Erickson et al, 2008; Szperl et al, 2011). Progress in understanding how mutations in MYO5B cause the dramatic defects in humans has been hampered by the limited availability of patient material and the absence of suitable animal models (van der Welde et al, 2013). Possibly, clues could be obtained from analysis of other congenital diarrheal disorders (CDDs). CDDs have been classified based on the genes and cellular processes involved. MVID has been assigned to the category of enterocyte differentiation and polarization (Canani and Terrin, 2011). FHL5 is a rare immunological disorder, not typically perceived as a CDD. Interestingly, a subset of FHL5 patients suffers from enterocyte abnormalities that are remarkably similar to MVID and which persist after hemopoietic stem cell transplantation (Stepensky et al, 2013). FHL5 is defined by mutations in STXBP2 (Zur Stadt, 2009, Cote et al, 2009), encoding syntaxin-binding protein-2/munc18-2. Munc18-2 forms a complex with (the major apical syntaxin) syntaxin-3, SNAP23 and cellubrevin that is responsible for fusion of docked transport vesicles at the BB (Galli et al, 1998; Riento et al, 1998).

In terms of a CDD, FHL5 would thus best be assigned to the category of enterocyte differentiation and polarization, like MVID. Given the striking gut-related similarities between MVID and FHL5, we explored functional relationships between MVID-associated MYO5B mutations and the BB vesicle fusion machinery in enterocytes.

Results and Discussion

Munc18-2, syntaxin-3, SNAP23 and cellubrevin-positive transport vesicles were absent from their location at the BB (Figure 1A1-D1) in MVID enterocytes. Instead, the proteins were retained intra-cellularly (Figure 1A2-D2, S1A). A fraction of syntaxin-3 co-distributed with rab11a (Figure 1E1-3) suggesting that the regulatory complex is in part held up in recycling endosomes. In accord, knockdown of myosin Vb in Caco-2 cells (FigureS1E)
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Figure 1. Myosin Vb loss-of-function perturbs the subcellular distribution of the BB vesicle fusion machinery. A-D) Localization of munc18, syntaxin-3, SNAP23 and cellubrevin at the BB (A1-D1, respectively) of control enterocytes and in intracellular compartments in MVID enterocytes (A2-D2 [arrows], respectively, dotted line: cell surface). E) Distribution of syntaxin-3 with rab11a in MVID enterocytes. Box on E1 is enlarged in E2. E3 shows PDM values of E2 (see Methods). Asterisks in A-E indicate gut lumen. F) Syntaxin-3 is at the BB in control Caco-2 cells (F1-4) and mislocalized after myosin Vb knockdown (F5-8). In all images nuclei represent DAPI staining. G) Western blot of syntaxin-3 (syn3) in myosin Vb KD Caco-2 cells. H) Distribution of syntaxin-3 with actin-rich microvillus inclusions in MVID enterocytes. H2-4) enlargement of box in H1. H5) PDM values of H4 (see methods).
Figure 2. Syntaxin-3 loss-of-function perturbs BB microvilli development. A, B) Syntaxin-3 localizes to actin-rich BB microvilli in Caco-2 cells (A). Actin-rich microvilli appearance in control (B1,2) and myosin Vb-KD Caco-2 cells (B3,4). C, D) T567 phosphorylation of ezrin at the BB of control (C, D1-2) and myosin Vb KD Caco-2 cells (C, D3-4); (C [side views], D [top views of BB domain]). E) FHL5 munc18-2/STXBP2 mutants are impaired in syntaxin (STX)-11 and syntaxin (STX)-3 binding. F) Distribution of syntaxin-3 (1, 4), T567-phosphorylated ezrin (2, 5) and ezrin (3, 6) in FHL5 crypts (1-3) and villi (4-8 6). G, H) Quantitative image analysis of D and F, respectively. I) Protein interaction network around myosin Vb and munc18-2.

Similarly changed the distribution of munc18-2 (Figure S1 B,C) and syntaxin-3 (Figure 1F5-8, compare) to control in 1F1-4) and increased expression of syntaxin-3 (Figure 1G). Thus, myosin Vb regulates the distribution of key components of the BB vesicle fusion machinery between the endosomal system and BB, and the faithful BB targeting of cellubrevin-positive transport vesicles. Syntaxin-3 also associated with actin-rich microvillus inclusions in MVID enterocytes (Figure 1H1-5), like in Madin-Darby canine kidney cells where loss of cell polarity induced the association of syntaxin-3 with microvillus inclusions and mistargeting of apical membrane-directed trafficking to the inclusions (Low SH et al., 2000). These data strongly suggest that retention of syntaxin-3 in endosomes of MVID enterocytes diverts BB enzyme-carrying...
transport vesicles for fusion with the syntaxin-3 endosomes thereby creating microvillus inclusion bodies. Since microvillus inclusions in intestinal biopsies of MVID patients contain endocytosed tracers, they qualify as bonafide endocytic organelles (Reinshagen et al, 2002).

The molecular mechanisms that regulate the development of BB microvilli can be uncoupled from the general apical-basolateral polarity program (Ten klooster et al, 2009), both of which are affected in MVID enterocytes (Muller et al, 2008). While syntaxin-3 serves to deliver apical proteins to the BB (Breunza et al, 2000; Low et al, 2000), a potential role in BB microvilli development has not been explored. Syntaxin-3 localized to the BB (Figure 2A-C) and at tips of BB microvilli in Caco2 cells (Figure 2B1-2, arrows). Knockdown of syntaxin-3 (Figure 2A, S1E) reduced the number of actin-rich microvilli (Figure 2A, B3-4, c.f., and 2B1-2), expression of munc18-2 and the microvillus protein villin (Figure S1B, D and F, respectively). Importantly, syntaxin-3 knockdown also inhibited Threonine-567 phosphorylation of the actin-plasma membrane linker protein ezrin (Figure 2C, 2D1-4, 2), which is required for microvilli development (Saotome et al, 2004). Ezrin itself maintained its localization at the BB (Figure S1F). Ubiquitously expressed munc18-2 binds syntaxin-11 and regulates exocytosis in cytotoxic T lymphocytes and Natural Killer cells (Zur Stadt et al, 2009), a process that shares features with epithelial polarity and BB morphogenesis in enterocytes (Gao et al, 2010). Conceivably, munc18-2 could functionally associate with syntaxin-3 in enterocytes. Indeed, FHL5/munc18-2 mutants that did not interact with syntaxin-11 (zur Stadt et al, 2009; Cote et al, 2009) also were impaired in binding syntaxin-3 (Figure 2E). Moreover, in FHL5 enterocytes, syntaxin-3 was redistributed from the BB to intracellular compartments in the crypts (Figure 2F1, compare to control crypts in supplementary-Figure 1G), but not in the villi (Figure 2F4). Coinciding with the re-distribution of syntaxin-3, phosphorylation of Threonine-567 in ezrin was inhibited in the crypts (Figure 2F2, compare to control crypts in supplementary-Figure 1G) but not in the villi (Figure 2F5), whereas ezrin itself was present at the BB in both crypt and villi (Figure 2F3 and F6, respectively). Quantification revealed a significant reduction in the fraction of Threonine-567 phosphorylated ezrin at the FHL5 crypt BB (Figure 2H), showing that BB syntaxin-3 is required for microvilli development presumably through phosphorylation of ezrin. Thus, loss of munc18-2 and syntaxin-3 function phenocopies decreased ezrin Threonine-567 phosphorylation seen in MVID enterocytes and in Caco-2 cells after knockdown of myosin Vb, by inhibiting the targeting of ezrin-phosphorylating kinases to the BB (chapter 3).

Together, the data implicate intracellular retention of the BB membrane fusion machinery as a causative factor in the pathogenesis of MVID. Since all known BB proteins require syntaxin-3 for delivery to the BB, the intracellular retention of just syntaxin-3 due to the loss of myosin Vb function explains the global defect in the delivery of proteins to the BB. It also provides a mechanism for the appearance of microvillus inclusions and atrophy of BB and reveals a common molecular pathway that unifies a subset of congenital diarrheal disorders. Not all CDD and MVID patients have MYO5B mutations. It might be interesting therefore to focus on the pathway we uncovered here to identify mutated genes in CDD with unknown genetic etiology. Such information will also contribute in an important manner
to understanding basic molecular aspects of enterocyte function. For future reference we have included an author-unbiased protein interaction network around myosin Vb and munc18-2 (Figure 2I). This network includes, besides BB fusion proteins syntaxin-3, SNAP23 and cellubrevin, also components of the apical endosome targeting machinery including rab8a, the knockout of which causes MVID-like features in mice (Sato et al, 2007).

**Methods**

**Patient material**

Material was from previously described MVID patient (chapter 3) and FHL5 patients. The FHL5 patient carrying a homozygous STXBP2 mutation (c.693_695del3bp) in Exon 9 was described in Stepensky et al 2013. This mutation hampers the binding to syntaxin 11 completely as described in zur Stadt et al 2009. He is now 13 years old and one of the oldest FHL Type 5 patients with gastrointestinal manifestation alive after stem cell transplantation, but still needs total parental nutrition due to chronic diarrhea.

**Immunolabeling, microscopy and quantification**

Immunolabeling was performed as described previously (Chapter 3, 4). Images were made on Leica TCS Sp8 confocal microscope. Quantitative image analyses were done using ImageJ plugins as described previously. To quantify the T567 phosphorylation status of ezrin in Caco-2 cells, images were stacked in 3D, median filtered (3x3) and maximum intensity projections were generated from the top 10 microns. Mean fluorescence values from 4 areas were calculated. For FHL5 tissues, images of ezrin and T567-phosphorylated ezrin were taken at identical confocal settings. Mean fluorescence values for T567-phosphorylated ezrin and ezrin per cell were calculated in four crypt and five villus regions from separate images.

**Antibodies**

A cDNA fragment encoding the second domain of human munc18-2 was generated by PCR and ligated in the BamH1 site of pGEX5X2. An antibody against munc18-2 was made by immunizing rabbits with GST-munc18-2(161-229). The antibody immune-precipitated a band of ~70 kDa, that was not seen in lysates of cells that were treated with siRNA against munc18-2 or by pre-immune serum. The antibody against Cherry was made by immunizing rabbits with GST-Cherry. Other antibodies used were Ezrin (Tebu bio) 1:100), pERM (Cell Signaling 1:100), Actin (Sigma 1:1000), Syntaxin 3 (Synaptic Systems 1:200), Cellubrevin (Synaptic systems 1:200), rab11 (BD bioscience 1:50), Villin (BD Bioscience 1:100), Snap23 (Synaptic systems 1:200), munc 18-2 (this study).

**DNA methods**

pEGFP-munc18-2 was generously provided by Genevieve de Saint Basile (Necker Hospital Paris) and subcloned in pCherry using BamHI and EcoRI sites. FHL5 point mutants were
generated by site-directed mutagenesis on pCherry-munc18-2, transferred by Gateway cloning to the lentiviral expression plasmid pLNT-WPRE-SFFV and used to generate lentiviruses as before (Elstak et al, 2011). The shRNA target sequence of human MYO5B cDNA was made as described previously in chapter 3 and 4. Sequence for human syntaxin-3 was used from Day P. et al, 2011 and (target sequence AAGGGCCAACAACGTCCGGAA) was cloned into the shRNA expression vector pLKO.1 TRC. A scrambled sequence was used as controls. Lentivirus was made in HEK 293 cells as described previously. In pLKO system, cells were selected on puromycin (5µg/ml) and knockdown tested with western blot and qRT-PCR. qPCR on knockdown was performed as described before using primers (Fw TTGAGATTGCTATCGACAACAC, rw GTCAAGTTAAGCCGAGTTTCC) for syntaxin-3. In experiments, Caco-2 cells were plated on Transwell filters (Corning, 0.4-micron pore size) and 48 h later transduced with virus in the presence of polybrene for 16 h, and cultured for another 4 days.

Co-immunoprecipitation
HEK293T cell lines expressing wild type and mutant Cherry-munc18-2 were established by lentiviral transduction. Cells were transiently transfected with pGW1-Myc-syntaxin-3 or pGW1-myc-syntaxin-11 constructs using the calcium-phosphate procedure. Cells expressing myc-syntaxin-3 were lysed in 20 mM HEPES pH 7.5, 1% TX-100, 100 mM NaCl, while myc-syntaxin-11 lysates were prepared with RIPA buffer, both supplemented with Complete mini protease inhibitor cocktail (Roche). Cherry-tagged protein was immune-precipitated with Cherry antibodies and detection was with the monoclonal 9E10 against myc or the polyclonal 5 antibody against munc18-2 as described (Hoogenraad et al, 2010).

Protein interaction network
A protein interaction network around human myosin Vb and munc18-2 was constructed using STRING 9.05 at http://string-db.org. Used parameters: all active prediction methods, highest confidence (0.900) score, no more than 10 interactions shown, 20 additional (white) nodes. A confidence view was selected.

Cell experiments
Caco-2 cells were cultured as described previously 20. Short-hairpin target sequences for myosin Vb and syntaxin-3 and primers used for polymerase chain reactions are listed in supplementary-Table 2. Short-hairpin (Sh)RNAs were introduced into cells using a lentiviral system (supplementary-Methods).

Acknowledgements
We thank Genevieve de Saint Basile (National Institute of Health and Medical Research (INSERM) Unit 768, Paris, France) for the munc18-2 cDNA.


References


Supplementary figure 1. A) Sub-cellular redistribution of munc18-2, syntaxin-3, SNAP23 and cellubrevin in MVID enterocytes (solid and dashed arrows show intracellular and residual BB staining, respectively). B) Cartoon depicting the process of acquiring a maximum intensity projection (MIP) of staining in the apical domain of the cells. C) Top view MIP of munc18-2 labeling in control and myosin Vb KD Caco-2 cells. D) Top view MIP of munc18-2 labeling in control and syntaxin-3 KD Caco-2 cells. E) Myosin Vb and syntaxin-3 mRNA expression after knockdown of the respective genes in Caco-2 cells. F) Expression of villin and ezrin at the BB of control and syntaxin-3 KD Caco-2 cells, shown as x-z side views and top view MIPs of the apical domain of control and syntaxin-3 KD Caco-2 cells. G) Localization of T567-phosphorylated ezrin at the BB of control crypts in the small intestine. In all images nuclei are stained by DAPI (blue).