CHAPTER 2

Functional characterization of mutations in the myosin Vb gene associated with microvillus inclusion disease

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ABSTRACT

Background & Aims. Microvillus inclusion disease (MVID) is a rare autosomal recessive enteropathy characterized by intractable diarrhea and complete inability to absorb nutrients and, at the morphological level, by villus atrophy and the specific loss of the apical brush border in intestinal epithelial cells. Recently, various MYO5B gene mutations have been identified in MVID patients. Interestingly, several MVID patients showed only a MYO5B mutation in one allele (heterozygous) or no mutations in the MYO5B gene, illustrating the need to further functionally characterize the cell biological effects of the MYO5B mutations.

Methods. The genomic DNA of nine patients with different ancestry diagnosed with microvillus inclusion disease was screened for MYO5B mutations, and qPCR and immunohistochemistry on the material of two patients was performed to investigate resultant cellular consequences.

Results. We identified 8 new MYO5B mutations: three homozygous and five heterozygous mutations which include stop codons/nonsense mutations, missense mutations, splice site mutation, large deletion, and compound heterozygous mutations. MYO5B encodes for the recycling endosome-associated and actin filament-binding motor protein myosin Vb. In the patients’ enterocytes we observed an aberrant subcellular distribution of myosin Vb, and additional key proteins that interact with myosin Vb and/or control apical recycling endosome-mediated protein trafficking (Rab11a, FIP1, FIP5). We further noted an increase in the number of late endosomes and/or lysosomes.

Conclusions. Our functional analysis indicate that MYO5B mutations can be correlated with an aberrant subcellular distribution of the myosin Vb protein and apical recycling endosomes which, together with the reported compound heterozygous mutations, significantly strengthen the link between MYO5B and MVID.
INTRODUCTION

A structurally, compositionally, and functionally distinct plasma membrane at the apex of the intestinal epithelial cell monolayer provides a selective and protective barrier that regulates the uptake of nutrients from the lumen. The inability of intestinal cells to maintain an apical brush border and the consequences it has on food absorption becomes particularly apparent in patients diagnosed with microvillus inclusion disease (MVID; OMIM 251850).

Microvillus inclusion disease is a rare autosomal recessive disease presenting with severe intractable diarrhea and malabsorption in neonates (Phillips et al., 1985, 1992; Cutz et al., 1989; Sherman et al., 2004; Goulet et al., 2004). At the cellular level, variable brush border atrophy with accumulation of lysosomal granules and microvillus inclusions is observed in the apical cytoplasm of MVID enterocytes (Phillips et al., 1985, 1992; Rueemmele et al., 2006; Iancu et al., 2007). Periodic acid-Schiff-stained and other apical brush border components (e.g. CD10) are typically absent from the cell surface and accumulate in membranous compartments in the apical cytoplasm (Ameen & Salas, 2000; Groisman et al., 2002). In contrast to the apical proteins, basolateral proteins display a normal polarized distribution (Ameen & Salas, 2000; Michail et al., 1998) and enterocytes appear to be normally arranged in monolayer with distinguishable cell-cell adhesion junctions. Because of the specific loss of apical surface identity, MVID provides an outstanding opportunity to study the genetics and molecular dynamics that underlie apical surface development.

Defective intracellular trafficking of apical brush border proteins in MVID has been proposed (Ameen & Salas, 2000). The exact nature of such impairment remains obscure but may occur in either the biosynthetic pathway via which newly synthesized brush border proteins are delivered from the trans-Golgi network to the cell apex, or in the apical recycling route via which brush border proteins are recycled back to this surface domain (Hoekstra et al., 2004).
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MVID is often described in children born of consanguineous parents and this allowed Müller et al. (2008) to map the MVID locus to 18q21 using homozygosity mapping in an extended Turkish kindred. Mutation analysis of a positional candidate gene from the region of homozygosity, MYO5B, revealed an in-frame insertion in the MVID patients from the Turkish kindred. To date, 25 different nonsense, missense, splice site, or in-frame insertion mutations in the MYO5B gene (OMIM# 606540) have been identified in 28 MVID patients from consanguineous and unrelated marriages (Müller et al., 2008; Erickson 2008; Ruemmele et al., 2010). The MYO5B gene encodes myosin Vb, which is an actin filament-based motor protein that interacts with and regulates among others the subcellular spatial distribution of recycling endosomes that express small GTPase proteins such as Rab11a on their cytoplasmic surface.

In several MVID patients MYO5B mutations were found in only one allele (heterozygous) or no MYO5B mutation was found (Müller et al., 2008). Moreover, although knockdown of myosin Vb in human epithelial colorectal adenocarcinoma (Caco-2) cells recapitulates most of the cellular phenotypes of MVID (Ruemmele et al., 2010), it is not known whether myosin Vb mRNA and protein expression and myosin Vb function is affected in MVID patients. These current gaps in our knowledge prevents MYO5B gene screening as a diagnostic tool for this difficult to recognize rare disease, and prevents reliable genetic counseling and prenatal screening. Supporting evidence that MYO5B mutations have consequences for the expression and/or function of the myosin Vb protein in MVID enterocytes as well as mutational analyses of additional MVID patients are therefore imperative. In this study we have used duodenal biopsies to demonstrate that MVID-associated MYO5B mutations affect the expression and function of the myosin Vb protein in MVID enterocytes. In addition, we have performed mutation analyses of 9 additional MVID patients of various ethnic backgrounds and report 8 new MYO5B mutations: three homozygous and five heterozygous mutations which include stop codons/nonsense mutations, missense mutations, splice site mutation, large deletion, and compound
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heterozygous mutations, and we have explored the potential connection to a defective apical endosomal recycling system in MVID etiology.

MATERIALS AND METHODS

Description of patients and clinical history

Nine patients in whom histological examination of small intestine mucosa confirmed the diagnosis of MVID were included in this study. Patients 1-6 were collected from a larger patient cohort that received a bowel transplant via the Liver/Small Bowel Transplant Program of the University of Nebraska Medical Center (USA). Patient 1 is a 1-year-old Hispanic male with early-onset MVID from reported non-consanguineous parents. His brother died of MVID at 21 months of age. Patient 2 is a 3-year-old Hispanic female with early-onset MVID of consanguineous parents (first cousins). She has two sisters with MVID one of which is Patient 3. Patient 3 is a 5-year-old Hispanic female with early-onset MVID from consanguineous parents (first cousins). She has two sisters with MVID one of which is Patient 2. Patient 4 is a 1-year-old Navajo Indian male with early-onset MVID of related parents (died of sepsis). Patient 5 is a 12-year-old Navajo Indian female with early-onset MVID from related parents (died of sepsis with multi-organ system failure). She has two healthy sisters. Patient 6 is a 0-year-old Caucasian female with early onset MVID from reported non-consanguineous parents (died of sepsis from aspergillus and continuing acute rejection). She has one healthy sibling and two siblings died with unknown cause. Patient 7 is a 1-year-old Polish-Caucasian female with early-onset MVID from reported non-consanguineous parents. Patient 8 is a 5-year old Moroccan boy with early-onset MVID from consanguineous parents (first-degree cousins). Patient 9 is a 5-year-old Dutch-Caucasian boy from unrelated parents who was diagnosed with late-onset MVID. Unaffected parents and siblings of Patient 7, 8 and 9 were also recruited and, after informed consent, saliva samples were collected and genomic DNA was extracted. In addition, available duodenal tissue from Patient 7, 8 and 9 and age-
matched normal control patients were obtained and processed for immunohistochemistry. Also two 2-year-old Dutch girls (twins) of non-consanguineous parents who presented severe secretory diarrhea and nutrient malabsorption directly after birth, but did not display the diagnostic light and electron microscopical hallmarks of MVID, were included in the study (Patients 10 and 11). Written consent was obtained for all patients. This study has been reviewed and approved by the University Medical Center Groningen review board.

**DNA and RNA isolation**
DNA was isolated from peripheral blood samples using standard laboratory procedures. DNA and RNA from saliva were also collected and isolated (OrageneDNA and OrageneRNA, DNA Genotek Inc, Ottawa, Canada). RNA from in liquid nitrogen snap-frozen biopsy samples was isolated after homogenization using 1 mm glass beads using Trizol (Invitrogen, Carlsbed, CA). Concentration and purity were determined with NanoDrop ND-1000 (Isogen Life Science, De Meern, The Netherlands).

**Homozygosity mapping**
Genome-wide homozygosity mapping was performed by hybridizing 200ng of genomic DNA isolated from peripheral blood of patients to a panel of 6,090 SNP markers on an Infinium HumanLinkage-12 BeadChip (Illumina, San Diego, CA).

**RT-PCR**
Real-time PCR reaction was performed for the quantification of MYO5B. RNA was isolated from duodenum biopsies of twelve controls and Patients 8 and 9. cDNA was generated with a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) using 1µg total RNA. Primers were designed with Primer Express v.3 (Applied Biosystems); RT_MYO5Bfor: TTGGAAGTGTGGCGATTCAG; RT_MYO5Brev: GCAGTCGGCAGAAGTTGCTT. For GUSB expression, we used a TaqMan Pre-
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Developed Assay (Applied Biosystems). Reactions consisted of 1xSYBR Green (or Universal) PCR Mastermix, 1mM of each primer and 1µl cDNA. Cycling conditions were 50ºC for 2 min., 95ºC for 10 min. and 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Results were analyzed using SDS v.2.3 (Applied Biosystems).

Expression array

For the expression profile of the apical recycling endosome-related RAB genes we used the Human Ref-8 v2 expression array (Illumina, San Diego, CA) according to manufacturer’s protocol. The quality and concentration of the RNA was determined with the 2100 Bioanalyzer (Agilent, Santa Clara, CA) using the Agilent RNA 6000 Nano Kit. RNA from each sample was used for amplification and labeling with the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX). BeadStudio Expression module v3.2.7 (Illumina) was used for first line quality check, background correction, and normalization of the data.

Sequencing

The MYO5B coding region and splice sites were PCR amplified and directly sequenced in all three probands. Their relatives and approximately 50 control individuals (~100 chromosomes) were screened for the detected mutations. Primers for PCR amplification (Suppl. Tables 1 and 2 (Appendix 1) show primers used for amplification of genomic DNA and cDNA, respectively) were designed using Primer3 (Rozen & Skaletsky, 2003) on the genomic sequence of MYO5B (NC_000018.8) and its mRNA (NM_001080467). The PCR reaction was performed with 50ng genomic DNA in 20µl reaction volume which included 1xPCR buffer-A (GE Healthcare, Piscataway/NJ), 2.5mM dNTPs, 1mM primers (Eurogentec, San Diego, CA), 0.5 U Taq polymerase (GE Healthcare). For exons 1, 2, 12, 17, and 18, PCR reaction was performed with 150ng of genomic DNA in 25µl reaction volume with 1xPCR buffer (Buffer-B) made of 0.1M Tris-HCL (pH8.8), 0.1M MgCl₂, 0.01M mercaptoethanol, 0.05M ethylenediamine-tetraacetic acid/0.1M (NH₄)₂SO₄. The PCR
conditions differed with respect to the annealing temperatures and buffers used. Initial
denaturation at 95°C for 5 min. (4 min. Buffer-B); 40 cycles (33 cycles Buffer-B) of
denaturation at 95°C for 30s (1 min. Buffer-B), annealing for 30s (1 min. Buffer-B),
and extension at 72°C for 30s (2 min. Buffer-B); final extension at 72°C for 5 min. (7
min. Buffer-B). PCR products were purified (37°C for 15 min., 80°C for 15 min.)
with ExoSap-IT (USB, Cleveland, Ohio) and Sephadex columns. Sequencing
reactions were performed using BigDye terminator mix (Applied Biosystems).
Sequences were read on a 3730 DNA analyzer and 3130 Genetic analyzer (Applied
Biosystems) and we aligned sequencing data with control and reference sequences
using ContigExpress software (Invitrogen, Carlsbed, CA).

Deletion detection
While searching for a maternally transmitted mutation in Patient 7, a Mendelian
inconsistency in the inheritance of the exon 11 variant c.1367A>G was observed: the
mother appeared to be homozygous A/A while the proband was homozygous G/G.
This could possibly point towards a maternal transmission of a deletion. In order to
detect large deletions in MYO5B, real-time quantitative PCR was used to determined
copy numbers of the exons. Reactions consisted of 1xSYBR Green PCR Mastermix
(Applied Biosystems), 1mM of each primer (Suppl. Tab. 2, Appendix 1) and 25ng of
genomic DNA. Cycling conditions were 50°C for 2 min., 95°C for 10 min. and 40
cycles of denaturation at 95°C for 15s, and annealing for 1 min.

Immunohistochemistry
Duodenal biopsies of MVID Patients 8 and 9 and age-matched controls were fixed in
paraffin, and cut in 3μm thick sections. Slides were dried overnight in 60°C and
deparaffinized in xylol-100%-96%-70% ethanol and demiwater. Epitopes were
retrieved by protease digestion or in citric acid pH 6.0 (autoclaved; 5 min., 120°C).
Endogenous peroxidase was deactivated with 3.5% H₂O₂. Following blocking of non-
specific binding sites in 4% normal-goat-serum, slides were incubated with primary
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antibodies, washed, and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies. Diaminobenzidine was used as a substrate for peroxidase. Hematoxyline was used to stain the nuclei. Slides were dehydrated with ethanol, dried and mounted. Antibodies used: polyclonal antibodies raised against a synthetic peptide derived from the C-terminal hyper variable region of the human Rab11a sequence (Zymed Laboratories Inc); polyclonal antibodies against Rip11/FIP5 (Prekeris et al., 2000); polyclonal antibodies raised against a synthetic peptide corresponding to C- or N-terminal residues (amino acids 1093-1112 or 23-41, respectively) of human myosin Vb (Antagene Inc; 60B923) that recognizes a single band of the appropriate molecular mass of \~214kDa on Western blot; Horseradish peroxidase-conjugated donkey anti-rabbit, sheep anti-mouse antibodies (GE Healthcare). Immunohistochemistry images show villous cells.

**RESULTS**

**Confirmation of MVID diagnosis**

Available duodenal tissue from Patient 8 (MVID early onset) and 9 (MVID late onset) and age-matched normal control patients were obtained and processed for electron microscopy and immunohistochemistry. At the cellular level, both patients displayed, with some variability, the characteristic histological and ultra-structural features of MVID, including villous atrophy, accumulation of periodic acid-Schiff (PAS)-positive material and metalloendopeptidase (CD10) in the apical cytoplasm of the intestinal epithelial cells (Fig. 1A, B). At the ultrastructural level, atrophy of apical brush border is observed (Fig. 1C c1, c3), together with a typical accumulation of electron-dense structures (Fig. 1c2), and microvilli-like inclusions in the apical cytoplasm (Fig. 1c3, c4).

**Homozygosity mapping for identification of \textit{MYO5B} mutations in MVID patients**

Independently of Müller et al. we have performed homozygosity mapping using the
Figure 1. Immunohistochemical and ultrastructural characteristics of MID enterocytes. MID enterocytes (here depicted from Patient 8) show cytoplasmic accumulation of periodic acid-Schiff (PAS)-positive material (A) and the brush border protein CD10 (B, arrows). At the ultrastructural level, loss of the apical brush border identity of the intestinal epithelial monolayer is observed (c1) together with the accumulation of electron dense lysosomal structures (c2) and microvillus inclusions in the apical cytoplasm (c3, c4).

DNA of Patient 8, who is the son of first-degree cousins. Homozygous regions over considerable genetic distances and physical sizes were located (Fig. 2C and Suppl. Tab. 3, see Appendix 1). Comparison of these positional candidate regions with the genomic locations of known apical recycling endosome-associated and functional candidate proteins confirmed MYO5B as a positional-functional candidate gene in this
The mutations in *MYO5B* gene in MVID

Patient (Fig. 2C). Sequencing of the *MYO5B* gene in Patient 8 revealed a homozygous stop codon in exon 33 (p.Q1456X; c.4366C>T) (Fig. 2A).

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**Figure 2. Identification and mutation analysis of *MYO5B* as the gene responsible for MVID.**

A). Pedigrees and *MYO5B* point mutations in the three families with MVID patients (Moroccan, Dutch, Polish, from left to right). Haplotype analysis in Patient 7 and her family was indicative for a deletion in *MYO5B* on the maternal chromosome. Patient 8 revealed a homozygous stop codon in exon 33 (c.4366C>T, p.Gln1456X) which removes the terminal Rab11a-binding sites (1799-1814). The c.1540T>C substitution in Patient 9 is a *de novo* mutation on the paternal chromosome (indicated with asterisk). B). Nine novel mutations found in MyosinVb MVID patients. Mutations in the tail domain lead to premature transcript termination (stop codon), while in the head domain, most of mutations are substitutions, leading to replacement of highly conserved aminoacids. C). Results of homozygosity mapping in Patient 8, the child of a consanguineous couple of Moroccan descent. The position and length of the homozygous segments along the genome are indicated together with the location of candidate genes from the apical recycling endosome pathway. D) Deletion mapping results of Patient 7. The maternally derived deletion, spanning exons 2–12, was determined by quantitative PCR using DNA from the proband’s brother (2 copies for each exon) to normalize the signal.
Eight new MYO5B mutations associated with nine microvillus inclusion disease patients

MYO5B is composed out of 40 coding exons which were separately amplified and subjected to sequence analysis. All eleven patients were included in the mutation analysis by direct sequencing of the entire gene in both forward and reverse directions. Six patients revealed homozygous mutations. Patient 6 revealed one heterozygous change, while Patient 7 and 9 carry compound heterozygous mutations (Table 1). Patients 10 and 11, who presented nutrient malabsorption and intractable secretory diarrhea after birth but were not diagnosed with MVID, did not reveal MYO5B mutations.

Patient 1 carries a homozygous non-conservative missense mutation in exon 8 (c.946G>A, p.Gly316Arg), which replaces a small aliphatic glycine (conserved in myosin Va and Vc; Suppl. Fig. 1, Appendix 1) with a large and charged arginine in the protein’s conserved head domain region. In Patients 2 and 3 we found a shared homozygous deletion in exon 19 (c.2330_del G; Suppl. Fig. 2, Appendix 1). This mutation disturbs the reading frame and leads to a premature stop codon (p.Gly777AsnfsX6; Suppl. Fig. 3, Appendix 1) in the first calmodulin-binding IQ1 motif of myosin Vb. Any resultant protein will therefore not be able to dimerize and function as a processive motor protein, and lacks the entire cargo-binding tail domain. Patients 4 and 5 are homozygous for a non-conservative missense mutation in exon 16 (c.1979C>T, p.Pro660Leu) (results not shown). This mutation was recently described (Erickson et al., 2008) in 7 Navajo MVID patients. In Patient 6 we found one heterozygous mutation in exon 19 which results in a premature stop codon (c.2246C>T, p.Arg749X) in the head domain of myosin Vb (p.Arg749 is conserved in myosin Va and Vc; Suppl. Fig. 4, Appendix 1). Resultant protein will not be able to dimerize and function as a processive motor protein, and lack the entire cargo-binding domain.

| Table 1. Summary of 9 new MYO5B mutations associated with MVID in this study. Abbreviations: NMD, nonsense-mediated RNA decay; PMT, prematurely terminated protein; Rab11a BD (Rab11a binding domain). |

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<table>
<thead>
<tr>
<th>Subject</th>
<th>Ancestry</th>
<th>Parental consang.</th>
<th>Sex/ onset</th>
<th>MYO5B mutation</th>
<th>Homo/ heterozygous</th>
<th>MYOVb domain</th>
<th>Predicted effect on RNA and/or protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hispanic</td>
<td>n.r.</td>
<td>male/ early</td>
<td>946 G&gt;A (G316R, exon8)</td>
<td>homozygous</td>
<td>head</td>
<td>nonconservative substitution, evolutionary conserved</td>
</tr>
<tr>
<td>2, 3</td>
<td>Hispanic</td>
<td>yes</td>
<td>female/ early</td>
<td>2330_del G (out of frame, leads to stop codon, exon 19)</td>
<td>homozygous</td>
<td>neck (IQ1)</td>
<td>nonconservative substitution, evolutionary conserved, PMT, loss of dimerization and cargo-binding domains</td>
</tr>
<tr>
<td>4, 5</td>
<td>Navajo Indian</td>
<td>yes</td>
<td>male/ early; female/ early</td>
<td>1979C&gt;T (P660L, exon 16)</td>
<td>homozygous</td>
<td>head</td>
<td>nonconservative substitution, evolutionary conserved</td>
</tr>
<tr>
<td>6</td>
<td>Caucasian</td>
<td>no</td>
<td>female/ early</td>
<td>c.2246 C&gt;T (R749X, exon 19)</td>
<td>compound heterozygous</td>
<td>neck (IQ1)</td>
<td>nonconservative substitution, evolutionary conserved, PMT, loss of dimerization and cargo-binding domains</td>
</tr>
<tr>
<td>7</td>
<td>Polish</td>
<td>no</td>
<td>female/ early</td>
<td>DIVS1_IVS12 (deletion exon 2-12)</td>
<td>compound heterozygous</td>
<td>head</td>
<td>shortened protein, in frame deletion residues 10-515</td>
</tr>
<tr>
<td>7</td>
<td>Polish</td>
<td>no</td>
<td>female/ early</td>
<td>1367A&gt;G (N456S; exon 11)</td>
<td>compound heterozygous</td>
<td>head</td>
<td>nonconservative substitution, evolutionary conserved</td>
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<tr>
<td>8</td>
<td>Moroccan</td>
<td>yes</td>
<td>male/ early</td>
<td>4366C&gt;T (Q1456X, exon 33)</td>
<td>homozygous</td>
<td>tail</td>
<td>NMD; PMT, loss of distal Rab11a BD</td>
</tr>
<tr>
<td>9</td>
<td>Dutch</td>
<td>no</td>
<td>male/ late</td>
<td>1540T&gt;C (C514R, exon 12)</td>
<td>compound heterozygous</td>
<td>head</td>
<td>nonconservative substitution, evolutionary conserved</td>
</tr>
<tr>
<td>9</td>
<td>Dutch</td>
<td>no</td>
<td>male/ late</td>
<td>IVS33+3753G&gt;C (splicing, intron 33)</td>
<td>compound heterozygous</td>
<td>tail</td>
<td>partial intron 33 insertion, NMD; PMT, loss of distal Rab11a BD</td>
</tr>
</tbody>
</table>
tail domain. For Patient 7, 8 and 9, we also obtained DNA samples from unaffected siblings and/or parents. Patient 7 reveals a compound heterozygous mutation, which includes a paternal allele with a non-conservative asparagine-to-serine (c.1367A>G, p.Asn456Ser) substitution in exon 11 of the head domain (Fig. 2A) (conserved in myosin Va and Vc; Suppl. Fig. 5, Appendix 1), together with a missense variant p.Met1688Val (c.5062A>G) in exon 37 (p.Met1688 is substituted in \textit{MYO5A} and \textit{MYO5C}; Suppl. Fig. 6, Appendix 1). p.Met1688Val represents an infrequent polymorphism, as it was found in Polish and Dutch controls with allele frequencies of 5.8% (6/104) and 1.7% (2/116), respectively. When searching for a maternally transmitted mutation in Patient 7, a Mendelian inconsistency in the inheritance of the exon 11 variant c.1367A>G was observed: the mother appeared to be homozygous A/A while the Patient was homozygous G/G (Fig. 2A). This could possibly point towards a maternal transmission of a deletion. Using real-time PCR to determine the copy number of the \textit{MYO5b} gene, we found that the maternal allele in patient 7 contained a deletion involving exons 2–12 of \textit{MYO5B} (Fig. 2D). Sequencing of \textit{MYO5B} in Patient 8 revealed a homozygous stop codon in exon 33 (c.4366C>T, p.Gln1456X) (Fig. 2A) which removes the terminal Rab11a-binding sites (1799-1814) (Fig. 2B). Sequencing of \textit{MYO5B} in Patient 9 showed that this patient is a compound heterozygote carrying a \textit{de novo} non-conservative substitution mutation in exon 12 (c.1540T>C, p.Cys514Arg), and a maternally derived mutation in intron 33 (c.4460-1G>C) that destroys the canonical splice acceptor (SA) site (Fig. 2A, B). Intron 33 harbors three clusters of potent candidate cryptic SA-sites (Suppl. Fig. 7, Appendix 1). PCR on the patient’s intestinal cDNA with primers for intron 33 and exon 35 demonstrated retention of >100bp of intron 33 immediately upstream of exon 34. This ‘extended exon 34’ contains nine stop codons, at least one in each of the three reading frames (Suppl. Fig. 8, Appendix 1). The p.Cys514 residue forms part of the helix-turn-helix motif in the motor domain that is associated with actin-binding (Holmes et al., 2004; Coureux et al., 2004; Tang et al., 2007; Cecchini et al., 2008) (Fig. 2E). All mutations identified in this study are listed in Table 1. The positions of all mutated
The mutations in MYO5B gene in MVID

residues in the crystal structure of the myosin Vb head domain are depicted in Fig. 2E.

Re-sequencing mutation-containing exons revealed that none of the identified mutations were detected in 50 ethnically matched controls, or have been reported as known variants (in HapMap, dbSNP, and the 1000 genome database), unless stated otherwise.

Mutations affect the mRNA expression level of the myosin Vb in MVID enterocytes

We analyzed the expression levels of myosin Vb mRNA from duodenal biopsies of Patients 8 and 9 by real-time PCR and compared these to control patients. In Patient 8, myosin Vb mRNA expression was reduced by 50% when compare to 14 non-MVID control patients (Fig. 3A), which is in agreement with the identified nonsense mutation p.Gln1456X, which is predicted to result in nonsense-mediated RNA decay (Isken & Maquat, 2000). In Patient 9 myosin Vb mRNA levels were comparable to controls (Fig. 3A). The aberrant expression and inadequate apical concentration of apical recycling endosome-associated proteins near the brush border may influence the production of their cognate transcripts through direct or indirect feedback mechanisms. The genes belonging to the apical recycling endosome showed either a 1.4–1.5–fold increase (RAB11A, RAB11-FIP1), or 0.7-fold reduction (RAB11-FIP5) in expression (Fig. 3B). The MYO5B-associated RAB8A also showed a 1.4-fold increased expression. Other Rab11a-binding genes showed no (RAB11-FIP2, RAB11-FIP3) or a slight (RAB11-FIP4) increase in expression. The previously reported apical recycling endosome-related RAB genes RAB11B, -17, -18, -20, and -25 were not differentially expressed (results not shown).

MVID enterocytes show aberrant expression of myosin Vb and other apical recycling associated proteins.

We also analyzed the cellular expression pattern of myosin Vb protein in duodenal biopsies of Patients 7, 8 and 9 and age-matched control. The myosin Vb protein is
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Figure 3. Mutations affect the mRNA expression level of the myosin Vb in MVID enterocytes. (A) Analysis of the expression level of myosin Vb mRNA in control, Patient 8 and 9 duodenal biopsies by real-time PCR. In Patient 8, myosin Vb mRNA expression was reduced by 50% when compared to 14 non-MVID control patients, which is in agreement with the identified nonsense mutation p.Gln1456X that is predicted to result in nonsense-mediated RNA decay (Isken & Maquat, 2000). In Patient 9 myosin Vb mRNA levels were comparable to controls. (B) Expression levels of the genes belonging to the apical recycling endosome showed either a 1.4-1.5-fold increase (RAB11A, RAB11FIP1), or 0.7-fold reduction (RAB11FIP5) in expression. The MYO5B-associated RAB8A also showed a 1.4-fold increased expression. Other Rab11a-binding genes showed no (RAB11FIP2, RAB11FIP3) or a slight (RAB11FIP4) increase in expression.

present in the villous enterocytes and mainly concentrates at their apical aspect below the brush border of control enterocytes (Fig. 4a, arrow). In contrast, no or little specific myosin Vb signal was detected in the enterocytes of Patient 7 (data not shown), Patient 8 and 9 (Fig. 4b and 4c respectively). The MYO5B mutations did not involve residues that were used in the synthetic peptides to generate these antibodies, and the antibodies should recognize the mutant protein if present.

The lack of clear myosin Vb signal in MVID enterocytes may reflect the absence of the protein (in accordance with the reduced myosin Vb mRNA levels in Patient 8; see above), and/or may reflect a dispersion of remaining myosin Vb protein throughout the cells rendering myosin Vb below the detection limit. Antibodies
The mutations in MYO5B gene in MVID

available to us were not suitable for immunoblot analyses and further studies are needed to determine the myosin Vb expression level in MVID enterocytes. Nevertheless, it is clear that the spatial organization of myosin Vb is severely perturbed in MVID enterocytes.

Myosin Vb regulates the subcellular positioning of recycling endosomes by binding to small GTPase Rab proteins such as Rab11a at the cytosolic surface of endosomes and attaching these endosomes to and moving them along actin filaments (Lapiere et al., 2001; Swiatecka-Urban et al., 2007; Nedvetsky et al., 2007; Roland et al., 2007; Hoekstra et al., 2004; van IJzendoorn, 2006). Alterations in the typical spatial organization of recycling endosomes in MVID enterocytes can therefore be used as a read-out for altered myosin Vb function. To address this, the expression and distribution of recycling endosome-associated proteins Rab11a (Lapiere et al., 2001; Swiatecka-Urban et al., 2007; Nedvetsky et al., 2007; Roland et al., 2007; Hoekstra et al., 2004; van IJzendoorn, 2006) and the Rab11a effector proteins FIP1C (RCP) and FIP5 (Rip11) (Prekeris et al., 2000) was investigated in duodenal biopsies of Patient 7 (data not shown), Patient 8 and 9. The recycling endosome-associated proteins Rab11a, FIP1C and FIP5 accumulate just below the enterocytes brush border close to the apical membrane in control duodenal tissue, similar to myosin Vb (Fig. 4d, 4g, 4j respectively). In contrast, in MVID enterocytes, Rab11a and FIP5 did not accumulate in the apical region and, instead, no specific staining pattern could be observed (Fig. 4e-f and 4-1). Interestingly, FIP1C yielded a variable pattern (Fig. 5h, 5i). Whereas virtually no FIP1C was detected in Patient 7 (data not shown) and Patient 8 (Fig. 4h), enterocytes from Patient 9 showed a prominent subapical accumulation of FIP1C (Fig. 4i). These distinct staining patterns between patients may reflect the different MYO5B mutations identified in them (see Discussion). Sequence analysis revealed no functional mutations in the coding regions of the genes RAB11A, RAB11FIP1, RAB11FIP3, and RAB11FIP5 in Patients 7, 8 and 9, except for single nucleotide polymorphisms in Rab11a (summarized in Suppl. Tab. 4, Appendix 1).

Early endosomal antigen 1 (EEA1), a marker of early sorting endosomes typically
excluded from myosin Vb-positive recycling endosomes, and the late endosome- and liposome-associated protein LAMP-1 displayed comparable staining patterns in controls and MVID Patients 7, 8 (data not shown) and in Patient 9 (Fig. 5, arrows). The distribution of the Golgi complex was also apparently unaffected in MVID enterocytes, although it appeared somewhat more concentrated in the supranuclear region (Fig. 5).

**MYO5B mutations substitute or delete amino acids that are important for the function of myosin Vb**

Myosin Vb is an actin-based molecular motor protein with binding sites for discrete small GTPase Rab proteins at its C-terminus. These Rab proteins are typically present on the cytosolic surface of transport vesicles and/or endosomes. As such, myosin Vb regulates vesicular trafficking and/or endosome dynamics.

The N456S mutation in Patient 7 involves a highly conserved asparagine residue preserved in *MYO5A* and *MYO5C*. The methionine of the M1688V variant is less conserved and substituted in *MYO5A* and *MYO5C* (Suppl. Fig. 5, Appendix 1). N456 is located in the “relay” helix of the motor domain, where its side chain makes two hydrogen bonds with the main chain of the switch-II loop (Fig. 6B, D). Together with switch-I and the P-loop, which are also found near N456, the relay helix and switch II form a set of conserved motifs shared in all myosins that participate in coupling changes in the ATPase active site to conformational changes in the actin-binding and force-generating domains (Coureux et al., 2004; Tang et al., 2007; Cecchini et al., 2008). N456 and its interactions with switch II are conserved in both myosin V and myosin II, and the residue is proposed to have a pivotal role in motor function as mediator of allosteric communication (Tang et al., 2007; Cecchini et al., 2008). As the N456S substitution in human myosin Vb will lead to disruption of at least one of the two hydrogen bonds with the switch-II loop, it is plausible that this mutation causes malfunctioning of the myosin motor. Although N456S likely represents the causative mutation, we cannot formally rule out a contributing effect from the p.M1688V
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Figure 4. MVID enterocytes show aberrant expression of myosin Vb and other apical recycling associated proteins. Duodenal biopsies of MVID Patient 8, Patient 9 and age-matched control were immunohistochemically labeled with antibodies against the N-terminus of myosin Vb (a,b,c), Rab11a (d,e,f), RCP/FIP1C (g,h,i) and Rip11a/FIP5 (j,k,l). The accumulation of myosin Vb, Rab11a and FIPs in the apical cytoplasm in control enterocytes (arrows) is lost in MVID enterocytes. The positive RCP/FIP1C staining in Patient 9 is explained in the text. Scale bar - 10µm.

Polymorphism. The maternally transmitted deletion of exons Q1456X mutation is predicted to be degraded by nonsense-mediated mRNA decay (Isken & Maquat, 2000), thus cannot bind Rab11a but might maintain actin-binding capacity.

The mutated C514 residue in Patient 9 is conserved in all 30 species examined (phylum chordate), as well as in the human paralogs *MYO5A* and *MYO5C* (Fig. 6A).
Figure 5. The Golgi apparatus, early endosomes and lysosomes are intact in MVID enterocytes. Immunohistochemistry staining of duodenal enterocytes of MVID Patient 9 with anti-Giantin (marker for Golgi), anti-EEA1 (early endosomal antigen 1) and anti-LAMP1 (lysosomal marker) displayed comparable staining pattern to the age-matched controls. The antibody (ab) control stainings (non-immune first antibody) were prepared on biopsy material from Patient 9. Scale bar - 10µm.

C514 forms part of the helix-turn-helix motif in the motor domain that is associated with actin-binding (Holmes et al., 2004). Inspection of crystal structures of the closely related myosin V motor domain from chicken (Coureux et al., 2004) reveals this residue to be important for stabilizing the helix-turn-helix region and for anchoring this to the lower-50K subdomain (Fig. 6B, C). Rigid-body-like rotations of the lower- and upper-50K subdomains in the motor allow a switch between states of strong and weak actin-binding affinity, one of the hallmarks of the motility mechanism of myosins (Coureux et al., 2004). It is conceivable that the C514R mutation destabilizes the functional conformation of the helix-turn-helix motif and/or causes its detachment.
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from the main body of the myosin motor, both of which would interfere with the processes of actin binding and release. If the maternally transmitted mutated allele gives rise to a stable truncated peptide (note that the transcript is predicted to be degraded by nonsense-mediated mRNA decay (Isken et al., 2000), this peptide will lack the terminal Rab11a-binding site.

**DISCUSSION**

**New mutations in MyoVb in MVID**

We have analyzed the sequence of *MYO5B* in 9 MVID patients and identified 8 new mutations (~25% of all reported mutations) including a large deletion, a single nucleotide deletions, two missense mutations, and one nonsense mutation. We also report two additional compound heterozygous *MYO5B* mutations. In two patients we found a homozygous missense mutation that has been described previously (Erickson et al., 2008). Our study adds 8 mutations to the 25 earlier reported by Müller and colleagues (24 mutations/21 patients) (Müller et al., 2008), Ruemmele et al., 2010) and Erickson and colleagues (one mutation shared by 7 Navajo patients) (Erickson et al., 2008), yielding a total of 33 distinct *MYO5B* mutations in 37 MVID patients that have been identified to date. With our data, providing 25% of all currently reported *MYO5B* mutations and patients, we make a first analysis of the current *MYO5B* mutation spectrum. Of the 33 thus far published *MYO5B* mutations, 24 are localized in the N-terminal head domain that includes actin-binding and ATP catalytic sites, 2 in calmodulin-binding IQ motifs that form the light chain-binding lever arm domain, 1 in a potential coiled-coil regions that mediates the association of the heavy chain into dimers, and 6 are localized in the cargo-binding globular tail domain. All of the *MYO5B* mutations are distinct from those reported in *MYO5A* and other nonconventional myosins. Furthermore, the reported heterozygous mutations are exclusively found in patients of Caucasian origin (Polish, Irish, French, and USA), and include at least one nonsense mutation or large deletion. Interestingly, all but one
Figure 6. Roles of the mutated residues linked to microvillus inclusion disease in class V myosins. A) Evolutionary conservation of the myosin Vb residues substituted in the p.N456S (c.1367 A>G) (Patient 7) and p.C514R (c.1540T>C) (Patient 9) mutations. B) Ribbon diagram of the nucleotide-free structure of the motor domain of chicken myosin V (rigor-like/strong actin-binding state, PDB code: 1OE9), indicating the locations of residues N456 and C514 (MYO5B mutated residues identified in this study) and residues V108, R219, W375, C454 and R656 (MYO5B mutated residues identified by Müller et al., 2008). The mutations reported by Müller et al. (2008) are located in or very near the actin-binding site (W375X), the relay helix (C454insKFC), switch I (R219H) and the ATP binding site (V108G and R656). The upper- and lower-50K subdomains are indicated and colored in green and dark grey, respectively. The helix-turn-helix
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missense mutations cluster in the myosin Vb head domain, whereas the nonsense, splice-site and deletions/insertions are found randomly in the motor, lever arm, and tail domain. While some of the mutations are predicted to result in nonsense-mediated RNA decay (e.g. the homozygous p.Gln1456X mutation in Patient 8 and the c.4460-1G>C mutation in Patient 9), which is supported by the observed reduction in myosin Vb mRNA levels in Patient 8, other *MYO5B* mutations involve residues that are important for the function of the myosin Vb protein. Indeed, the N456 residue mutated in Patient 7, for instance, is part of a set of conserved motifs shared in all myosins that participate in coupling changes in the ATPase active site (P-loop and switch I, Fig. 6B, D) to conformational changes in the actin-binding and force-generating domains, and proposed to have a pivotal role in motor function as mediator of allosteric communication (Holmes et al., 2004; Coureux et al., 2004; Tang et al., 2007; Cecchini et al., 2008).

It is encouraging that all MVID patients (except for one (Müller et al., 2008)) that have been screened thus far carry mutations in their *MYO5B* gene, and the discovery of additional compound heterozygous mutations by Ruemmele and colleagues (2010) and us (this study) significantly strengthens the correlation between *MYO5B* and...
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MVID. This correlation is further supported by a recent study by Ruemmele and colleagues (2010) in which knockdown of myosin Vb in human epithelial colorectal adenocarcinoma (Caco-2) cells recapitulates most of the cellular phenotypes of MVID, and by our observation that MYO5B mutations were not found in two patients that presented with secretory diarrhea and malabsorption after birth but were not diagnosed with MVID. A firm association of MYO5B mutations with MVID is a major advance in the diagnosis of this rare but fatal disease, in which variable phenotypes are seen among patients. It will also facilitate reliable genetic counseling and prenatal screening. Because total parenteral nutrition and bowel transplants are, at best, non-permanent solutions for treating this devastating disease, the continuing identification of MYO5B mutations will pave the way for the development of alternative therapeutic strategies.

Loss of MyoVb affects location of other apical recycling associated proteins

Because MVID involves a specific loss of apical surface identity in enterocytes without visible defects of the basolateral cell surface or monolayer arrangement, it provides an outstanding model for studying brush border development and endosomal trafficking in a human context. We can also see how mutations in MYO5B can perturb these processes.

A main function of myosin Vb is to regulate the subcellular distribution and positioning of recycling endosomes. It does so by interacting with small GTPase Rab proteins such as Rab11a and its effectors (FIP1 and FIP5) at the cytosolic surface of recycling endosomes and coupling these endosomes to and positioning them along actin filaments.

Our previous studies in cultured epithelial cells have demonstrated that a loss of Rab11a-FIP5 function prevents internalized proteins from recycling back to the cell surface (Prekeris et al., 2000), while a loss of Rab11a-FIP1 function in the apical cytoplasm leads to the redirection of internalized brush border proteins into the late endosomal/lysosomal route (Peden et al., 2004). This is in full agreement with the
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electron microscopy observations, which revealed a significant accumulation of late endosomes/lysosomes at the apex of MVID enterocytes.

We demonstrate that the typical and myosin Vb–controlled accumulation of Rab11a- and FIP5-positive recycling endosomes in the apical cytoplasm of the cells is abolished in MVID enterocytes. Interestingly FIP1, in contrast to Rab11a and FIP5, retained its subapical localization in Patients 7 and 9, but not in Patient 8. While this suggests that the intracellular distribution of FIP1 may be differently regulated when compared to that of Rab11a and FIP5, the differential staining pattern of FIP1 between patients likely reflects the specific \textit{MYO5B} mutations carried by these patients, and may offer a tool for future genotype-phenotype analyses.

The \textit{MYO5B} mutations thus impair the spatial distribution and, likely, the functioning of the apical recycling endosome system. It should be noted that our conclusions are based on three cases of this rare disease and that future experiments are necessary to further consolidate these.

**Working model of MVID**

We propose that mutations in \textit{MYO5B} cause structural defects in the myosin Vb protein that prevent brush border proteins to be delivered from the apical endosomal system to the apical cell surface. These proteins, instead, accumulate in late endosomes/lysosomes. Our current working model is depicted in Figure 7. Previous \textit{in vitro} studies by us and others led to the proposal that Rab11a, myosin Vb, and the apical recycling endosomal system are functionally coupled to the development of functional apical surface domains (van IJzendoorn & Hoekstra, 1998, 2000; Wakabayashi et al., 2005; Li et al., 2007). The impairment of these two intertwined processes in MVID patients and the causative relation with \textit{MYO5B} mutations, as demonstrated in this study, is the first proof of this hypothesis.

In addition to Rab11a, Rab8a also shows binding affinity for the globular tail of myosin Vb, with each GTPase defining different recycling pathways (Roland et al., 2007). \textit{Rab8A}-deficient mice showed mislocalization of apical peptidases and
transporters and displayed phenotypes similar to those of MVID patients (Sato et al., 2007). However, no mutations were detected in RAB8A in MVID patients (Sato et al., 2007). It is conceivable that different mutations in the tail domain of myosin Vb may affect the binding capacity for either Rab11a or Rab8a GTPases, or both. This may result in comparable, but not necessarily similar phenotypes. The exocytic transport route, involving Rab4-, Rab8-, and Rab11-positive endosomes (Cramm-Behrens et al., 2008), or the recycling of basolateral plasma membrane proteins (Müller et al., 2008), could also be perturbed by MYO5B mutations. Further in-depth analysis of MYO5B mutations and their molecular consequences are warranted to expand our understanding of how they are related to the phenotypic variations observed in MVID patients. Furthermore, proven MVID patients lacking bi-allelic MYO5B mutations,
like the ones reported by Müller et al. (2008), should be tested for possible mutations in the above Rab GTPases as they may mimic clinically the phenotype of MYO5B mutations.

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