Identification and characterization of a novel family of membrane magnesium transporters, MMgT1 and MMgT2

Angela Goytain and Gary A. Quamme

Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada

Submitted 6 June 2007; accepted in final form 28 November 2007

Goytain A, Quamme GA. Identification and characterization of a novel family of membrane magnesium transporters, MMgT1 and MMgT2. Am J Physiol Cell Physiol 294: C495–C502, 2008. First published December 5, 2007; doi:10.1152/ajpcell.00238.2007.—Magnesium is an essential metal, but few selective transporters have been identified at the molecular level. Microarray analysis was used to identify two similar transcripts that are upregulated with low extracellular magnesium. The corresponding cDNAs encode proteins of 131 and 123 amino acids with two predicted transmembrane domains. The two separate gene products comprise the family that we have termed “membrane Mg2+ transporters” (MMgTs), because the proteins reside in the membrane and mediate Mg2+ transport. When expressed in Xenopus laevis oocytes, MMgT1 and MMgT2 mediate Mg2+ transport as determined with two-electrode voltage-clamp analysis and fluorescence measurements. Transport is saturable Mg2+ uptake with Michaelis constants of 1.47 ± 0.17 and 0.58 ± 0.07 mM, respectively. Real-time RT-PCR demonstrated that MMgT mRNAs are present in a wide variety of cells. Subcellular localization with immunohistochemistry determined that the MMgT1-hemagglutinin (HA) and MMgT2-V5 fusion proteins reside in the Golgi complex and post-Golgi vesicles, including the early endosomes in COS-7 cells transfected with the respective tagged constructs. Interestingly, MMgT1-HA and MMgT2-V5 were found in separate populations of post-Golgi vesicles. MMgT1 and MMgT2 mRNA increased by about threefold, respectively, in kidney epithelial cells cultured in low-magnesium media relative to normal media and in the kidney cortex of mice maintained on low-magnesium diets compared with those animals consuming normal diets. With the increase in transcripts, there was an apparent increase in MMgT1 and MMgT2 protein in the Golgi and post-Golgi vesicles. These experiments suggest that MMgT proteins may provide regulated pathways for Mg2+ transport in the Golgi and post-Golgi organelles of epithelium-derived cells.

We have shown that cellular Mg2+ control is predominantly through differential gene expression leading to synthesis of Mg2+-responsive proteins (20). Indeed, we used this approach to identify genes encoding novel Mg2+ transport proteins (8). In the present study, we used microarray analysis to screen for magnesium-regulated transcripts in epithelial cells. This revealed two transcripts whose relative levels were dramatically altered by extracellular magnesium. Thus, these transcripts potentially represented a species of RNA whose synthesis was regulated by changes in magnesium. The corresponding cDNAs showed that they represented a novel gene family comprising two members that we have termed membrane Mg2+ transporter 1 (MMgT1) and 2 (MMgT2) because they are membrane proteins and mediate Mg2+ uptake when expressed in Xenopus laevis oocytes. Subcellular localization with immunohistochemistry demonstrated that MMgT1 and MMgT2 fusion proteins are principally found in the Golgi complex and post-Golgi vesicles. Consistent with the observation that low magnesium increases MMgT1 and MMgT2 transcripts, there was an apparent increase in the respective proteins in the Golgi and post-Golgi vesicles. Also evident was the observation that MMgT1 and MMgT2 partially sorted to different post-Golgi organelles. We conclude that the differential expressions of MMgT1 and MMgT2 are involved in the control of Mg2+ metabolism within the Golgi complex and post-Golgi vesicles.

MATERIALS AND METHODS

Animal preparation and cell culture. Male mice were maintained for 3 days on a low-magnesium diet (ICN diet no. 902205, Nutritional Biochemicals, Cleveland, OH) or on this diet supplemented with 0.05% MgSO4, which was comparable with commercial mouse chow. The plasma magnesium concentrations of the two groups were not different (low magnesium: 0.87 ± 0.06 mM vs. normal magnesium: 0.88 ± 0.07 mM), but the mean urinary magnesium concentration was significantly lower (3.9 ± 2.9 mM) in animals maintained on the magnesium-restricted diet compared with control mice (15.8 ± 1.9 mM). This is in agreement with our early study (26). Urinary volumes and sodium and calcium excretions were not different between the two groups. The protocol was approved by the Committee on Animal Care of the University of British Columbia.

Mouse distal convoluted tubule (MDCT) cells were derived and immortalized by Pizzonia and colleagues (19). The MDCT cell line has been extensively used by us to study the hormonal and nonhormonal control of renal magnesium transport (4). Either MDCT cells or monkey kidney COS-7 cells were grown in basal DMEM-Ham’s F-12 (1:1) media (GIBCO) supplemented with 10% FCS (Flow Laboratories, McLean, VA), 1 mM glucose, 5 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified environment...

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5% CO₂ – 95% air at 37°C. Where indicated, subconfluent MDCT cells were cultured in Mg²⁺-free media (Stem Cell Technologies, Vancouver, BC, Canada) for 16 h before being harvested. Other constituents of the Mg²⁺-free culture media were similar to the complete media.

Oligonucleotide microarray analysis. Microarray analysis was performed according to the protocol recommended by Affymetrix (http://www.affymetric.com) using MG U74 Bv2 and MG U74 Cv2 arrays (Affymetrix, Santa Clara, CA) as described previously by us (8). DNA fragments representing transcripts that were upregulated with low magnesium were selected and prioritized according to properties characteristic of membrane transport proteins.

Quantitative analysis of MMgT transcripts by real-time RT-PCR. PCR products were quantified continuously with AB7000 (Applied Biosystems) using SYBR green fluorescence according to the manufacturer’s instructions. The primer sets for mouse MMgT1 were 5’-GCTTGTAGGTGTCGGCTTT-3’ (forward) and 5’-TGAAGGCCGGAACACGACT-3’ (reverse), and those for mouse MMgT2 were 5’-AGAGAACGGGTGTCCTCC-3’ (forward) and 5’-ACAGTCGTAGCCAGACGTT-3’ (reverse). Relative amounts of MMgT RNA were normalized to mouse β-actin transcripts.

Genomic sequence analysis. MMgT cDNA sequences were determined by standard methods. Database searching and alignments were performed using BLAST. Nonredundant and expressed sequence tag databases were sourced. Protein homology searches were performed by comparing the amino acid query sequence against the SWISSPROT database. Full-length MMgT cDNA sequences have been deposited in the GenBank database (Accession Nos. human: Eu069461, mouse: Eu069461).

Protein motifs were identified using BLASTP and the SWISSPROT database. Membrane topology was predicted by SOSUI and Kyte-Doolittle hydrophobicity analysis.

Plasmid construction and generation of expression constructs. Mouse MMgT1 cDNA was purchased from RIKEN (Rik960048L06), and MMgT2 was from IMAGE (IRAV 4459181). MMgT1 contained the entire coding region of the mMMgT1 cDNA flanked by 47 bp of untranslated 5’-nucleotide sequence and 205 bp of untranslated 3’-sequence, and MMgT2 possessed 122 5’-untranslated and 96 3’-untranslated sequences. Isolated clones were sequenced to confirm sequence integrity.

For immunolocalization experiments, MMgT1 cDNA was subcloned into the pcDNA3.1 vector containing a COOH-terminal hemagglutinin (HA) tag, and MMgT2 was subcloned into the pcDNA3.1 vector with a COOH-terminal V5 tag. PCR primers were performed using 5’-CAAGGTACCTGAATCAGTGCGCCGTCG-3’ (forward) and 5’-CTTCGGTACCATGGTGGCGTGGCTG-3’ (reverse) as indicated in the manuscript. Mouse MMgT1-HA and/or MMgT2-V5 fusion constructs were transiently transfected with MMgT1-HA and/or MMgT2-V5 fusion constructs into HepG2 cells using Lipofectamine 2000 (Invitrogen).

Identification of MMgT1 and MMgT2 as magnesium-responsive genes. With the knowledge that differential gene expression is involved with selective control of epithelial cell magnesium conservation, our strategy was to use microarray analysis to identify cDNAs that were upregulated with low magnesium (8). Two similar cDNA fragments were identified by increases in transcripts that conformed to full DNA sequences that might encode related hypothetical proteins. Based on electrophysiological and fluorescence properties and the subcellular location of the encoded proteins, we designated proteins as MMgT1 or MMgT2, respectively. In confirmation of the microarray data, MMgT1 mRNA was increased ~2.5-fold in immortalized MDCT epithelial cells (n = 11 independent preparations) cultured in low magnesium compared with normal animals and cells, respectively. The respective increases in MMgT2 transcripts were a 1.5-fold increase in the kidney cortex and 3.0-fold increase in MDCT cells.

Characterization of MMgT genes. Full-length mouse MMgT cDNAs were identified by BLAST searches of the GenBank database. Full-length MMgT1 possessed 80% amino acid identity to MMgT2 (Fig. 1A). MMgT1 and MMgT2 comprise 131 and 123 amino acids, respectively (Fig. 1A). Both MMgTs were highly conserved among the human, mouse, rat, and pig. To date, there is no reported MMgT2 in the human genome.
Mouse MMgT1 is 97% similar to human MMgT1, and mouse MMgT2 is 81% similar to human MMgT1. Hydrophobicity plots using the SOUSI program predicted only two putative transmembrane helices (Fig. 1, B and C). Human MMgT1 is located on chromosome Xq26.3. The respective chromosomal locations in the mouse were XA5 for MMgT1 and 11B2 for MMgT2, and those in the rat were Xq36 for MMgT1 and 10q23 for MMgT2.

MMgT1 and MMgT2 were present in a wide variety of tissues (Fig. 1D). MMgT1 transcript was abundant in the heart muscle and kidney with less transcript in the liver and brain. There was very little MMgT1 RNA in the intestine and colon. By comparison, MMgT2 was high in the brain and kidney with less transcript in the heart, colon, and liver. As with MMgT1, there was little MMgT2 transcript in the intestine. Renal tissue consistently possessed the most MMgT1 and MMgT2 RNA, and distal convoluted tubule cells had the greatest levels of those tissues tested.

**MMgT mediates Mg^{2+} transport in expressing Xenopus oocytes.** To determine if the MMgTs cDNA encoded functional Mg^{2+} transporters, we prepared the respective cRNA, injected it into *Xenopus* oocytes, and performed two-microelectrode voltage-clamp analysis and microfluorescence experiments. The electrophysiological data gave evidence for a rheogenic process with large inward currents in either MMgT1 (Fig. 2A) or MMgT2 (Fig. 2B) cRNA-injected oocytes, whereas there were no appreciable currents in control H$_2$O- or total poly(A) RNA-injected cells from the same batch of oocytes. The reversal potentials shifted right with increasing external Mg^{2+} concentrations, similar to that predicted by the Nernstian relationship for Mg^{2+} transport (indicated by arrows; Fig. 2, A and B). Mouse MMgT-mediated Mg^{2+}-induced
uptake was saturable: the $K_m$ for MMgT1 was $1.47 \pm 0.17$ mM ($n = 32$; Fig. 2C), and the $K_m$ for MMgT2 was $0.58 \pm 0.07$ mM ($n = 29$; Fig. 2D). $K_m$ values did not vary with voltage in each case. In support of the conclusions using voltage-clamp analysis, we performed flux experiments with fluorescence spectrometry using the Mg$^{2+}$-sensitive dye mag-fura-2 (10). Currents were initially measured at resting potentials with oocytes in Mg$^{2+}$-free solutions and then in solutions contain-
ing 2.0 mM MgCl₂ to confirm that oocytes were expressing the respective MMgTs. Oocytes were then voltage clamped at −70 mV (Fig. 2E). Fluorescence was determined throughout the experiment. The 340-to-385-nm fluorescence ratio reflects the change in free Mg²⁺ concentration within the oocyte. The 340-to-385-nm ratio, i.e., intracellular Mg²⁺ content, increased in MMgT1- and MMgT2-expressing oocytes but not in control H₂O-injected oocytes (Fig. 2E). Moreover, the increase in Mg²⁺ concentration was associated with the simultaneously measured currents, indicating that Mg²⁺-evoked currents were mediated by Mg²⁺ flux for both MMgT1 (Fig. 2F) and MMgT2 (Fig. 2G).

Mg²⁺-evoked currents were not altered with deletion of external sodium by substitution with choline (106 ± 8% of control currents determined with −125-mV clamp; n = 3) or replacement of chloride with nitrate (97 ± 6% of control, n = 3), suggesting that transport does not depend on extracellular sodium or chloride. Niflumic acid (0.5 mM), an anion transport inhibitor, did not affect Mg²⁺ currents (94 ± 11% of control, n = 3).

A variety of other divalent cations was used to determine the selectivity of the expressed MMgT-mediated transport. MMgT1 mediated Sr²⁺, Fe²⁺, Co²⁺, and Cu²⁺ transport, whereas MMgT2 mediated Sr²⁺, Co²⁺, Cu²⁺, Ba²⁺, Mn²⁺, and Ni²⁺ transport in addition to Mg²⁺ (Fig. 2D). None of the divalent cations tested elicited detectable currents in water-injected oocytes. Notably, Mn²⁺ elicited currents in MMgT2-expressing oocytes but not in MMgT1-expressing cells. The differential selectivity for Mn²⁺ was also evident using fluorescence measurements (Fig. 2E). Mn²⁺ did not quench mag-fura-2 fluorescence in MMgT1-expressing cells but markedly quenched fluorescence in MMgT2-expressing oocytes, as would be expected if MMgT2 but not MMgT1 mediated Mn²⁺ transport. These experiments clearly indicate that MMgT1 and MMgT2 mediate Mg²⁺ transport in MMgT1- and MMgT2-expressing oocytes. Additional electrophysiological experiments demonstrated that 0.2 mM Mn²⁺ inhibited Mg²⁺-mediated currents in MMgT1-expressing oocytes, whereas Ni²⁺ and Gd³⁺ did not affect Mg²⁺ transport (data not given). Accordingly, MMgT1-mediated transport is substrate selective and inhibitable, confirming properties of membrane transporters. Of the cation substrates tested here, only Mg²⁺ is found at the physiological concentrations used in these experiments, so we conclude that MMgT1 and MMgT2 are principally Mg²⁺ transporters.

Finally, we used immunofluorescence to show that MMgT1 and MMgT2 fusion proteins are localized in surface membranes of MMgT1- and MMgT2-expressing oocytes (Fig. 2J). The surface staining was consistent with the results of the functional experiments. No staining was present in native water-injected oocytes.

**Subcellular localization of MMgT.** To investigate the subcellular localization of MMgT1 and MMgT2 proteins, we performed immunofluorescence using anti-HA and anti-V5 antibodies in kidney COS-7 cells transfected with MMgT1-HA and MMgT2-V5 constructs, respectively. The MMgT1-HA fusion protein extensively colocalized with GM130 (a cis-Golgi matrix protein), suggesting that MMgT1 is principally resident in the Golgi complex (Fig. 3A). However, there was also apparent staining of post-Golgi vesicles with partial overlap with Rab5, a marker of early recycling endosomes (Fig. 3B). There was no colocalization with endoplasmic reticulum (ER) or late endosome/lysosome markers. The MMgT2 fusion protein demonstrated a similar distribution, predominately in the Golgi, with appreciable amounts in post-Golgi vesicles, including partial staining of the punctate structures representing the early recycling endosomes (Fig. 4).

To determine the subcellular distribution of protein with changes in magnesium, COS-7 cells were cultured in nominally magnesium-free media for 12 h, and localization was determined with immunofluorescence as described above. There appeared to be an increase in MMgT1 (Fig. 3C) and MMgT2 (Fig. 4C) proteins in both Golgi compartments and post-Golgi vesicles. This observation is consistent with the increase in the respective transcripts in epithelial cells in response to magnesium deficiency.

Finally, we determined if there was colocalization of MMgT1 and MMgT2 proteins within epithelium-derived cells. COS-7 cells were transfected with both fusion constructs, and the subcellular localization was determined with immunofluorescence. As expected, there was colocalization of MMgT1 and MMgT2 fusion proteins within the Golgi complex (Fig. 5A). However, it was of interest that there was only partial colocalization within the post-Golgi vesicles, which suggests that they
Fig. 4. Subcellular localization of MMgT2. Immunofluorescence staining of MMgT2-V5 transfected COS-7 cells. A and B: cells were cultured in media containing normal magnesium concentrations, fixed, and incubated with V5 antibody (left) and GM130 (A, middle) or Rab5 (B, middle). The respective overlays are shown on the right. MMgT2 protein was distributed to the Golgi complex and post-Golgi vesicles including in the partial labeling of early endosomes. A similar pattern of MMgT2 distribution was observed as with the MMgT1 protein, as shown in Fig. 3. C: COS-7 cells were cultured in low magnesium, fixed, and incubated with HA antibody (left) and GM130 (middle). The overlay is shown on the right. As with MMgT1, there was an apparent increase in MMgT2 protein in the Golgi and post-Golgi vesicles in magnesium-depleted cells.

Fig. 5. Subcellular colocalization of MMgT1 and MMgT2 fusion proteins. COS-7 cells were transfected with both MMgT1-HA and MMgT1-V5 epitope-tagged constructs, and immunofluorescence staining was performed with HA (left) and V5 (middle) antibodies. Merged images are presented on the right. A: cells cultured in normal media with magnesium. B: cells cultured in low magnesium. Both proteins were evident in the Golgi complex, but, of note, they appeared to localize to different post-Golgi vesicles in normal and Mg2+-depleted cells.

DISCUSSION

In summary, we show here that MMgT1 and MMgT2 proteins are novel membrane Mg2+ transporters that probably reside in the Golgi and post-Golgi vesicles. Not only do MMgTs mediate Mg2+ transport, but they are regulated by the available Mg2+ concentration in that the respective transcripts and possibly proteins are increased in response to diminished Mg2+ levels. A number of points are of interest. First, MMgT1 and MMgT2 transcripts are present in a wide variety of tissues, suggesting a housekeeping role for the two transporters in cellular Mg2+ metabolism. Second, the proteins comprise only two predicted transmembrane domains (TMDs), implying that they might form functional oligomers. It suggests that they may form homooligomeric and perhaps heterooligomeric units, as it is generally thought that proteins with only two TMDs are not sufficient to form a functional transporter. Many membrane receptors, transporters, and other integral membrane proteins require dimerization or higher oligomerization for their activity. As either MMgT1 or MMgT2 form functional transporters when expressed alone in oocytes, they must at least act as homomultimeric proteins, but they may form heterooligomers and thereby might have different functional and morphological properties. The fact that both MMgT1 and MMgT2 fusion proteins colocalize within the Golgi might suggest they undergo heterooligomerization. The redundancy of two Mg2+ transport proteins in the same subcellular compartment is also of interest as it may be important in maintaining the cellular Mg2+ balance but also lend itself to mutationally induced dominant negative inhibition of function. Finally, the presence of MMgT fusion proteins in post-Golgi vesicles suggests that they might play additional roles downstream of the Golgi complex. They partially colocalize to the early endosomes, but a significant amount of protein traffics to other post-Golgi vesicles. Interestingly, they appear to sort to separate organelar compartments, suggesting that they may ultimately subserve different functional roles in Mg2+ metabolism.

Intracellular Mg2+ concentration is in the order of 0.5 mM, but it is not homogeneously distributed across the cell. Using spatial imaging with mag-fura-2, we have shown that there are relatively higher levels in the perinuclear region comprising the ER, Golgi, and other organelles. Mg2+ plays an important role in the ER and Golgi as it is essential in protein assembly, lipid biosynthesis, and vesicle trafficking. These biochemical processes are catalyzed by specific enzymes that require specific luminal Mg2+ levels for optimal function. It is now known that the Golgi and post-Golgi vesicles possess numerous ionic transporters to maintain ideal Ca2+, Mn2+, Zn2+, H+, K+, and ATP levels (1, 2, 6, 11, 13–15, 17, 27, 32). Accordingly, it is not surprising that there are dedicated transporters to maintain optimal Mg2+ concentrations in these organelles. The novel
MMgT1 and MMgT2 proteins described here provide the first Mg\(^{2+}\) transporters to be identified in the Golgi complex and post-Golgi vesicles.

Numerous mammalian divalent metal transporters have been molecularly identified and extensively studied, but few have been shown to mediate Mg\(^{2+}\) transport. The first mammalian Mg\(^{2+}\) transporter to be identified was MR2, a mitochondrial protein encoded by nuclear DNA (33). In the transient receptor potential melastatin (TRPM) family of cation channels, the ubiquitous TRPM7 has been shown to mediate plasma membrane Mg\(^{2+}\) transport and to be essential for cellular viability (16, 18). Another member of this family, TRPM6, forms a major Mg\(^{2+}\) transporter in the plasma membrane of intestinal and kidney epithelial cells (3, 25, 29). Using differential gene expression, we (8) have recently identified a novel family of Mg\(^{2+}\) transporters, designated MagT, that was regulated at the translational and protein level by cellular Mg\(^{2+}\) balance (personnel observations). More recently, by applying the same microarray platform, we (7) have shown that the solute carrier SLC41, a family of proteins comprising three gene products (SLC41A1, SLC41A2, and SLC41A3), mediates Mg\(^{2+}\) transport when expressed in Xenopus oocytes. Additionally, we have shown that the ancient conserved domain protein (ACDP) family, composed of ACDP1, ACDP2, ACDP3, and ACDP4, is differentially expressed in response to Mg\(^{2+}\) content and the expressed proteins mediate Mg\(^{2+}\) transport (9). Mutations in ACDP1 are thought to underlie urofacial syndrome, although the alteration has not been identified (30). Finally, we (10) have shown that the nonimprinted in Prader-Willi/Angelman (NIPA) family of genes encode plasma membrane Mg\(^{2+}\) transport proteins (10). Mutations in NIPA1 lead to a loss of function defect which provides the basis for hereditary spastic paraplegia (22, 23). The role of each of these transporter families in the control of cell Mg\(^{2+}\) is currently under active investigation. Nevertheless, there is now abundant evidence for a number of unique mammalian Mg\(^{2+}\) transporters. In the present study, we report the identification of another family of Mg\(^{2+}\) transporters that appear to be predominately located in the Golgi complex but might play an important role in post-Golgi vesicles.

GRANTS

This work was supported by Canadian Institutes of Health Research Grant MOP-53288 (to G. A. Quamme).

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