Hypomagnesemia with Secondary Hypocalcemia due to a Missense Mutation in the Putative Pore-forming Region of TRPM6*§

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Hypomagnesemia with secondary hypocalcemia is an autosomal recessive disorder caused by mutations in the TRPM6 gene. Current experimental evidence suggests that TRPM6 may function in a specific association with TRPM7 by means of heterooligomeric channel complex formation. Here, we report the identification and functional characterization of a new hypomagnesemia with secondary hypocalcemia missense mutation in TRPM6. The affected subject presented with profound hypomagnesemia and hypocalcemia caused by compound heterozygous mutation in the TRPM6 gene: 1208(−1)G > A affecting the acceptor splice site preceding exon 11, and 3050C > G resulting in the amino acid change (P1017R) in the putative pore-forming region of TRPM6. To assess the functional consequences of the P1017R mutation, TRPM6P1017R and wild-type TRPM6 were co-expressed with TRPM7, resulting in the amino acid change (P1017R) in the putative pore-forming region of TRPM6. To assess the functional consequences of the P1017R mutation, TRPM6P1017R and wild-type TRPM6 were co-expressed with TRPM7 in Xenopus oocytes and HEK 293 cells, and currents were assessed by two-electrode voltage clamp and whole cell patch clamp measurements, respectively. Co-expression of wild-type TRPM6 and TRPM7 resulted in a significant increase in the amplitude of TRPM7-like currents. In contrast, TRPM6P1017R suppressed TRPM7 channel activity. In line with these observations, TRPM7, containing the corresponding mutation P1040R, displayed a dominant-negative effect upon co-expression with wild-type TRPM7. Confocal microscopy and fluorescence resonance energy transfer recordings demonstrated that the P1017R mutation neither affects assembly of TRPM6 with TRPM7, nor co-trafficking of heteromultimeric channel complexes to the cell surface. We conclude that a functional defect in the putative pore of TRPM6/7 channel complexes is sufficient to impair body magnesium homeostasis.

Mg²⁺ plays a vital role in virtually all cellular pathways as a cofactor of enzymes, an essential structural element of proteins and nucleic acids, and a modulator of receptors and ion channels (1–4). At present, the molecular mechanisms controlling Mg²⁺ homeostasis are poorly understood. Thus, clinical and molecular characterizations of hereditary disorders associated with Mg²⁺ handling provide a promising point of entry to investigate the molecular components pertinent to cellular Mg²⁺ handling.

Recently, it was discovered that loss-of-function mutations in the gene of melastatin-related transient receptor potential cation channel 6 (TRPM6)² result in hypomagnesemia with secondary hypocalcemia (HSH) (5–8). HSH is an autosomal recessive disorder characterized by low serum Mg²⁺ levels due to defective intestinal absorption and increased renal Mg²⁺ wasting (3, 9, 10). Hypocalcemia results from a secondary insufficiency of the parathyroid glands in the presence of profound hypomagnesemia. Supplementation with high Mg²⁺ doses compensates for the Mg²⁺ deficiency of HSH patients (3, 9, 10).

TRPM6 belongs to the melastatin-related group of the TRP ion channel family (11, 12). Like other TRP channels, TRPM proteins contain six transmembrane helices (S1–S6) flanked by cytoplasmic N and C termini; TRPMs most likely function as tetrameric channel complexes (12). Hydrophobic segments located between the S5 and S6 helices of four channel subunits are thought to contribute to a channel pore. In contrast to other known ion channels, TRPM6 and its closest family member, TRPM7, display the unique structural feature of being cation channels fused to Ser/Thr kinase domains at their C termini (13).

TRPM7 is a ubiquitously expressed protein, which is essential for Mg²⁺ homeostasis. Disruption of the TRPM7 gene in DT40 chicken lymphocytes and the zebrafish Danio rerio, resulted in Mg²⁺ deficiency (14, 15). Functional characterization of heterologously expressed TRPM7 revealed a constitutively active cation channel permeable to a broad range of divalent cations, including Mg²⁺ and Ca²⁺ (16–18). TRPM7 channel activity is tightly regulated by the intracellular free con-

² The abbreviations used are: TRPM6, melastatin-related transient receptor potential cation channel 6; TRPM7, melastatin-related transient receptor potential cation channel 7; TRPC6, canonical transient receptor potential cation channel 6; HSH, hypomagnesemia with secondary hypocalcemia; KvAP, a voltage-dependent K⁺ channel from the thermophilic archaea-bacterium Aeropyrum pernix; GFP, enhanced green fluorescent protein; CFP, enhanced cyan fluorescent protein; YFP, enhanced yellow fluorescent protein; FRET, fluorescence resonance energy transfer; DIC, differential interference-contrast; HEK 293 cells, human embryonic kidney 293 cells; MIC, Mg²⁺-inhibited cation; RT, reverse transcription; PBS, phosphate-buffered saline.

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centrations of Mg$^{2+}$ ([Mg$^{2+}$]), MgATP ([MgATP]) and a growing number of other stimuli (19–25). Annexin A1 and myosin II have been identified as physiological substrates of the TRPM7 kinase domain (23, 26). However, the functional interplay between kinase and channel domains in TRPM7 is far from being understood (15, 20, 27, 28).

Conflicting findings have been reported concerning functional characteristics of TRPM6 expressed in heterologous cell systems. According to Voets et al. (29), TRPM6 forms homooligomeric channel complexes with biophysical characteristics similar to those of TRPM7. More recently, Li et al. (30) described a number of TRPM6 properties different from TRPM7, including single channel conductance, divalent cation permeability, and pH sensitivity. In contrast, two other studies suggest that TRPM6 functions only in association with TRPM7 by means of heterooligomeric channel complex formation (8, 28). Consistently, in DT40 cells, which are Mg$^{2+}$-deficient due to a genetically disrupted TRPM7 gene, recombinant human TRPM6 (unlike TRPM7) is unable to compensate for TRPM7 (28). Within the formed channel complex, TRPM6 potentiates TRPM7 channel activity (8) and is able to cross-phosphorylate TRPM7, but not vice versa (28). Moreover, in terms of biophysical characteristics, heterooligomeric TRPM6/7 channels were found to be different from TRPM7 homooligomers (30). At present, the stoichiometry of TRPM6/7 in native channel complexes remains unknown.

Most HSH mutations in TRPM6 introduce stop codons, deletions of exons, frame shifts, or affect splice sites, thus resulting in complete loss of function (7). Thus, it remains elusive whether a lack of channel and/or kinase activities of TRPM6 is responsible for the HSH phenotype. Therefore, the molecular analysis of HSH mutations affecting distinct domains of TRPM6/7 (for instance, the kinase domain and pore-forming segments) should be instrumental in dissecting the specific role of different TRPM6 domains for Mg$^{2+}$ handling. For example, we recently discovered the disease-causing molecular mechanism of a TRPM6 missense mutation, which disrupts the heteromeric assembly of TRPM6 and TRPM7 thereby eliciting HSH (8).

Here, we report the identification and functional characterization of a novel point mutation in the TRPM6 gene. We disclose a novel molecular mechanism underlying HSH: The P1017R mutation neither affects the expression of the TRPM6 protein, nor its co-assembly with TRPM7, but impairs channel activity of TRPM6/7 complexes by dominant-negative suppression. The effect exerted by TRPM6-P1017R strongly supports the notion that both channel subunits contribute to functional TRPM6/7 complexes. Thus, a suppression of cation fluxes via bi-functional TRPM6/7 complexes is sufficient for the development of HSH.

**EXPERIMENTAL PROCEDURES**

**Subjects, Genotyping, and Clinical Studies**—Identification and clinical characterization of an HSH subject (Family F14) was reported previously (7). Subsequent genotyping, reported here, was performed using direct sequencing of the entire coding region of the TRPM6 gene from genomic DNA extracted from blood leukocytes as described (5, 7). Assessment of renal magnesium handling, as well as determination of serum [Mg$^{2+}$] and [Ca$^{2+}$] levels were performed as reported (5, 7).

**Molecular Biology and Generation of TRPM6 and TRPM7 Antibodies**—Human TRPM6 (TRPM6, variant a, AY333285) and mouse TRPM7 (NM_021450) cDNAs were cloned into the pcDNA3.1/V5-His Ta-TOPO eukaryotic expression vector (Invitrogen) as described previously (8). TRPM6-P1017R and TRPM7-P1040R mutations were introduced by site-directed mutagenesis using the QuickChange system (Stratagene). To generate TRPM6 and TRPM7 C-terminal fused to enhanced green fluorescent protein (EGFP), enhanced yellow fluorescent protein (YFP) and enhanced cyan fluorescent protein (CFP), STOP codons were replaced by Xhol restriction sites through site-directed mutagenesis followed by in-frame subcloning of YFP cDNAs. Previously, we demonstrated that YFP tags do not influence trafficking and channel properties of TRPM6 and TRPM7 (8). Expression of TRPM6 and TRPM7 in *Xenopus laevis* oocytes was performed using cDNAs subcloned into the pOGII vector (a Bluescript derivative with the 5’- and 3’-untranslated regions of *Xenopus β*-globin). All cDNA constructs used in the present work were confirmed by sequencing. A polyclonal TRPM6 antiserum was obtained as recently reported (8). To generate a polyclonal TRPM7-specific antibody, rabbits were immunized with the following peptide coupled to keyhole limpet hemocyanin: H$_2$N-DSPFVDS-KAALLP-COOH (Eurogentec, Brussels). Subsequently, the TRPM7-specific antibody was purified by peptide affinity chromatography.

**Structural Analysis of the TRPM6 Pore-forming Region**—Three-dimensional coordinates of the S1–S6 segment of the human TRPM6 protein (Q9B8X4) were acquired from MODBASE (saliab.org/modbase), a data base of annotated comparative protein structure models (31). The pore-forming segment of TRPM6 (amino acids 872–1074) was matched to the structure of KvAP, a voltage-dependent K$^+$ channel from the thermophilic archae-bacterium *Aeropyrum pernix* (32), PDB code 1orq (chain C, amino acids 28–240). Accordingly, the 1orq template was applied for comparative modeling, and annotated coordinates were used to generate molecular graphics images with the University of California at San Francisco Chimera package (33). For simplicity, only α-carbon traces of the S5–S6 regions from TRPM6 and KvAP are shown (Fig. 1B). For sequence alignment ClustalW (www.ebi.ac.uk/clustalw/) was used.

**Cell Culture, Transient Expression, and Generation of Stable Cell Lines**—Human embryonic kidney (HEK) 293 cells were maintained at 37 °C under 5% CO$_2$ in Earle’s minimal essential medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin (PAA Laboratories, Pasching, Austria). Cells were transiently transfected using the FuGENE6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Stable cell lines expressing TRPM6 and TRPM7 were generated using an ecdyson-inducible expression system (Invitrogen) according to the supplier’s instructions. Briefly, TRPM6 and TRPM7 cDNAs were subcloned into the pIND(SP1)/Hygro expression vector (Invitrogen) and transfected into the Ecr293 cell line (Invitrogen). Stable transfectants were selected by culturing in 400 μg/ml hygromycin B (Invitrogen), propagated,
and analyzed for ponasterone A-inducible expression by RT-PCR and Western blotting. Two clones were selected for further analysis: M6pIND (clone 2-1, expressing human TRPM6) and M7pIND (clone 2-19, expressing mouse TRPM7). The M6pIND and M7pIND cell lines were maintained at 37 °C under 5% CO₂ in Dulbecco’s modified Earle’s medium (PAA Laboratories) supplemented with 10% fetal calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin, 400 µg/ml hygromycin B, and 400 µg/ml Zeocin (Invitrogen).

Western Blotting—M6pIND and M7pIND cells grown in 35-mm dishes to ~80% confluence were induced by different concentrations of ponasterone A (Invitrogen) as indicated in the figure legends. 24 h after induction, cells were washed twice with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3). Next, 1 ml of 2× Laemmli buffer was added and samples were heated for 5 min at 65 °C. Cell lysates (20 µl) were subjected to SDS-PAGE (8%) and blotted on nitrocellulose membranes (Protran, Whatman). After incubation in blocking buffer (5% nonfat dry milk in PBS) for 1 h at room temperature, blots were probed either with anti-TRPM6 antiserum (1:1000) or anti-TRPM7 antibody (2 µg/ml) and blocked for 1 h with 10% (v/v) normal goat serum in PBS at room temperature. The TRPM7-specific antibody (0.3 µg/ml) or the TRPM6-specific antiserum (1:1000) were applied. The secondary antibody (0.5 µg/ml) was goat anti-rabbit IgG conjugated to Alexa488 (Molecular Probes). Each incubation was performed in PBS containing 5% (v/v) normal goat serum for 1 h at room temperature followed by a triple washing step with PBS. After the final washing step, coverslips were placed on glass slides using mounting medium (DakoCytomation), and examined using a LSM510 META confocal laser scanning microscope as indicated above.

Fluorescence Resonance Energy Transfer (FRET) Recordings—Our static FRET protocol was based on donor (CFP) fluorescence recovery after acceptor (YFP) bleach as reported previously (8, 34). Briefly, HEK 293 cells grown on 25-mm glass coverslips in 35-mm dishes were transiently transfected with 4 µg/dish plasmid DNAs (1 µg of plasmid cDNA encoding the CFP-tagged TRPM channel subunit and 3 µg of plasmid encoding the respective YFP-tagged subunit). Cells were examined 40–48 h after transfection at room temperature in HEPES-buffered saline (140 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 5 mM glucose, 0.1% bovine serum albumin, pH 7.4) with an Olympus IX70 inverted microscope equipped with a polychrome IV monochromator (TILL Photonics), an Olympus 63×/1.4 UAPo objective, a Sensicam charge-coupled device camera, and a Lambda 10-2 emission filter wheel (Sutter Instruments). FRET efficiencies were determined by monitoring the increase in the CFP (FRET donor) fluorescence emission during selective YFP (FRET acceptor) photobleaching (8, 34). In each experiment, data from four to six single cells were averaged. Means ± S.E. were calculated from three to four independent transfections for each combination of CFP- and YFP-fused TRPM6/7 subunits. Statistical analysis was performed using Student’s t test.

Electrophysiological Techniques—M6pIND or M7pIND cells grown in 35-mm dishes to ~70% confluence were induced with 5 µM ponasterone A for 18–24 h before performing electrophysiological experiments. For transient expression of TRPM6P1040R-YFP, TRPM6P1017R-YFP, TRPM7P1040R, and TRPM7P1017R-YFP in the pcDNA3.1/V5-His vector (Invitrogen) or TRPM7P1040R in pIRE2-EGFP vector (Clontech) in M7pIND cells, corresponding plasmid DNAs (2 µg/dish) were transfected with the FuGENE6 transfection reagent. ~24 h after transfection, culture media were replaced by fresh media containing 5 µM ponasterone A. Only GFP- or YFP-positive cells were measured 18–24 h after induction. Whole-cell patch clamp recordings were carried out at room temperature (22 °C). Cells were superfused with bath solution: 140 mM NaCl, 5 mM CsCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM HEPES, titrated to pH 7.4 with NaOH. Data from whole-cell recordings were collected with an EPC10 patch clamp amplifier (HEKA) using the Pulse software (HEKA). Patch pipettes were made of borosilicate glass (Science Products) and had resistances between 1.8 and 3.1 MΩ when filled with intracellular solution: 120 mM CsCl, 10 mM NaCl, 0.635 mM CaCl₂ (5.5 mM free Ca²⁺, calculated with the CaBuf program (ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip)), 10 mM 1,2-bis(2-aminophenoxo)ethane-N,N,N',N''-tetraacetic acid tetrakis, 1 mM
HEDTA, 10 mM HEPES, titrated to pH 7.2 with CsOH. The liquid junction potential was 5.1 mV and was corrected for by the Pulse v8.7 software. Current-voltage relationships were recorded during voltage ramps from 100 to 100 mV with a slope of 0.5 V s⁻¹ applied at a frequency of 2 Hz. Using the Pulse software, series resistance and capacity were estimated and corrected automatically before each ramp. Series resistance compensation of 80% was used to reduce voltage errors in all experiments. Data were acquired at a frequency of 5 kHz after filtering at 1.67 kHz. Statistical analysis was performed using Student’s t test.

Expression of TRPM Proteins in X. laevis Oocytes—TRPM6 and TRPM7 cDNAs inserted into the pOGII expression vector were transcribed in vitro with the mMessage mMachine system (Ambion). 10 ng of cRNA for each TRPM construct was injected into defolliculated Xenopus oocytes. For co-expression experiments, a 1:1 ratio of different cRNAs (10 ng for each TRPM) was used. In a subset of experiments, 10 ng of TRPM7 cRNA were co-injected with 5 ng of wild-type TRPM6 plus 5 ng of TRPM6P1017R cRNAs. Oocytes were kept in ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES (pH 7.4), 2.5 mM sodium pyruvate, 0.5 mM theophylline, and 20 μg/ml gentamicin at 16 °C. 2–5 days after injection, two-electrode voltage-clamp measurements were performed with a GeneClamp 500 amplifier (Axon Instruments) at room temperature. Currents were recorded in ND96 solution without sodium pyruvate, theophylline, and gentamicin. Data were reproduced in at least four different batches of oocytes derived from different frogs. Statistical analysis was performed on current recordings derived from one batch of oocytes with at least seven oocytes per data point using Student’s t test.

RESULTS

Identification and Clinical Characterization of a Novel HSH Missense Mutation—Recently, we described the genotyping and clinical characterization of an HSH patient (F14.1) with a heterozygous mutation in the TRPM6 gene (7). In brief, the patient was born to non-consanguineous parents and presented at the age of 7 months with generalized convulsions. HSH was diagnosed based on profound hypomagnesemia (0.29 mM serum [Mg²⁺]) and hypocalcemia (1.6 mM serum [Ca²⁺]) as compared with normal serum levels of these cations (0.7–1.1 mM [Mg²⁺] and 2.2–2.9 mM [Ca²⁺]). Sequence analysis revealed a splice site mutation at the acceptor splice site preceding exon 11 [1208(+)G→A] of the TRPM6 gene (7).

Here, we extended the genotyping analysis of the F14 family (Fig. 1A) and detected a second affected allele: a single nucleotide exchange (3050C→G) leading to a non-conservative amino acid exchange from proline to arginine at position 1017 (P1017R). The patient F14.1 presented with cerebral seizures due to severe hypomagnesemia (0.29 mM) and currently...
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receives continuous oral magnesium supplementation at a typical dosage for HSH patients (0.6 mmol/kg/day). As expected, each of the mutations was observed in a heterozygous state in the parents (Fig. 1A). Notably, the father carrying the allele P1017R presented with a normal serum [Mg²⁺] level (0.93 mmol/L) and fractional magnesium excretion (2.4%). Thus, we classified the patient as compound heterozygous, with a phenotype indistinguishable from previously described HSH patients (5, 7).

The identified HSH mutation affects a residue located between the predicted S5 and S6 domains of TRPM6 (supplemental Fig. S1). This region displays only weak sequence homology to other TRP channels (35). Therefore, we took advantage of the MODBASE resource to map P1017 in the annotated three dimensional coordinates of the TRPM6 S5–S6 segment (see “Experimental Procedures”). As shown in Fig. 1 (B and C), TRPM6 has amino acid and predicted structural similarity to a voltage-gated K⁺ channel from the archae-bacterium Aeropyrum pernix (KvAP) (32). The comparison of TRPM6 and KvAP suggested that P1017 may be located in the center of the putative pore helix (Fig. 1B). This domain is thought to be an essential structural element of the channel pore in a large number of ion channels (35). Consequently, we hypothesized that the substitution of P1017 by a positively charged Arg could change the conformation of the pore helix and thus interfere with ion permeation properties of TRPM6.

TRPM6P1017R Suppresses TRPM7-mediated Currents in Xenopus Oocytes—As described previously, TRPM6 interacts with its close homologue TRPM7 for efficient membrane insertion and formation of functional channel complexes. We therefore co-expressed cRNAs of TRPM6P1017R or wild-type TRPM6 with TRPM7 cRNA in Xenopus oocytes, the expression system of choice to obtain a precise stoichiometric cRNA ratio (1:1) for subsequent protein expression. As determined by two-electrode voltage-clamp measurements, expression of TRPM7 resulted in inward and outward currents at negative and positive voltages, respectively (Fig. 2, A and B). In line with our previous observations, TRPM6 expressed alone did not induce ion currents significantly different from uninjected control oocytes. Co-injection of cRNAs encoding wild-type TRPM6 and TRPM7 significantly increased current amplitudes. In contrast, co-expression of TRPM6P1017R not only failed to increase TRPM7 currents, but rather resulted in a substantial suppression of TRPM7-mediated currents (Fig. 2, A and B). These data are compatible with the assumption that P1017 of TRPM6 contributes to the ion channel pore of heteromeric TRPM6/ TRPM7 complexes and mutation of P1017 exerts a dominant-negative effect on TRPM7.

As reported above, the father who carries one mutant (3050C > G) allele did not display a detectable magnesium deficiency. To mimic this situation in our expression system, we co-injected 10 ng of TRPM7 cRNA with a cRNA mixture of the two TRPM6 variants (5 ng of TRPM6wt plus 5 ng of TRPM6P1017R). We observed that co-expression of TRPM6wt masked the suppressive effect of TRPM6P1017R on TRPM7 (Fig. 2C). Thus, in vivo and in vitro data suggest that in the father’s heterozygous state the channel subunit encoded by the wild-type allele is able to overcome the negative effect of TRPM6P1017R providing an explanation for the lack of the HSH symptoms in the patients’ father.

Heteromeric Channel Complexes Formed by TRPM7 and TRPM6P1017R in HEK 293 Cells Lack Channel Activity—To obtain independent evidence for a role of the TRPM6P1017R mutation, we generated two HEK 293 cell lines, M6pIND and M7pIND, which express TRPM6 and TRPM7 under the control of a ponasterone A-inducible promoter as demonstrated by Western blotting, RT-PCR analysis, and immunofluorescent staining (Figs. 3A, 3B, 4A, and 4B). In accord with our experiments with Xenopus oocytes, whole-cell patch clamp recordings revealed different biophysical characteristics of M6pIND and M7pIND cells (Figs. 3C and 4C). TRPM7 expression yielded large outwardly rectifying currents with a reversal potential close to 0 mV. Inward currents, although small, were significantly increased (Fig. 3C). Consistently, immunofluorescent staining of the ponasterone A-induced M7pIND cells using the TRPM7-specific antibody invariably detected the channel subunits on the cell surface (Fig. 3B). Similar to recent observations (36), expression of TRPM7 substantially
changed cell morphology without alteration in cell capacitance, an indicator of the cell surface area (supplemental Fig. S2). Ponasterone A-stimulated M7PIND cells displayed a decrease in motility and, thereby, formation of dense colonies (Fig. 3B, supplemental Fig. S2). These findings are compatible with the notion that TRPM7-mediated ion fluxes are involved in the observed morphological alterations of M7PIND cells.

Unlike TRPM7, the expression of TRPM6 neither elicited cation currents, nor induced detectable changes in cell morphology or the capacitance of M6PIND cells (Fig. 4, B and C, supplemental Fig. S2). Moreover, immunostaining of M6PIND cells using the TRPM6-specific antiserum revealed predominant localization of the channel subunits in intracellular membrane compartments (Fig. 4B) compatible with a lack of TRPM6-mediated currents. To exclude that M6PIND cells expressed TRPM6 at low levels insufficient for the detection of channel activity, we transiently transfected M6PIND cells with a plasmid vector coding for TRPM6 and assessed expression levels of the protein using the TRPM6-specific antiserum. Fig. 4D shows that the two expression systems yielded a comparable amount of TRPM6 protein. As in the inducible expression system, we cannot detect TRPM6-mediated currents in transiently transfected HEK 293 cells when compared with untransfected controls (Fig. 5C). Thus, our studies with three different expression systems suggest that TRPM6, in contrast to TRPM7, does not efficiently form functional homooligomeric channel complexes.

We found that current densities recorded with stimulated M7PIND cells were only 1.8 times lower than those detected in HEK 293 cells (n = 9) transfected with plasmid vector coding for TRPM7 (1053 ± 204 pA/picofarad, three independent transfections). These observations are well compatible with our Western analyses to monitor expression levels of ponasterone A-induced and transiently expressed TRPM7 (Fig. 3D). Thus, M7PIND cells appear to

FIGURE 3. Characterization of the M7PIND cell line. A, dose dependence of ponasterone A-induced TRPM6 expression as demonstrated by Western blotting (left panel) and RT-PCR analysis (right panel). An antibody against TRPM7 and TRPM7-specific PCR primers was used to demonstrate a ponasterone A-inducible expression of TRPM7. An antibody against β-actin and β-actin-specific PCR primers were used for corresponding input controls. Positions of TRPM7- and β-actin-specific bands are indicated by arrows. B, immunofluorescence staining of M7PIND cells using the TRPM7-specific antibody before (left panel) and after (right panel) the addition of ponasterone A to the culture media. Representative confocal images of Alexa488 fluorescence (Alexa488) and their overlay with corresponding DIC images (Overlay) are depicted. Arrows indicate the cell surface; scale bars, 10 μm. C, dose dependence of ponasterone A-induced TRPM7 currents. Three exemplary current-density voltage relationships for 0, 1, and 5 μM ponasterone A are displayed (left panel). The inset shows the three corresponding time courses for inward and outward current increases at holding potentials of −100 and +100 mV. The arrow indicates the zero current level. The right panel shows the current density analysis for different ponasterone A incubations. The current densities were determined from the maximal current over 600 s at ±100 mV; bars represent means ± S.E. Numbers above the bars indicate the total number of recorded cells and the number of independent inductions; *, p < 0.05; **, p < 0.01 compared with 0 μM ponasterone A. D, Western blot analysis of M7PIND cells transiently transfected with 2 μg of plasmid DNA coding for TRPM7-YFP (M7-YFP) or wild-type TRPM7 (M7), (−) and (+) indicate the addition of 5 μM ponasterone A (Pon.) to the culture media. An antibody against TRPM7 was used to detect expression of wild-type or YFP-tagged TRPM7. An antibody against β-actin was used for a corresponding loading control. Positions of TRPM7, TRPM7-YFP, and β-actin-specific bands are indicated by arrows.

be well suited for a functional assessment of TRPM6 channel subunits. To this end, we transiently expressed TRPM6 variants in M7PIND cells (Fig. 5A) and found that, unlike TRPM6WT, TRPM6P1017R caused a suppression of TRPM7-mediated channel activity (Fig. 5A).
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P1017 in TRPM6 is a conserved residue in the TRPM gene family (supplemental Fig. S1). Three-dimensional modeling of the pore-forming region of TRPM7 mapped the corresponding P1040 in TRPM7 to an equivalent position to that of TRPM6P1017R, TRPM7P1040R completely suppressed channel activity of wild-type TRPM7 in the ponasterone A-stimulated M7pIND cells (Fig. 5B).

TRPM7 is considered to be the molecular substrate of Mg2+-inhibited cation (MIC) currents readily detectable in various cells when cytoplasmic [Mg2+] is lowered during whole-cell recordings, the commonly used maneuver to detect TRPM7-mediated currents in heterologous expression systems (16, 37). We asked whether TRPM6P1017R and TRPM7P1040R would be able to suppress native MIC currents. Similar to other reports (16, 36), our recordings on untransfected HEK 293 cells revealed TRPM7-like inward and outward currents (Fig. 5C). In fact, unlike TRPM6M6pIND, transient expression of both mutants caused a substantial reduction of TRPM7-like currents. Interestingly, the suppressive effect of TRPM7P1040R was significantly more pronounced than that of TRPM6P1017R (Fig. 5C). Altogether, our biophysical experiments provide an independent line of evidence that TRPM6P1017R and TRPM7P1040R function as dominant-negative channel subunits.

FIGURE 4. Characterization of the M6pIND cell line. A, dose dependence of ponasterone A-induced TRPM6 expression as demonstrated by Western blotting (left panel) and RT-PCR analysis (right panel). An antiserum against TRPM6 and TRPM6-specific PCR primers were used to demonstrate a ponasterone A-inducible expression of TRPM6. The antibody against β-actin and the β-actin-specific PCR primers were used for corresponding input controls. Positions of TRPM6- and β-actin-specific bands are indicated by arrows. B, immunofluorescence staining of M6pIND cells using the TRPM6-specific antiserum before (left panel) and after (right panel) the addition of ponasterone A to the culture media. Representative confocal images of Alexa488 fluorescence (Alexa488) and their overlay with corresponding DIC images (Overlay) are depicted. Scale bars are 10 μm. C, dose dependence of ponasterone A-induced TRPM6 currents. Three exemplary current-density voltage relationships for 0, 1, and 5 μM ponasterone A are displayed (left panel). The inset shows the three corresponding time courses for inward and outward current increases at −100 and +100 mV. The arrow indicates the zero current level. The right panel shows the current density analysis for different ponasterone A incubations. The current densities were determined from the maximal current observed over 600 s at +100 mV; bars represent means ± S.E. Numbers above the bars indicate the total number of recorded cells and the number of independent inductions. D, Western blot analysis of TRPM6 expression in M6pIND cells transiently transfected with 2 μg of plasmid DNA coding for wild-type TRPM6. (−) and (+) indicate transient transfection of TRPM6 (M6) or the addition of 5 μM ponasterone A (Pon.) to the culture media. An antiserum against TRPM6 was used to detect expression of TRPM6. An antibody against β-actin was used for a corresponding loading control. Positions of TRPM6 and β-actin-specific bands are indicated by arrows.

P1017 in TRPM6 is a conserved residue in the TRPM gene family (supplemental Fig. S1). Three-dimensional modeling of the pore-forming region of TRPM7 mapped the corresponding P1040 in TRPM7 to an equivalent position to that of TRPM6.
may interfere with gating/permeation mechanisms of the ion channel and, thus, the mutant channel may well be present on the cell surface (38–41).

To elucidate the mechanisms responsible for TRPM6<sup>P1017R</sup> malfunction we used fluorescence imaging techniques to monitor the assembly of channel subunits. Previously, we and others (8, 34, 42) demonstrated that FRET is a reliable approach to study homo- and heteromultimerization of TRP channels, including TRPM6 and TRPM7. Therefore, we labeled TRPM6 and TRPM7 variants with YFP and CFP tags, co-expressed them in HEK 293 cells, and recorded a static FRET signal from the homo- and heteromultimers formed (Fig. 6A). We found that wild-type and mutated subunits were indistinguishable in FRET efficiencies (human TRPC6 was used as negative control).

Next, we used confocal microscopy to test whether TRPM6<sup>P1017R</sup> affects the trafficking of TRPM7 subunits to the plasma membrane and, thereby, suppresses its channel activity. To this end, we co-expressed TRPM7 fused to YFP with untagged TRPM6<sup>P1017R</sup> and compared its subcellular localization with TRPM7-YFP expressed alone (Fig. 6B). We observed that in both cases TRPM7-YFP was detectable at the cell surface. Moreover, imaging of HEK 293 cells transfected with TRPM7<sup>P1040R</sup>-YFP demonstrated the correct localization of the mutant protein at the cell surface (Fig. 6B). Alternatively, we used a TRPM7-specific antibody for immunofluorescence staining of HEK 293 cells expressing wild-type or mutated TRPM7 (supplemental Fig. S3). This approach also demonstrated a correct cell surface localization of TRPM7<sup>P1040R</sup>.

As shown previously (8), the subcellular localization of TRPM6 labeled with YFP can be used to monitor its assembly with TRPM7 in living HEK 293 cells. TRPM6-YFP expressed alone is retained in intracellular membrane compartments (Fig. 6C) analogous to the localization of the untagged protein in M6<sup>IND</sup> cells (Fig. 4B). Co-expression of TRPM6-YFP or TRPM6<sup>P1017R</sup>-YFP with untagged TRPM7 resulted in co-trafficking of both proteins to the cell surface (Fig. 6C). Thus, in terms of functional and trafficking
characteristics, TRPM6<sup>P1017R</sup> is clearly different from the previously described mutant HSH allele, TRPM6<sup>S141L</sup> (8). The latter mutant was not able to enhance TRPM7 channel activity due to its inability to assemble with TRPM7 and to be co-trafficked to the plasma membrane. Collectively, these findings are in line with the hypothesis that deficient ion permeation through TRPM6<sup>P1017R</sup>/TRPM7 complexes is the primary mechanism responsible for the development of HSH.

**DISCUSSION**

Here we report the genotyping, and in vitro functional analysis of a second point mutation in the TRPM6 gene associated with HSH. The newly discovered missense mutation causes the first known amino acid exchange in the channel pore-forming segment of TRPM6. The affected patient presented with a typical HSH phenotype, including hypomagnesemia as well as the secondary symptoms, hypocalcemia and generalized convulsions. In clinical terms, patients carrying the newly discovered mutant allele are indistinguishable from previously described HSH individuals completely lacking TRPM6 protein expression (5–7).

The mutated P1017 is located between the predicted S5–S6 helices of TRPM6, a segment with relatively low primary amino acid sequence homology to other TRP channels. Therefore, we took advantage of the structural prediction of this segment and mapped P1017 to the putative pore helix, a conserved element of the pore structure in many ion channels (35). Remarkably, the three-dimensional model of the TRPM6 S5–S6 region is well compatible with the recent structural prediction of the equivalent region of a more distantly related family member, TRPM4 (43, 44). Moreover, the model-based structural analysis of the S5–S6 segment of TRPM6 suggested particular amino acids to be involved in folding of the putative pore loop, which, like in other channels (35), are assumed to be critical determinants of ion selectivity. In fact, systematic site-directed mutagenesis of corresponding amino acid residues in TRPM7 resulted in substantial changes in its permeation characteristics.  

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![Diagram](image-url)
Based on the structural prediction, we hypothesized that the P1017R mutation in TRPM6 may alter the overall folding of the pore helix, thereby affecting its ion permeation characteristics. To evaluate the missense mutation functionally, we used two experimental systems: Xenopus oocytes and HEK 293 cells. In both expression models TRPM6, when expressed alone, did not show a significant level of channel activity, whereas co-expression of wild-type TRPM6 and TRPM7 resulted in amplification of TRPM7-mediated currents. However, unlike wild-type TRPM6, TRPM6\textsuperscript{P1017R} caused a suppression of TRPM7-mediated ion currents. Confocal microscopy of living HEK 293 cells showed that TRPM6\textsuperscript{P1017R}/TRPM7 complexes were normally co-targeted to the cell surface. Thus, biophysical and trafficking characteristics of TRPM6\textsuperscript{P1017R} are noticeably different from the TRPM6\textsuperscript{S141L} allele described previously: the S141L mutation severely impaired trafficking of TRPM6 without detectable impact on TRPM7 channel activity (8). Together, we conclude that TRPM6\textsuperscript{P1017R} was efficiently assembled with TRPM7 but exerted a dominant-negative effect on channel activity primarily due to functional defects in the channel pore of the heterotetramer.

The affected proline in TRPM6 is a highly conserved residue in TRPM proteins. Accordingly, the incorporation of a HSH-like mutation in the closely related TRPM7 channel had functional consequences similar to those observed in TRPM6\textsuperscript{P1017R}/TRPM7\textsuperscript{P1040R} was properly targeted to the cell surface where it elicited a dominant-negative effect on ion currents mediated by wild-type TRPM7. Thus, these experiments provide additional evidence in favor of our molecular explanation for the functional defect in TRPM6\textsuperscript{P1017R}.

None of the HSH families with loss-of-function mutations in the TRPM6 gene examined so far did display a haploinsufficiency in heterozygous subjects, i.e. only a complete lack of TRPM6 causes clinical symptoms of HSH (7). However, it is likely that mutations affecting exon splicing sites or truncating the coding sequence due to a frameshift (7) result in expression of dominant-negative channel subunits. If so, such a situation would be similar to the P1017R allele. Of note, our genetic and clinical data indicate clearly that the patient’s father carrying a single mutation in the gene (P1017R) has normal renal magnesium handling. A plausible explanation for the lack of symptoms in the patient’s father is that TRPM6\textsuperscript{P1017R} is not able to completely suppress the function of proteins produced by the wild-type allele. Apparently, a functional role of TRPM6 may be maintained by a relatively low amount of the protein, a phenomenon frequently observed with genes associated with autosomal recessive disorders (38–41, 45). In fact, our experiments with Xenopus oocytes showed that TRPM6\textsuperscript{wt} co-injected with TRPM6\textsuperscript{P1017R} at a 1:1 ratio reversed the dominant negative effect of TRPM6\textsuperscript{P1017R} on TRPM7. The following scenario can be proposed to explain this observation. Wild-type TRPM6 may heteromultimerize more efficiently than the mutant protein. Thus, the actual number of functional channel complexes on the cell surface may be different from that calculated from a stoichiometric expression ratio of TRPM6\textsuperscript{wt}/TRPM6\textsuperscript{P1017R} in the heterozygote. Also, we cannot exclude that our heterologous expression system does not comprehensively reproduce the pathophysiological situation in native epithelial cells. Yet, different isoforms of TRPM6 (8) and TRPM7 (17) may shape the net effect of P1017R in vivo. It is also possible that, in the heterozygous state, the expression of both alleles may be up-regulated raising the number of heterooligomers containing only wild-type subunits to a sufficient level.

Apart from the clinical importance, analysis of the TRPM6 and TRPM7 mutants highlighted a number of issues concerning the function of these proteins. First, we found that transiently expressed TRPM6\textsuperscript{P1017R} and TRPM7\textsuperscript{P1040R} are able to suppress native TRPM7-like, Mg\textsuperscript{2+}-inhibited (MIC) cation currents in HEK 293 cells. In line with recent data obtained with a short hairpin RNA approach (36), these results provide independent evidence that TRPM7 channel complexes are molecular substrates of endogenous MIC channels. Second, the dominant-negative effect exerted by TRPM6\textsuperscript{P1017R} on endogenous MIC channels supports our previous notion (8) that in TRPM6/7 heterooligomers both channel subunits contribute to the channel pore. Third, analyses of HSH missense mutations affecting different functional modules of TRPM6 may provide valuable information permitting to dissect the biological role played by its channel and kinase domains. At present, the functional interplay between channel and kinase activities of TRPM6/7 complexes remains elusive. Our functional analysis of TRPM6\textsuperscript{P1017R} indicates that suppression of the channel function in TRPM6/7 complexes is sufficient to elicit the characteristic HSH phenotype. This finding is well compatible with the biophysical analysis of heterologously expressed TRPM7 lacking the kinase domain or the TRPM7 mutants deficient in the kinase activity (15, 27, 46). These studies showed that principally TRPM7 kinase activity is not required for channel activity. Moreover, wild-type and “kinase-dead” TRPM7 variants were found to be equivalent in their ability to rescue the viability and Mg\textsuperscript{2+} deficiency of DT40 lymphocytes carrying a genetically disrupted TRPM7 gene (15). More recently, Clark et al. (23) reported that TRPM7-mediated Ca\textsuperscript{2+} influx is required for kinase domain-dependent actomyosin contractility. In summary, our analysis of the new missense HSH mutation in TRPM6, together with in vitro experiments on TRPM7, suggest that in TRPM6/7 the kinase domains may function downstream of their channel activity. Accordingly, TRPM6\textsuperscript{P1017R} and TRPM7\textsuperscript{P1040R}, characterized here, represent valuable molecular tools to further test this hypothesis.

Recent reports on the functional characterization of TRPM7 and TRPM6 yielded controversial results. Interestingly, small variations in the composition of pipette solutions used for whole-cell recordings of TRPM7-mediated currents are sufficient to produce contradictory observations (16, 17, 46, 47). It is still a moot issue whether TRPM6 homomultimers form functional ion channels in native cells. Here, we used two alternative heterologous expression systems, Xenopus oocytes and HEK 293 cells, and could not detect significant TRPM6 channel activity, fully consistent with the channel’s predominant intracellular localization. These findings are in line with our previous observations (7, 8, 12) and with a recent report of Schmitz et al. (28) who demonstrated that TRPM6 requires TRPM7 for efficient cell surface localization. However, studies of two other groups (29, 30) reported that transfection of a plasmid vector coding for human TRPM6 resulted in the formation of func-
tional ion channels. Notably, attempts to record native currents mediated solely by TRPM6 homooligomers have not been successful (30). Therefore, we used an independent approach to functionally evaluate TRPM6 and TRPM7 proteins. There is evidence to show that TRPM7-mediated cation entry regulates adhesion and spreading of cells (23, 36). In accord with these findings (36), we observed that ponasterone A-induced expression of TRPM7, in contrast to TRPM6, resulted in HSH. The dominant-negative effect exerted by TRPM6 P1017R provides strong evidence to show that TRPM7-mediated cation entry regulates adhesion and spreading of cells. Hence, the different morphological characteristics of TRPM6- and TRPM7-expressing cells corroborate our biophysical and trafficking data demonstrating that TRPM6 subunits are very inefficient in the formation of functional homooligomers.

We cannot exclude that a specific overexpression maneuver or application of “chemical chaperone” strategies may overcome the intracellular retention mechanism of TRPM6. However, a growing number of examined tissues and cell lines invariably show co-expression of TRPM6 with TRPM7 (4, 8, 48–50) raising the question whether TRPM6 can function alone. The specific and efficient assembly of TRPM6 with TRPM7, reproduced in various alternative expression models, most probably reflects the intrinsic mode of TRPM6 function as a subunit of TRPM6/7 complexes. Such a scenario would agree well with an indispensable role of TRPM7 in DT40 lymphocytes (15).

In conclusion, we identified a second point mutation in TRPM6, P1017R, resulting in HSH. The dominant-negative effect exerted by TRPM6 P1017R provides strong evidence to conclude that both channel subunits, TRPM6 and TRPM7, contribute to a common channel pore in TRPM6/7 complexes. Dominant-negative inhibition by mutant channel subunits represents a novel molecular mechanism underlying HSH. Our findings indicate that impairment of channel activity in TRPM6/7 complexes is sufficient for the development of Mg2+ deficiency in HSH patients.

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**Novel Missense Mutation in TRPM6**

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