The Channel Kinases TRPM6 and TRPM7 Are Functionally Nonredundant*

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TRPM7 and its closest homologue, TRPM6, are the only known fusions of an ion channel pore with a kinase domain. Deletion of TRPM7 in DT40 B-lymphocytes causes growth arrest, Mg\(^{2+}\) deficiency, and cell death within 24–48 h. Amazingly, in analogy to TRPM6-deficient patients who can live a normal life if provided with a Mg\(^{2+}\)-rich diet, TRPM7-deficient DT40 B-lymphocytes show wild type cell growth if supplied with 5–10 mM Mg\(^{2+}\) concentrations in their extracellular medium. Here we have investigated the functional relationship between TRPM6 and TRPM7. We show that TRPM7 deficiency in DT40 cells cannot be complemented by heterologously expressed TRPM6. Nevertheless, both channels can influence each other’s biological activity. Our data demonstrate that TRPM6 can modulate TRPM7 function. In conclusion, although TRPM6 and TRPM7 are closely related and deficient in either one of these molecules severely affects Mg\(^{2+}\) homeostasis regulation, TRPM6 and TRPM7 do not appear to be functionally redundant but rather two unique and essential components of vertebrate ion homeostasis regulation.

The TRPM proteins are a recently identified subgroup of ion channels comprising eight members. They belong to the growing transient receptor potential (TRP) superfamily of cationic channels (1–5). TRPM6 (ChaK2) and TRPM7 (TRP-PLIK, ChaK1, and LTRPC7) have been shown to be involved in regulating Mg\(^{2+}\) homeostasis in vertebrates (6–10). Both channels show the unique functional duality of being ion channels and kinases, since they include an active Thr/Ser kinase at their C terminus, which belongs to the atypical family of eukaryotic α-kinases (11, 12). Genomic studies revealed the existence of six α-kinases in mammals, including TRPM6 and TRPM7. These kinases show no sequence homology to conventional protein kinases. Structural analyses of the TRPM7 kinase domain revealed similarities to the fold of protein kinase A family members (13).

TRPM6 was first identified by Ryazanova et al. (14), who screened for homologues of eukaryotic elongation factor 2 kinase, as well as by two other groups who showed that mutations in the TRPM6 gene locus are linked to an autosomal recessive form of familiar hypomagnesemia with secondary hypocalcemia (HSH), by performing a candidate gene approach (15, 16). Affected HSH patients suffer from neurologic symptoms, including seizures and muscle spasms during infancy. The lifelong oral administration of high doses of magnesium is sufficient to ensure a complete relief of the clinical symptoms. Although the pathophysiology of HSH is largely unknown, several studies indicate a primary defect in intestinal Mg\(^{2+}\) transport (17, 18). Recent biophysical analyses by one group have shown striking parallels between gating mechanisms and ion selectivity profiles of TRPM6 and TRPM7, since TRPM6 was shown to be regulated by intracellular Mg\(^{2+}\) levels and to be permeable for Mg\(^{2+}\) and Ca\(^{2+}\) (19). In contrast, another group using imaging approaches reports that TRPM6 is only present at the cell surface when associated with TRPM7, leading to increased Mn\(^{2+}\) entry as assessed by fura-2 fluorescence quenching measurements (20).

TRPM7 was originally cloned by three different research groups; Clapham and co-workers (21) identified the TRPM7 kinase domain in a yeast two-hybrid screen using the C2 domain of phospholipase Cβ as a bait, and Ryazanova et al. (14) cloned TRPM7 as already described for TRPM6 by screening data bases for homologues of human eukaryotic elongation factor 2 kinase, whereas we cloned mouse and human TRPM7 using a bioinformatics approach aimed at identifying novel ion channels expressed in the immune context (3, 22, 23). Electrophysiological characterization of TRPM7 overexpressed in HEK-293 cells has revealed outward rectifying currents with data suggesting nonselective conduction of Na\(^{+}\) and Ca\(^{2+}\) in one study (21) and a selectivity toward divalent cations such as Ca\(^{2+}\) and Mg\(^{2+}\) in another (23). Despite earlier controversy about the role of the TRPM7 kinase domain in regulating channel gating (21, 23), it seems in more recent reports that the kinase domain is not required for channel activity and does not function as a gating domain (22, 24, 25). Matsushita et al. (24) furthermore identified two phosphorylation sites upstream of the kinase domain via mass spectrometry at Ser\(^{1511}\) and Ser\(^{1567}\) of mouse TRPM7 and showed that mutations of these sites as well as of a position required for phototransferase activity (D1775A) did not affect mTRPM7 channel gating in Chinese hamster ovary cells. The authors conclude that TRPM7 channel function is dissociated from its kinase activity (24). In contrast, we found previously that mutations affecting TRPM7 phototransferase activity also impact channel function by modulating its dose-response curve to intracellular Mg\(^{2+}\) and MgATP (22). Another recent study identified annexin 1 as an endogenous substrate of TRPM7 kinase (26). The biological role of annexin 1 phosphorylation via TRPM7 is currently unknown, but this is the first indication that TRPM7 can influ-
ence cellular processes by modulating the activity of proteins that are substrates of its kinase domain.

Deletion of TRPM7 in chicken DT40 B-lymphocytes is lethal, a rarely observed phenotype in ion channel knock-out models. Both the viability and the proliferation of TRPM7-deficient B-cells are rescued by supplementing the extracellular growth medium with 5–10 mM Mg\(^{2+}\) concentrations, in analogy to TRPM6-deficient patients who live a normal life if provided with a Mg\(^{2+}\)-rich diet, linking TRPM7 and Mg\(^{2+}\) to cell proliferation and cell death. Another recent report indicates that TRPM7 is involved in anoxic neuronal death (27).

In this study, we document that TRPM6 and TRPM7 are not functionally redundant, since TRPM7 deficiency in DT40 cells cannot be complemented by heterologously expressed TRPM6. This is analogous to TRPM6 patients for whom TRPM7 is not able to complement for TRPM6 deficiency, despite the ubiquitous expression pattern of TRPM6. We show that TRPM6 and TRPM7 can influence each other’s biological activity. TRPM6 and TRPM7 can be efficiently co-immunoprecipitated, and TRPM6 requires TRPM7 for surface expression. Using a phosphothreonine-specific antibody, we have found that TRPM6 can phosphorylate TRPM7, but not vice versa. Coexpression studies of TRPM6 and TRPM7 in DT40 cells indicate that TRPM6 might modulate TRPM7 function in a Mg\(^{2+}\)-dependent manner.

**MATERIALS AND METHODS**

**Molecular Biology**—Human TRPM7 WT and TRPM7Δkinase cDNA cloning and the establishing of stable inducible expression of the corresponding proteins in HEK-293 T-Rex cells (Invitrogen) have been previously described (22). For generation of TRPM6 WT (GenBank\(^{TM}\) accession number NM_017662; cDNA was kindly provided by Bernd Nilus and colleagues), TRPM6 K1804R and TRPM6Δkinase (stop codon amino acid position 1735) expression constructs, TRPM6, and TRPM6 mutants were cloned into the pcDNA5/TO plasmid, allowing the doxycycline-inducible expression of N-terminally FLAG-tagged channels. The TRPM6-KR mutant (K1804R) was generated by site-directed mutagenesis (QuikChange\(^{TM}\), Stratagene) following the standard protocol. pcDNA5TO plasmids encoding TRPM6 WT and mutant channels were transfected into HEK-293 cell lines with stable expression of TRPM7 WT and TRPM7Δkinase. TRPM6 WT was also transfected into HEK-293 T-Rex WT cells, in order to generate a cell line overexpressing TRPM6 alone. The predicted DNA sequences of all constructs were verified by sequencing.

**Immunoblotting**—0.5–1 \( \times 10^7 \) HEK-293 cells with inducible expression of the indicated channels were plated, and expression was induced by adding doxycycline for 24–48 h. Anti-FLAG or anti-HA (Sigma) immunoprecipitations were performed from lysates (the phosphatase inhibitor calyculin A (10 \( \mu \)M; Cell Signaling) was added to the lysis buffer for phosphorylation experiments) of 10\(^7\) HEK-293 cells. Immunoprecipitated proteins were washed three times with lysis buffer, separated by SDS-PAGE using 6% polyacrylamide gels, transferred to a polyvinylidene difluoride membrane, and analyzed by anti-FLAG, anti-HA, or anti-Thr(P) (Cell Signaling) immunoblotting. Blots were sometimes reprobed after stripping. Several different Ser(P) antibodies were also tested (Clontech, Sigma, and Cell Signaling) but without success.

For surface labeling experiments, intact cells were incubated with biotin (Pierce). After inactivation of residual biotinylation reagent, the cells were lysed and immunoprecipitated with anti-FLAG antibody. The anti-FLAG immunoprecipitates were washed extensively and analyzed by Western blotted with streptavidin-horseradish peroxidase (Sigma).

**In Vitro Phosphorylation Assays of hTRPM6 and hTRPM7 Channels**—For in vitro assays, hTRPM6 or hTRPM7 WT and mutant channels were immunoprecipitated with anti-FLAG- or anti-HA-coated beads. The beads were incubated in a total volume of 40 \( \mu \)l of reaction buffer (50 \( \mu \)M Tris-HCl, pH 7.2, 0.1 (v/v) \( \beta \)-mercaptoethanol, 10 \( \mu \)M calyculin A, 100 \( \mu \)M Mg-ATP, and containing varying MgAc concentrations). Phosphorylation was analyzed by anti-Thr(P) immunoblotting.

**Cell Culture**—The transiently or stably transfected HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and basiclinid S (5 \( \mu \)g/ml; Invitrogen) to maintain the pcDNA6TR plasmid allowing stable expression of the Tet repressor. Zeocin (400 \( \mu \)g/ml; Invitrogen) was added for HEK-293 cell lines transfected with pcDNA4TO constructs and hygromycin (100 \( \mu \)g/ml; Calbiochem) when pcDNA5TO constructs were used. Protein overexpression was induced by adding doxycycline (100–1000 ng/ml) to the growth medium.

The TRPM7-deficient DT40 cell line complemented with stable overexpression of WT TRPM7 (cWT TRPM7, also cWT M7), and mutant channels in an inducible fashion has been described previously (22). These cell lines (TRPM7\(^{-/-}\)-DT40 and cWT TRPM7) were cultured in chemically defined medium + 10 mM MgCl\(_2\) for normal cell maintenance and generation of TRPM7-expressing TRPM7\(^{-/-}\)-DT40 cells. To perform Mg\(^{2+}\)-dependent measurements and growth curves, serum- and Mg\(^{2+}\)-free medium was used as indicated (chemically defined HyQ CCM1 and customized Mg\(^{2+}\)-free HyQ CCM1 media were purchased from Hyclone and supplemented with 1% chicken serum (Sigma)).

The TRPM7\(^{-/-}\)-DT40 cell line and a TRPM7-deficient cell line complemented with hTRPM7 WT were transfected with the same pcDNA5/TO construct encoding FLAG-tagged TRPM6 WT as the one used for transfection of HEK-293 cells. Two cell clones with relatively matched inducible TRPM6 expression as assessed by anti-FLAG immunoblotting were chosen for further analysis.

**Phosphoamino Acid Analysis**—For in vitro assays, hTRPM6 or hTRPM7 WT and mutant channels were immunoprecipitated with anti-FLAG- or anti-HA-coupled beads. The beads were incubated in 50 mM HEPES-KOH (pH 7.4), 10 mM MgCl\(_2\), 4 mM MnCl\(_2\), 100 \( \mu \)M ATP, 20 \( \mu \)Ci [\( \gamma \)-\( ^{32} \)P]ATP (specific activity of 3000 Ci/mmol). The total volume of the reaction was 50 \( \mu \)l. After incubation at 30 °C for 30 min, Laemmlli buffer was added, and the samples were boiled. The samples were separated by 6 or 8% SDS-PAGE and transferred to a polyvinylidene difluoride Immobilon-P membrane (Millipore Corp.) by semidry transfer. The portion of membrane with phosphoprotein was excised and incubated in 6 \( \text{M} \) HCl at 110 °C for 1.5 h. After incubation, the samples were dried with a Speedvac evaporator, and the dried material was dissolved in water. Nonradioactive phosphoserine, phosphothreonine, and phosphopyrosine were added to the samples. Phosphoamino acids were separated by two-dimensional electrophoresis on thin layer cellulose plates 10 \( \times \) 10 cm (cellulose on glass; Merck). The first dimension was performed in pH 1.9 electrophoresis buffer containing 0.58 \( \mu \)M formic acid and 1.36 \( \mu \)M acetic acid at 1000 V for 20 min. The second dimension was performed in pH 3.5 electrophoresis buffer containing 0.87 \( \mu \)M acetic acid, 0.5% (v/v) pyridine, and 0.5 \( \text{mM} \) EDTA at 1000 V for 8 min. The TLC plates were stained with 0.2% ninhydrin in ethanol and then exposed to film.

**Mg\(^{2+}\)-Measurements**—Cytosolic [Mg\(^{2+}\)] was monitored in a cuvette-based assay by detecting changes in Mag-fura fluorescence using the QM-6/2003 fluorometer from PTI (Photon Technology International). Standard labeling protocols were used (on the World Wide Web, see probes.invitrogen.com/). Briefly, cells were plated for 18–24 h prior to analysis and either left untreated or treated with 1 \( \mu \)g/ml doxycycline in chemically defined medium supplemented with 10 \( \mu \text{M} \) MgCl\(_2\) and 1% chicken serum or in the same medium without MgCl\(_2\). The cells were

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then loaded with Mag-Fura (Molecular Probes) for 30 min at 37 °C in Mg²⁺-free buffer (135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 5.6 mM glucose, 10 mM Hepes, 0.1% bovine serum albumin), washed, and measured by exposure every 2 s to 340/380-nm excitation accompanied by monitoring 510-nm emission light for the period of time. The ionophore 4-bromo-A-23187 (1 mM final concentration, Sigma) and MgCl₂ (10 mM final concentration) were added directly into the cuvette as indicated.

RESULTS

**Analysis of TRPM6/TRPM7 Association and Cell Surface Expression**—TRPM6 and its closest relative TRPM7 are the only known channel kinases. Both have been linked to Mg²⁺ homeostasis regulation in vertebrates. In analogy to other ion channel families with a similar membrane topology (voltage-gated K⁺ regulation in vertebrates. In analogy to other ion channel families known channel kinases. Both have been linked to Mg²⁺-TRPM6 and its closest relative TRPM7 are the only known channel kinases. Both have been linked to Mg²⁺ homeostasis in vertebrates. In analogy to other ion channel families with a similar membrane topology (voltage-gated K⁺ channels, cyclic nucleotide gated channels), the pore of TRP family are known to heteroassociate, we analyzed the interaction between human FLAG-tagged TRPM6 and HA-tagged TRPM7 proteins by co-immunoprecipitation. To this aim, we generated HEK-293 cell lines with stable, doxycycline-inducible co-expression of these N-terminally tagged versions of TRPM6 and TRPM7. To ensure that FLAG-TRPM6 was not nonspecifically precipitated using the anti-HA antibody, we generated a stable HEK-293 cell line inducibly expressing FLAG-TRPM6 alone (Fig. 1A). No FLAG-reactive band could be seen on Western blots when immunoprecipitated with anti-HA (Fig. 1B, top panel, middle section). In contrast, in the presence of HA-TRPM7, FLAG-TRPM6 can clearly be detected using the same conditions (Fig. 1B, top panel, left section). Conversely, HA-TRPM7 could be co-immunoprecipitated when using anti-FLAG beads recognizing FLAG-TRPM6 (Fig. 1B, bottom panel, right section). We therefore concluded that TRPM6 and TRPM7 can associate and probably form heteromeric channels.

Since TRPM7 has been shown to be ubiquitously expressed, as opposed to TRPM6, which appears to be predominantly expressed in the kidney and intestine, we investigated the possibility that the association between TRPM7 and TRPM6 might influence surface expression levels of TRPM6. To experimentally assess this hypothesis, we surface-labeled intact FLAG-TRPM6-expressing cells, as well as cells co-expressing FLAG-TRPM6/HA-TRPM7 with biotin. After lysing the cells in both labeling reactions, we immunoprecipitated TRPM6 using anti-FLAG-coated beads. The samples were subsequently analyzed by Western blot, first by developing with streptavidin-horseradish peroxidase to visualize protein surface expression and, following a stripping step, with FLAG antibody to assess total levels of FLAG-TRPM6 (Fig. 1C). Although the FLAG-TRPM6 expression level is substantially higher in the “TRPM6 alone” cells, the corresponding streptavidin signal is very low and is probably the result of a small proportion of the cells being already disrupted during the labeling process. On the other hand, biotinylation of TRPM6 was much more efficient when co-expressed with TRPM7 (Fig. 1C, top left) and is labeled to the same extent as a band with a slightly smaller molecular weight. This band probably corresponds to co-precipitated, surface-expressed TRPM7 proteins. This observation suggests that TRPM7 promotes the trafficking of associated TRPM6 subunits to the cell surface.

**Effects of TRPM6 Overexpression on Cell Growth and on Mg²⁺ Homeostasis in TRPM7-deficient DT40 B-cells—** TRPM7 deficiency is lethal in the avian DT40 B-cell line, unless the growth medium is supplemented with millimolar amounts of Mg²⁺. Remarkably, human patients suffering from the recessive hereditary disease HSH experience debilitating seizures, leading to death in the first year of age, unless their nutrition is supplemented with Mg²⁺. These patients have been shown to bear mutations in the TRPM6 alleles but still express TRPM7 (also in the kidneys), indicating that TRPM7 and TRPM6 are not functionally redundant. To further test this idea, we used the TRPM7⁻/⁻ DT40 B-cell line to assess the ability of TRPM6 to complement for the absence of TRPM7 under conditions of low extracellular Mg²⁺. We first performed reverse transcription-PCR analysis to look for endogenous TRPM6 expression in DT40 cells and found no transcript of the chicken TRPM6 homologue (data not shown). Next, we established stable doxycycline-inducible expression of hTRPM6 in the TRPM7⁻/⁻ DT40 cells.

**Figure 1. Analysis of heterologous expression, association, and surface expression of hTRPM6 and hTRPM7.** A, analysis of protein expression of FLAG-hTRPM6 channels. HEK-293 T-REX cells (Invitrogen) with stable expression of the Tet repressor were transfected with FLAG-tagged human TRPM6 WT, and cell clones were selected for stable, doxycycline (Dox)-inducible protein expression (+, induced; −, noninduced). FLAG-tagged TRPM6 was immunoprecipitated (IP) and subsequently analyzed by immunoblotting using an anti-FLAG antibody. B, FLAG-hTRPM6/HA-hTRPM7 association. HEK-293 cells stably co-expressing FLAG-tagged hTRPM6 WT (M6) and HA-tagged hTRPM7 WT (M7) or expressing FLAG-tagged hTRPM6 WT alone as a control were analyzed. Stable protein expression was induced by adding doxycycline to the growth medium (+/−). Proteins in cell lysates were immunoprecipitated with anti-FLAG or anti-HA antibodies and analyzed by immunoblotting with anti-FLAG antibody. The blot membrane was subsequently stripped and reprobed with anti-HA antibody. C, analysis of cell surface expression of TRPM6/7 channels. 10⁴ HEK 293 cells stably expressing the indicated channel(s) were induced with doxycycline to express the proteins for 24–48 h before being subjected to surface labeling with biotin. The channels were subsequently immunoprecipitated and analyzed by immunoblotting with streptavidin-horseradish peroxidase (HRP) to detect surface-expressed proteins. After stripping, the membrane was reprobed with anti-FLAG in order to detect total levels of FLAG-hTRPM6. These results are representative of at least three separate experiments.
Functional Comparison of TRPM6 and TRPM7

Whereas hTRPM7 can complement for TRPM7 deficiency, hTRPM6 does not improve cell growth under low Mg\(^{2+}\) conditions in the absence of TRPM7 (Fig. 2A). Since we demonstrated above that TRPM6 and TRPM7 associate, we constructed a supplementary DT40 cell line starting with the TRPM7\(^{-/-}\) cells complemented with hTRPM7 and added simultaneous stable inducible expression of hTRPM6. Surprisingly, despite the comparatively lower level of FLAG-TRPM6 protein expression (Fig. 2B), coexpression of hTRPM6 severely decreases the capability of hTRPM7 to rescue the growth defect resulting from the genomic disruption of TRPM7 in a medium that has a defined ionic composition and contains no Mg\(^{2+}\) except for the slight amount of Mg\(^{2+}\) included in the 1% chicken serum (Fig. 2C). The same observation was made comparing two independent TRPM6/TRPM7-coexpressing DT40 clones. This cell growth phenotype correlates with the relative levels of free Mg\(^{2+}\) in these different cell lines as assessed with the fluorescent Mg\(^{2+}\) dye Mag-fura after culturing the cells for 24 h in the same Mg\(^{2+}\)-free medium as the one used for the cell growth experiments (Fig. 2D).

Already at base line, the TRPM7-deficient DT40 cells were reproducibly showing the lowest 340/380 fluorescence ratio, reflecting the lower levels of cytosolic free Mg\(^{2+}\) in these cells as compared with the same TRPM7\(^{-/-}\) cells complemented with hTRPM7. Paralleling the growth behavior shown in Fig. 2C, the TRPM6/TRPM7-complemented DT40s show an intermediary phenotype. Following the addition of 10 mM MgCl\(_2\) into the cuvette, the TRPM7 KO cells show the least recovery of their [Mg\(^{2+}\)]\(_i\). Subsequently, 4-bromo-A-23187 was given to the same samples in order to cause the progressive release of endogenous Mg\(^{2+}\) from intracellular stores, since this cell-permeable ionophore has been shown to exhibit low activity for Ca\(^{2+}\). Again, the weakest increase in cytosolic [Mg\(^{2+}\)] was registered in the TRPM7-deficient cells and the strongest was registered in the hTRPM7-complemented cells, with the TRPM6/TRPM7 DT40s in between. Over the complete course of the experiment, the 340/380 fluorescence ratio increased 35% in the TRPM7-KO cells, 42% in the TRPM7/7TRPM6 cells, and 58% in the TRPM7-complemented cells. This suggests that the intracellular stores were as expected, containing the least Mg\(^{2+}\) in the TRPM7\(^{-/-}\) cells, and confirms that TRPM6 has a detrimental effect on TRPM7-dependent Mg\(^{2+}\) homeostasis regulation in DT40s under hypomagnesic conditions. Interestingly, this effect was not seen when physiological amounts of Mg\(^{2+}\) (1–2 mM) were added to the growth medium.

Phosphoamino Acid Analysis of TRPM6 and TRPM7 Wild Type Channels—Since both TRPM6 and TRPM7 have phosphotransferase activity and can be autophosphorylated, we next conducted studies aimed at comparing the phosphoamino acid composition of full-length TRPM6 and TRPM7. The TRPM6 and TRPM7 kinase domains are 78% identical over a stretch of 280 aa (hTRPM6 aa 1711–1990, hTRPM7 aa 1556–1834), except for the last ~30 amino acids that show no homology. Interestingly, the region upstream of the kinase domain, which has been shown to be phosphorylated in TRPM7 (13, 24), is the only domain that is not conserved at all between the two sister channels. Despite these differences in protein sequence, the relative amounts of phosphothreonine to phosphoserine are very similar in immunoprecipitated wild type FLAG-TRPM6 and HA-TRPM7 following a 10-min-long in vitro incubation at 30°C with 20 μCi of [γ-\(^{33}\)P]ATP, as assessed by two-dimensional electrophoresis on thin layer cellulose plates after acidic heat denaturation of the proteins (Fig. 3). Both TRPM6 and TRPM7 exhibit no tyrosine phosphorylation, an expected result, since their kinase subdomains belong to the atypical family of Thr/Ser α-kinases that also includes the prototypical mammalian eukaryotic elongation factor 2 kinase. Threonine and serine phosphorylation can clearly be detected in both TRPM6 and TRPM7. The proportion of Thr(P) to Ser(P) appears to be very similar in both channels and was 70% Ser(P) to 30% Thr(P) (±4%) in two separate experiments.

Cross-phosphorylation Studies between Wild Type and Kinase Mutant TRPM6 and TRPM7 Subunits—It is currently unknown whether TRPM6 and TRPM7 autophosphorylation is an intramolecular or intermolecular event between associated subunits. We therefore sought to determine if TRPM6 and TRPM7 monomers are capable of phosphorylating associated subunits in trans. Since it has been reported that the main TRPM7 phosphorylation sites for its own kinase are in a protein stretch upstream of the kinase domain, we generated TRPM6 and TRPM7 constructs that are lacking the phosphotransferase domain but retain this phosphorylation stretch. We have previously shown that this kinase-deleted TRPM7 version fails to show autophosphorylation. The corresponding TRPM6 truncation was analyzed using the same in vitro phosphorylation protocol and representation of the phosphorylated channels by Western blot using a phosphothreonine-specific antibody, following immunoprecipitation of FLAG-TRPM6 kinase channels. As expected, the deletion of the TRPM6 kinase leads to a clear lack of autophosphorylation, despite high protein expression levels of FLAG-TRPM6 kinase, as opposed to the full-length protein, which clearly shows phosphorylation (Fig. 4A). The difference in molecular weight between the WT channels and the kinase-deleted mutants is sufficient to obtain a reliable separation of these two channel versions on an SDS-polyacrylamide gel. By coexpressing the full-length versions of TRPM6 or TRPM7 with their corresponding kinase-deleted truncation, the ability of the WT proteins to phosphorylate Δkinase subunit(s) associated with them can therefore be investigated. This approach revealed that both TRPM6 (Fig. 4B) and TRPM7 (Fig. 4C) wild type subunits are capable of cross-phosphorylating their corresponding kinase-deleted version. This observation implies that in the context of the TRPM6/TRPM7 heteromer, there might be cross-phosphorylation events between associated subunits of these two ion channels. Next, we therefore conducted a similar series of experiments, but this time TRPM6 WT was coexpressed with TRPM7Δkinase, and vice versa. Using the same phosphothreonine-specific antibody, we found that although TRPM6 can efficiently phosphorylate TRPM7Δkinase (Fig. 5A), the contrary is surprisingly not true, since we could not detect TRPM6Δkinase phosphorylation by adding TRPM7 WT (Fig. 5B), despite a very strong autophosphorylation of HA-TRPM7 WT. This absence of phosphorylation signal is not due to a lack of association between TRPM7 and TRPM6Δkinase, since HA-TRPM7 WT can be easily co-immunoprecipitated with FLAG-TRPM6Δkinase (Fig. 5B). To ensure that the deletion of the entire TRPM6 kinase is not affecting the conformation of the TRPM6/TRPM7 heteromer, indirectly contributing to the inability of TRPM7 to cross-phosphorylate TRPM6Δkinase, a phosphotransferase-deficient TRPM6 point mutant was generated. To this aim, a conserved lysine residue located at amino acid position 1804, which in analogy to the TRPM7 structure is thought to play a crucial role in binding MgATP in the kinase domain, was mutated to arginine (K1804R). This TRPM6 mutant corresponds to the TRPM7-K1648R mutant, which we have previously shown to be very weakly autophosphorylated. Importantly, the TRPM7-K1648R mutant channels fully activate under conditions of low intracellular Mg\(^{2+}\), but significantly higher Mg\(^{2+}\)/MgATP concentrations are needed to achieve a level of suppression comparable with TRPM7 WT (22). As expected, TRPM6-K1648R autophosphorylation cannot be detected when expressed alone (data not shown). Similarly to the observation made when coexpressing TRPM7 WT and TRPM6Δkinase, TRPM7 WT does not show phosphorylation of TRPM6-K1648R, although these two proteins can efficiently be co-immunoprecipitated (Fig. 5C). This result confirms the
FIGURE 2. Complementation studies of TRPM7−/− DT40 cells with hTRPM6. A, growth curves of TRPM6- or TRPM7-complemented TRPM7-deficient DT40 cells. Stable overexpression of HA-tagged hTRPM7 WT (cWT M7) or of FLAG-tagged hTRPM6 WT (M7 KO + M6) in TRPM7-deficient DT40 cells was induced by adding doxycycline (DX) to Mg2+-free chemically defined medium, which only contains 1% chicken serum (no fetal bovine serum). β, analysis of inducible protein co-expression of human FLAG-TRPM6/HA-TRPM7 channels in TRPM7-deficient DT-40 B-cell line (cWT M7 + M6). Equal numbers of cells of the hTRPM6/hTRPM7 coexpressing DT40 TRPM7−/− cell line cultured in the presence or absence of doxycycline (Dox) (+/-) were analyzed by immunoprecipitation (IP), followed by Western immunoblotting (WB) with anti-FLAG or anti-HA antibodies. C, growth curves of TRPM7−/− DT40 cells complemented with hTRPM7 or hTRPM6/hTRPM7. Stable overexpression of hTRPM7 WT (cWT M7) or of hTRPM6 WT and hTRPM7 WT combined (cWT M7 + M6) in TRPM7-deficient DT40 cells was induced by the addition of doxycycline to the Mg2+-free chemically defined growth medium, which contains only 1% chicken serum (no fetal bovine serum). Δ, intracellular free Mg2+ levels in TRPM7−/− DT40 cell lines either not complemented, complemented with hTRPM7 alone, or complemented with hTRPM6/hTRPM7 combined. The three DT40 cell lines were cultured in Mg2+-free, chemically defined medium for 12–16 h, and the health of the cells was checked visually. The cells were then loaded with the fluorescent dye Mag-Fura for 30 min at 37 °C in a Mg2+-free buffer, and the samples were analyzed in a cuvette-based assay using a fluorometer (PTI). Where indicated by the arrows, 10 mM final concentration of MgCl2 and a 1 mM concentration of the Mg2+-specific ionophore 4-bromo-A-23187 (depletion of the intracellular Mg2+ stores) was added. These data are representative of three separate experiments.
Functional Comparison of TRPM6 and TRPM7

Finding cross-phosphorylation between the sister channels TRPM6 and TRPM7 does not appear to be reciprocal under the chosen experimental conditions. Although it is conceivable that other residues (in particular serines) that are not recognized by the used phosphothreonine-specific antibody are being phosphorylated, our results demonstrate that one or several sites in TRPM6 kinase can only serve as substrate for TRPM6 WT, and not for TRPM7 WT, indicating that the TRPM6 and TRPM7 kinases have different substrate specificities (Fig. 6).

**DISCUSSION**

Besides the mitochondrial Mg$^{2+}$-transporter Mrs2 (28), the channel kinases TRPM6 and TRPM7 appear to be the first molecularly defined components of the mammalian Mg$^{2+}$ transport machinery (29). Currently, the only human patients with a characterized deficiency in one of the eight TRPM family members suffer from a severe and life-threatening form of HSH and have been shown to bear various mutations in the TRPM6 loci (15, 16). If diagnosed early based on the determination of homeostasis, TRPM7 shows similarity to TRPM7 upon overexpression of hTRPM6 WT. In vitro phosphorylation assays were performed as described above. Phosphorylation of the proteins was subsequently analyzed by Western immunoblotting with anti-Thr(P) antibody. Protein expression levels were verified after stripping of the membrane through reprobing with anti-FLAG antibody. C. analysis of in vitro phosphorylation of hTRPM6 kinase coexpressed with hTRPM7 WT. In vitro phosphorylation assays were performed as described above. Phosphorylation of the proteins was subsequently analyzed by Western immunoblotting with anti-Thr(P) antibody. Protein expression levels were verified after stripping of the membrane through reprobing with anti-FLAG antibody. C. analysis of in vitro phosphorylation of hTRPM6 kinase coexpressed with hTRPM7 WT. In vitro phosphorylation assays were performed as described above. Phosphorylation of the proteins was subsequently analyzed by Western immunoblotting with anti-Thr(P) antibody. Protein expression levels were verified using an anti-HA antibody. These data are representative of at least two separate experiments.

Intracellular Mg$^{2+}$ and MgATP. The biophysical characterization of TRPM6 is currently controversial; one group can measure currents similar to TRPM7 upon overexpression of TRPM6 by itself in HEK-293 cells, whereas another group could only detect a current amplification when TRPM6 and TRPM7 were co-expressed, but not with TRPM6 alone. Chubanov et al. (20) subsequently conducted FRET and imaging co-localization studies in HEK-293 cells and confirmed the hypothesis that TRPM6 requires TRPM7 for surface expression. In the present study, we chose a biochemical approach and performed co-immunoprecipitations of stably expressed epitope-tagged versions of TRPM6 and TRPM7 in HEK-293 cells and cell surface biotinylation experiments. Our results also support the idea that TRPM6 and TRPM7 associate and that trafficking of TRPM6 to the cell surface is strongly dependent on TRPM7 co-expression. In light of this observation, our finding that TRPM6 cannot compensate for TRPM7 deficiency in the DT40 model is an expected outcome. Currently, since TRPM7 is ubiquitous, no tissues have been found in which TRPM6 is expressed in the absence of TRPM7, but it is important to notice that the strong pheno-
TRPM6 and TRPM7 in the TRPM7⁻/⁻-deficient DT40 avian cell line leads to growth deficiency in media with a defined ionic composition and only trace amounts of Mg²⁺. Importantly, this inhibitory effect of TRPM6 on TRPM7 can only be seen under hypomagnesic conditions but not when physiological levels of Mg²⁺ (1–2 mM) were added to the extracellular medium. Probably because of the small size of the DT40s, cell surface experiments turned out to be technically challenging, and we therefore cannot exclude the possibility that intracellular retention of TRPM7 by TRPM6 might explain this observation. It is conceivable that this effect is cell type-specific, since DT40s are chicken B-lymphocytes that do not normally show TRPM6 expression. Future studies in other cell types and using similar hypomagnesic growth conditions will help determine whether TRPM6 exhibits this inhibitory effect on TRPM7 function in cell types where both sister channels are natively expressed.

Since our results support the idea that TRPM6 and TRPM7 can form heteromeric channels, and because both possess C-terminal kinase domains that are highly homologous to each other, we sought to examine the possibility that cross-phosphorylation events between associated TRPM6 and TRPM7 subunits can take place. X-ray crystallography studies of the isolated TRPM7 kinase have revealed that two kinase domains associate as a domain-swapped dimer (13). Furthermore, it has been recently shown that major autophosphorylation sites of the mouse TRPM7 kinase are two serine residues (24). One is located at the very N-terminal end of the kinase, Ser₁⁵₁⁻¹⁵₁³ (corresponds to Ser₁⁵₁⁻¹⁵₁³ in humans), and the other is slightly further upstream in a stretch that appears to be a linker region between the kinase and the adjacent domain, Ser₁⁵¹₁⁻¹⁵₁³ (Ser₁⁵¹₁⁻¹⁵₁³ in humans). Interestingly, both of these positions are not conserved in hTRPM6. Ser₁⁵⁶⁹ in is in a conserved segment but corresponds to a threonine residue in TRPM6 (Thr¹⁷₂⁴), and Ser₁⁵¹₃ lies in a region that shows no homology between the two sister channels, although it is noticeable that TRPM6 also contains multiple serine residues in this stretch. Despite these discrepancies in their primary
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sequence, we found that the overall ratio from ~70% phosphoserine to 30% phosphothreonine is very similar between wild type full-length TRPM6 and TRPM7 following in vitro phosphorylation of immunoprecipitated channels. In previous phosphoamino acid analyses of mTRPM7, maltose-binding protein or glutathione S-transferase fusions of soluble C-terminal fragments of mTRPM7 purified in *Escherichia coli* have been mostly used (14, 24), although in one set of data full-length mTRPM7 was used (24). These studies are in agreement that autophosphorylation on serine is largely predominant, and phosphothreonine is barely detectable. Using the complete human TRPM6 and TRPM7 channels overexpressed in a human cell line, we found that phosphothreonine is easily detectable, although still clearly less than phosphoserine. Furthermore, antibodies against phosphothreonine are extremely reliable in representing TRPM7/TRPM6 phosphorylation, as opposed to several commercially available phosphoserine antibodies, which in our hands all failed to detect the phosphorylated channels, suggesting that the structural context of these phosphoserines is not recognizable by these particular antibodies.

We subsequently designed experiments allowing us to investigate in trans phosphorylation events between channel subunits. In previous studies, we have characterized a kinase-deleted version of hTRPM7 (aa 1–1569), which retains ion channel activity, albeit to a much lower level than the full-length protein. It should also be mentioned that a slightly different truncation of mouse TRPM7 (aa 1–1599) failed to produce any detectable current in a different study, reflecting a difference in stability and/or trafficking of these two published TRPM7 kinase constructs. Here, we generated the hTRPM6 kinase-deleted mutant (aa 1–1725) corresponding to our hTRPM7 kinase and found as expected that it cannot autophosphorylate. By co-expressing the kinase-deleted mutants of TRPM6/TRPM7 with their full-length versions, we could unequivocally demonstrate that phosphorylation can occur in trans between associated channel subunits. This also holds true when TRPM6 is co-expressed with TRPM7 kinase, but surprisingly not in the reverse situation, since we were not able to show phosphorylation of TRPM6 kinase or of the phosphotransferase-deficient TRPM6 K1804R mutant by TRPM7 wild type, despite the clear heteromerization of these different channels (Fig. 6). This observation suggests that TRPM6 might influence the biological activity of TRPM7 via phosphorylation of one or several specific threonine residues but not vice versa. In this context, it is important to mention a very recent study from Hermosura et al. (34), who identified a hTRPM7 variant in a subset of patients with neurodegenerative disorders found in high incidence in indigenous populations of Western Pacific islands. The produced TRPM7 protein exhibits a single amino acid exchange at position 1428 from a threonine to an isoleucine residue. T1482I-TRPM7 channels were found to have an altered sensitivity toward intracellular Mg\(^{2+}\), resulting in lower currents under conditions of [Mg\(^{2+}\)], that are physiologically suboptimal (0.5 mM). This phenotype fits the epidemiological findings that link the occurrence of these neurological disorders to environments severely deficient in Ca\(^{2+}\) and Mg\(^{2+}\). Mechanistically, the authors suggest that Thr\(^{1482}\) phosphorylation affects the Mg\(^{2+}\) sensitivity of the TRPM7 channels, since the catalytic activity of the T1482I kinase is not affected, but the threonine autophosphorylation of the mutant channels appears to be markedly decreased. The T1482I mutation lies in the Ser/Thr-rich linker between the TRPM7 kinase and the domain upstream of it, a region that shows no conservation between TRPM6 and TRPM7, possibly explaining why we can detect threonine cross-phosphorylation of TRPM7 by TRPM6 but not the opposite. Although this is speculative, our findings might support a model where TRPM6 acts as a crucial modulator of TRPM7 biological activity, by tuning its Mg\(^{2+}\) sensitivity via phosphorylation.

**CONCLUSION**

In summary, the present study demonstrates that TRPM6 and TRPM7 can heteromerize and that TRPM6 can only traffic efficiently to the plasma membrane of HEK-293 cells when co-expressed with TRPM7. Although TRPM6 and TRPM7 are highly homologous sister channels that share the same overall structural features, including the presence of a highly conserved α-kinase domain at their C terminus, they do not appear to be functionally redundant. TRPM6-deficient patients show severe hypomagnesemia despite the presence of TRPM7, which is also expressed in kidney and intestine, the major organs responsible for Mg\(^{2+}\) homeostasis regulation, and as shown here, TRPM6 cannot compensate for TRPM7 deficiency in genetically engineered DT40 TRPM7\(^{-/-}\) cells. Furthermore, whereas TRPM6 is capable of phosphorylating threonine residues in TRPM7 subunits associated with it, the opposite is not true, implying that these two channels might play different roles in modulating each other’s biological activity as well as intracellular signaling events, consolidating the idea that TRPM6 and TRPM7 are both in their own way crucial components of mammalian ion homeostasis regulation.

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