Transcriptional Regulation of the Claudin-16 Gene by Mg^{2+} Availability

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Key Words

Hypomagnesemia • Hypermagnesemia • Tubular Mg^{2+} Transport • Promoter • Gene Expression • Paracellin-1

Abstract

Background/Aims: Renal tubular Mg^{2+} reabsorption is mediated predominantly by the tight junction channel protein claudin-16 which is encoded by the gene CLDN16. Hypermagnesemia decreases, whereas hypomagnesemia increases Mg^{2+} reabsorption. This study examines the role of claudin-16 in the adaptive response of the kidney to Mg^{2+} availability. **Methods/Results:** Mice received a low-, normal- or high Mg^{2+} diet for up to 3 days. Mg^{2+} -loaded animals displayed hypermagnesemia with increasing urine Mg^{2+}/Ca^{2+} levels paralleled by a decrease in claudin-16 protein and mRNA in the kidney. Mg^{2+} -deprived animals developed hypomagnesemia with decreasing urine Mg^{2+}/Ca^{2+} levels associated with an increase in claudin-16 protein and mRNA abundance. Mg^{2+} depletion markedly increased and Mg^{2+} load decreased endogenous claudin-16 mRNA levels in calcium-sensing receptor-transfected HEK293 cells compared with native HEK293 cells. The effect of Mg^{2+} availability on the human CLDN16 (hCLDN16) gene promoter

was examined. Using a 2.5kb hCLDN16 5'-flanking DNA sequence, we show that magnesium depletion increases and Mg^{2+} load decreases hCLDN16 promoter activity in transfected HEK293 cells. **Conclusions:** Changes in Mg^{2+} availability may influence claudin-16 mediated Mg^{2+} transport at the transcriptional level. The possible involvement of the cell membrane bound Ca^{2+}/Mg^{2+} sensing receptor or the potential role of a hypothetical Mg^{2+} response element on the CLDN16 promoter in the Mg^{2+} -induced response remains to be explored.

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Introduction

Magnesium is the most abundant divalent cation in the intracellular fluid and serves as a critical co-factor in a wide variety of biological processes [1]. Abnormalities in Mg^{2+} homeostasis are relatively common in clinical practice and often lead to neuromuscular disturbances, central nervous system manifestations and cardiovascular dysfunction [2-4]. In mammals, the kidney is the principal organ responsible for Mg^{2+} balance. Normally, more than 95% of the filtered Mg^{2+} is reabsorbed by the renal

tubule. The major site of Mg^{2+} reabsorption is the thick ascending limb of the loop of Henle (TAL), where 60-70% of the filtered load is reclaimed [3, 4]. Mg^{2+} (and Ca^{2+}) transport in this tubule segment occurs primarily through paracellular conductance driven by the lumen positive electrical potential [3, 4]. This transport process is mediated by the channel protein, claudin-16 (paracellin-1), which is a member of the claudin family of tight junction proteins [5, 6], and is expressed in the tight junction of the TAL and the distal convoluted tubule (DCT) [7, 8]. Human claudin-16, the first to be reported tight junction protein involved in ion resorption, is encoded by the gene hCLDN16 (also known as hPCLN-1, NCBI accession no NM-006580) [7], mutations in which cause familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) syndrome [7]. We have recently characterized the promoter region of hCLDN16 [9].

Renal Mg^{2+} transport also occurs via TRPM6, which is a member of the transient receptor potential (TRP) family [10], and is exclusively expressed in the DCT and small intestine [10, 11]. TRPM6 constitutes the apical Mg^{2+} entry channel in transcellular Mg^{2+} reabsorption [11]. Mutations in the human TRPM6 gene cause autosomal recessive hypomagnesemia with secondary hypocalcemia [10].

A variety of factors are known to modulate Mg^{2+} reabsorption in the renal tubule, among them Mg^{2+} restriction and Mg^{2+} load, which have been found to influence both transcellular Mg^{2+} transport in the DCT and paracellular Mg^{2+} flux in the TAL [12-17]. Micropuncture experiments in rat nephron demonstrated that a low Mg^{2+} diet leads to urinary Mg^{2+} retention due to increased Mg^{2+} reabsorption in the TAL [16, 17]. Culturing mouse distal convoluted tubule (MDCT) cells in Mg^{2+} -free medium increased their Mg^{2+} transport rate by a mechanism likely involving *de novo* transcription, as demonstrated by actinomycin D-induced inhibition of this transport [3, 12]. In contrast to the effect of Mg^{2+} deprivation, Mg^{2+} infusion [14] and acute elevation of Mg^{2+} concentration at the contraluminal membrane of the TAL in rats [15] inhibited Mg^{2+} reabsorption in this tubule segment. It has been recently demonstrated that Mg^{2+} restriction, but not Mg^{2+} load, modulates renal TRPM6 mRNA levels in mice [13]. Nevertheless, the molecular mechanisms responsible for the regulation of paracellular Mg^{2+} transport, the major means of renal tubular Mg^{2+} reabsorption, by Mg^{2+} availability, and the role of the tight junction protein channel, claudin-16, in this process have not been investigated.

In this study we aimed to examine the involvement

of claudin-16 in the adaptive response of mouse kidney and renal cell lines to changes in Mg^{2+} availability. We demonstrate that Mg^{2+} -deprived mice show an increase in both claudin-16 protein and mRNA in the kidney compared to mice receiving normal Mg^{2+} diet, whereas Mg^{2+} -loaded animals show the opposite, namely, a decrease in kidney claudin-16 protein and mRNA. In addition, we demonstrate a marked extracellular Mg^{2+} concentration-dependent change in endogenous claudin-16 mRNA levels in calcium-sensing receptor (CaSR)-transfected human embryonic kidney (HEK293) cells. Finally, using 2.5kb CLDN16 5'-flanking DNA sequence previously cloned and characterized by us [9], we show that Mg^{2+} depletion increases and Mg^{2+} load decreases hCLDN16 promoter activity in transfected HEK293 cells. Our findings provide the first direct evidence that paracellular, claudin-16-mediated Mg^{2+} transport is transcriptionally regulated by Mg^{2+} availability, a process possibly involving the CaSR.

Materials and Methods

Animal Studies

Eight week old male ICR mice were housed in individual metabolic cages, enabling urine collection. The animals were kept in a light and temperature controlled room and were fed (*ad libitum*) a low Mg^{2+} diet (0.012mg Mg^{2+} /gr) (Harlan Tekland, Madison, WI, USA), a control diet (1.51mg Mg^{2+} /gr) (Kofolk, Petach-Tikva, Israel) or a high Mg^{2+} diet (control chow + 50gr/l $MgSO_4$ in distilled water) for 1-3 days. Low Mg^{2+} and control mice had free access to distilled water (<0.24mg/li Mg^{2+} , 0 mg/li Ca^{2+}). Urine was collected every day from all metabolic cages. At 24 hour intervals mice were anesthetized by intraperitoneal administration of Sagatal (pentobarbitone sodium, Rhone Merieux, Essex, UK). Blood samples were collected by heart puncture. Serum and urine samples were analyzed for magnesium, calcium, sodium and creatinine content using standard automated techniques (Roche Modular P800). Kidneys were harvested for determination of claudin-16 protein abundance and claudin-16-, claudin-19-, and claudin-2 mRNA levels.

Immunofluorescence Microscopy

For immunofluorescence staining, cryosections (5 μ m) of mouse kidneys were fixed in ethanol and acetone. Sections were blocked in 1% BSA in PBS and exposed to rabbit anti-claudin-16 antibodies (Zymed Laboratories, San Francisco, CA, USA) either alone, or, for colocalization purposes, in combination with antibodies labeling specifically either the TAL or the tight junction. For this purpose, antibody to Tamm-Horsfall protein (a marker of the TAL), kindly provided by Dr. John R. Hoyer (Philadelphia, PA, USA), and antibody to occludin (a marker of the tight junction) (Santa Cruz Biotechnology, Santa Cruz, CA) were used.

Primary antibodies were diluted in PBS containing 1% BSA and applied to cryosections for 1 hour at room temperature. Sections were subsequently exposed to appropriate fluorescent secondary antibodies (FITC-conjugated and/or TR-conjugated) (Santa Cruz Biotechnology) in PBS containing 1% BSA for 1 hour. Stained sections were mounted in UltraCruz Mounting Medium containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclear staining (Santa Cruz Biotechnology). Immunostained slides were visualized with a Zeiss inverted Axiovert 135 microscope set (Carl Zeiss, Germany) with appropriate filters.

Quantitative Real Time PCR Analysis

Total kidney RNA was prepared using Tri Reagent (Sigma, St. Louis, MO, USA) followed by phenol/chloroform extraction and isopropanol precipitation. RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and further cleaned with RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription of 2 µg of total mouse kidney RNA was carried out using MMLV (Promega) with random hexamer primers. Quantitative PCR experiments were performed using TaqMan Universal PCR Master Mix (Roche Molecular Systems, NJ, USA) with Assay on Demand primers (Applied Biosystems, Foster City, CA) for gene expression analysis of mouse CLDN16 (Mm00475025_m1) with mouse 18S (Hs9999901_s1) or mouse TATA-binding protein (Mm00446973_m1) serving as a house-keeping gene of normalization. Additional primers used in mice were CLDN2 (encoding claudin-2) (Mm00516703_s1) and CLDN19 (encoding claudin-19) (Mm00462192_m1). All measurements were performed in triplicate in an ABI Prism 7000 Cycler (Applied Biosystems).

Cell Lines

HEK293 cells were grown and maintained in DMEM/F-12 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air-5% CO₂.

HEK293 cells stably transfected with the human CaSR (HEK293 - CaSR) (provided by Dr. A. Hofer, Harvard Medical School, Boston, MA) [18]) were grown and maintained in DMEM/F-12 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 IU/ml penicillin and 5 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air-5% CO₂.

Quantitative Real-Time PCR Analysis of Cell Lines

Confluent HEK293 or HEK293 - CaSR cells were planted in six-well dishes (4 x 10⁵ cells/well). After 24h in serum-containing regular media, cells were washed with PBS and placed in media with low (0 mM), normal (0.7 mM) or high (1.5 mM) Mg²⁺. Following 24h exposure to these media, cells were washed with PBS, total RNA was prepared, and reverse transcription was carried out as in mouse kidney (see earlier). Quantitative PCR experiments were performed using Assay on Demand of human CLDN16 (Hs00198134_m1) and human TRPM6 (Hs00214306_m1) with human TATA - binding protein (Hs99999910_m1) serving as a house-keeping gene for normalization.

Transient Transfections and Reporter Gene Assays

A plasmid, constructed in our laboratory [9], containing a 5'-flanking 2.5kb fragment of the CLDN16 promoter cloned upstream to a luciferase reporter gene in pGL3-basic (Promega), termed pJ2M, was used in these experiments. pJ2M was transiently transfected into HEK293 along with plasmid pCH110 (Pharmacia, Uppsala, Sweden), carrying a CMV- driven LacZ gene for normalization of transfection efficiency. pGL3 vector containing no insert was used as a negative control. Promoter activity was estimated from the ratio of hCLDN16 - driven luciferase activity in pGL3 - basic vector to promoter - less pGL3 - basic vector. Plasmids were purified using Nucleobond AX (Macherey Nagel, Duren, Germany). For transfections, cells were plated (5x10⁴/well) in 24-well dishes in serum containing medium. Transfections were performed using Eugene 6 (1.2 µl/well) (Roche) with 0.3 µg pJ2M or pGL3-basic and 0.3 µg pCH110. Forty eight hours after transfection, media were replaced with fresh media containing 0.2% bovine serum albumin (Sigma), in place of FCS, as well as different Mg²⁺ concentrations: 0mM (low), 0.7mM (normal) or 1.5mM (high) Mg²⁺ for 16-24 hours.

For enzymatic assays, cells were washed with PBS (150mM NaCl, 15mM sodium phosphate, pH 7.3) and lysed by incubating in 200 µl/well M-Per (Pierce) for 5 minutes at 37°C. Lysed cells were centrifuged and supernatant aliquoted (50 µl/well) into 96 well plates. Fifty microliters of Luciferase Assay Reagent (Promega) were automatically dispensed and the light intensity of the reaction immediately read in a luminometer (Lucy, Anthos, Austria) for a period of 10 seconds. Luciferase activity was normalized to β-galactosidase activity, which was measured in identical cell lysates as follows: 160 µl of ONPG substrate (Sigma) were added to 30 µl of cell lysate in each well of a 96 well plate. The reaction mixture was incubated for 30 minutes at 37°C, or until yellow color developed. β-galactosidase measurements were performed spectrophotometrically with a 405 nm filter. Measurements of luciferase and β-galactosidase were performed in duplicate.

Statistical Analysis

Data comparisons were made with nonparametric Mann-Whitney U-test using the SPSS Version 16 computer program. P<0.05 was considered significant.

Results

Effect of Dietary Mg²⁺ on Mineral Concentration in Serum and Urine of Mice

The dependence of serum and urine Mg²⁺ and Ca²⁺ concentrations on dietary Mg²⁺ intake in mice was examined (See Materials and Methods). As shown in Fig. 1, Mg²⁺-loaded animals displayed hypermagnesemia (A) with increasing urine Mg²⁺ concentrations (B), whereas Mg²⁺-deprived animals developed hypomagnesemia (A) with decreasing urine Mg²⁺ levels (B). It is noteworthy that the hypermagnesemia induced by Mg²⁺ load was

Fig. 1. Dependence of serum (A) and urine (B) Mg^{2+} levels and of serum (C) and urine (D) Ca^{2+} levels on dietary Mg^{2+} level in mice. Mice were fed a low Mg^{2+} diet, a control or a high Mg^{2+} diet for 1-3 days (see Materials and Methods). Blood and urine samples were analyzed for Mg^{2+} , Ca^{2+} and creatinine (Cr) concentrations. Values are means $\pm$ SE of 3-4 independent experiments, each performed on 5-6 mice. Low dietary Mg^{2+} intake decreased and high dietary Mg^{2+} intake increased serum (A) and urine (B) Mg^{2+} levels in mice. Both diets had no effect on serum Ca^{2+} levels (C). However, low dietary Mg^{2+} intake decreased and high dietary Mg^{2+} intake increased urine Ca^{2+} levels (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs normal Mg^{2+} .

much more pronounced than the hypomagnesemia resulting from Mg^{2+} deprivation. Since claudin-16 is known to transport Mg^{2+} as well as Ca^{2+} , the effect of dietary Mg^{2+} intake on serum and urine Ca^{2+} levels was also examined. From Fig. 1(C) it is apparent that Mg^{2+} load or depletion did not alter serum Ca^{2+} levels. Urinary excretion of Ca^{2+} , however, significantly increased in Mg^{2+} -loaded mice, and decreased with Mg^{2+} restriction (D). No dietary Mg^{2+} level-dependent changes in serum and urine Na^+ levels were found (data not shown).

Effect of Dietary Mg^{2+} Levels on Claudin-16 Protein Abundance in Mouse Kidney

To identify the nephron segment expressing claudin-16 in our mouse model, and to verify that claudin 16 is expressed in the tight junction, kidney sections from mice fed normal Mg^{2+} diet (control mice) were double stained with claudin-16 antibody in combination with either an antibody to Tamm Horsfal protein (THP) or an antibody to occludin. As seen in Fig. 2A, claudin-16 staining was apparent in THP-expressing nephron segments, indicating claudin-16 expression in the TAL (upper panel) and it colocalized with occludin indicating its expression in the

tight junction (lower panel). These findings are in line with previous studies demonstrating exclusive claudin-16 expression in the tight junction of the TAL of the rabbit [7] and rat [8].

Next, cryosections of kidneys harvested from mice after 3 days of normal, low or high dietary Mg^{2+} intake were probed with claudin-16 antibody. As shown in Fig. 2B, changes in Mg^{2+} availability affected the abundance of claudin-16. Kidneys of Mg^{2+} -loaded mice displayed significantly lower levels of claudin-16 protein compared to control kidneys, whereas Mg^{2+} -deprivation caused a marked increase in claudin-16 protein staining.

Effect of Dietary Mg^{2+} Levels on CLDN16 mRNA Expression in Mouse Kidney

Next, the effect of dietary Mg^{2+} intake on claudin-16 mRNA levels in mouse kidney was examined. The effect of Mg^{2+} availability on other members of the claudin family expressed in the kidney namely, claudin-19, known to participate in renal paracellular Mg^{2+} transport [19], and claudin-2, was examined as well. As seen in Fig. 3A, and in line with the changes in claudin-16 protein abundance (see earlier), Mg^{2+} -loaded animals displayed a

Fig. 2. A. Localization of claudin-16 protein in mouse kidney. Frozen sections of mouse kidneys were immunostained as follows: Upper Panel - anti claudin-16 antibody followed by FITC-conjugated secondary antibody (stained green) (a), anti Tamm-Horsfal protein (THP) followed by TR-conjugated secondary antibody (stained red) (c) and a merge of the two (colocalized proteins yield yellow stain) (b). Lower Panel - anti claudin-16 antibody followed by FITC-conjugated secondary antibody (green) (d), anti occludin followed by TR-conjugated secondary antibody (red) (f) and a merge of both (showing a yellow stain) (e). No staining was observed in all kidneys examined in the absence of primary and presence of secondary antibody (data not shown) indicating antibody specificity. B. Effect of dietary Mg^{2+} level on claudin-16 protein abundance in mouse kidneys. Cryosections of mouse kidneys after 3 days of low, normal or high dietary Mg^{2+} intake were probed with anti claudin-16 antibody followed by FITC-conjugated secondary antibody (green color). Nuclei were visualized by 4,6-diamidino 2-phenylindole dihydrochloride (DAPI) staining (blue color). No claudin-16 staining was observed in kidneys examined in the absence of primary and presence of secondary antibody (data not shown) indicating antibody specificity. Low Mg^{2+} increased and high Mg^{2+} decreased claudin-16 protein expression in kidney sections.

decrease of up to 50% in CLDN16 mRNA, whereas Mg^{2+} -deprived animals showed an increase of up to 95% in CLDN16 mRNA when compared to normomagnese-mic mice. The most prominent changes in CLDN16 mRNA levels occurred on day 3 of the experiment. In contrast, analysis of kidneys on day 3 revealed no effect of dietary Mg^{2+} content on claudin-19 and claudin-2 mRNA levels (Fig. 3B).

Effect of extracellular Mg^{2+} Concentration on Endogenous CLDN16 mRNA level in HEK293 Cells

We examined the effect of changes in extracellular Mg^{2+} concentration on endogenous CLDN16 mRNA level in native HEK293 cells as well as in HEK293 cells stably transfected with the human CaSR (HEK-CaSR) [18] (see Materials and Methods). As opposed to native

Fig. 3. Effect of dietary Mg^{2+} level on claudin-16-, claudin-19-, and claudin-2 mRNA levels in mouse kidney. A. Data represent claudin-16 mRNA levels in kidneys of mice receiving high or low Mg^{2+} diets for 3 consecutive days relative to claudin-16 mRNA in kidneys of mice fed a control diet, following normalization to 18S mRNA. Values are means $\pm$ SE of 3-5 independent experiments each performed in duplicate. Mg^{2+} -loaded animals showed a decrease of up to 50% in CLDN16 mRNA compared to normomagnesemic mice. Mg^{2+} -deprived animals displayed an increase of up to 95% in CLDN16 mRNA. Mg^{2+} level - induced changes were most prominent on day 3 of the experiment. B. Data represent claudin-16-, claudin-19-, and claudin-2 mRNA levels in kidneys of mice receiving high or low Mg^{2+} diet for 3 consecutive days relative to mRNA levels in kidneys of mice fed a control diet, following normalization to 18S RNA. Values are means $\pm$ SE of 3-5 independent experiments each performed in duplicate. As opposed to the marked changes in claudin-16 mRNA levels induced by low and high Mg^{2+} diets, dietary Mg^{2+} content did not affect claudin-19-, and claudin-2 mRNA levels. *** $p < 0.005$ vs control diet.

Fig. 4. Effect of extracellular Mg^{2+} level on CLDN16 mRNA and TRPM6 mRNA levels in HEK293 and CaSR - transfected HEK293 (HEK-CaSR) cells. Data represent CLDN16 (A) or TRPM6 (B) mRNA levels in cells exposed for 24 hours to media containing high or low Mg^{2+} concentrations relative to CLDN16 or TRPM6 mRNA, respectively, in cells exposed to normal (control) Mg^{2+} . Data are presented following normalization to TBP mRNA. Values are means $\pm$ SE of 3-5 independent experiments, each performed in triplicate. A. While low Mg^{2+} increased CLDN16 mRNA level by 55% and high Mg^{2+} had a negligible effect on this level in native HEK293 cells, low Mg^{2+} markedly increased (by 280%) and high Mg^{2+} decreased (by 30%) CLDN16 mRNA level in HEK-CaSR cells. B. Low Mg^{2+} increased TRPM6 mRNA level by 85% and high Mg^{2+} had a negligible effect on this level in native HEK293 cells. These Mg^{2+} - induced changes in TRPM6 mRNA were of similar magnitude in HEK-CaSR cells. * $p < 0.05$, *** $p < 0.005$ for values in HEK-CaSR vs HEK293 cells.

HEK293 cells, which do not express the CaSR [20-22], the latter cell line harbors this receptor and, hence, was used by us to probe the possible effect of the CaSR on the adaptive response to Mg^{2+} availability. The effect of extracellular Mg^{2+} concentration on endogenous TRPM6 mRNA level (previously shown to be upregulated by Mg^{2+} restriction *in vivo* [13]) was also examined in both

HEK293 and HEK-CaSR cells. As shown in Fig. 4A, exposure of native HEK293 cells to low Mg^{2+} medium for 24h increased CLND16 mRNA level by 55% when compared with conditions of normal Mg^{2+} . High Mg^{2+} concentration in the medium had a negligible effect on CLDN16 transcript level in native HEK293 cells. In contrast, Mg^{2+} depletion and Mg^{2+} load resulted in a 280%

Fig. 5. Effect of ambient Mg^{2+} on human CLDN16 (hCLDN16) promoter activity in HEK293 cells. Cells were transiently transfected with pGL3-basic or pGL3-basic containing the 2.5kb 5'-flanking region of hCLDN16 (pJ2M) followed by exposure to 0mM (low), 0.7mM (normal), or 1.5mM (high) Mg^{2+} for 24h. Subsequently, luciferase activity was measured. To control for transfection efficiency, cells were cotransfected with pCH110 and luciferase activity was normalized to β -galactosidase activity. Data are presented as fold change of reporter plasmid activity (calculated as % pGL3-basic vector activity) in cells exposed to low or high Mg^{2+} relative to activity in cells exposed to normal Mg^{2+} . Values are means $\pm$ SE of 3-5 independent experiments, each performed in quadruplicate. hCLDN16 promoter activity increased by two fold when exposed to low Mg^{2+} and decreased by 30% when exposed to high Mg^{2+} , compared to normal/control Mg^{2+} conditions. * $p < 0.05$ vs normal Mg^{2+} .

increase and a 30% decrease, respectively, in endogenous CLDN16 level in HEK293 - CaSR cells. As depicted in Fig. 4B, exposure of native HEK293 cells to low Mg^{2+} medium increased TRPM6 mRNA level by 85% when compared with conditions of normal Mg^{2+} . High Mg^{2+} had a negligible effect on TRPM6 mRNA level in these cells. However, in contrast to the findings on CLDN16 mRNA, no significant differences in TRPM6 mRNA levels were observed between HEK293 and HEK-CaSR cells exposed to either low Mg^{2+} or high Mg^{2+} (Fig. 4B).

Effect of Mg^{2+} Availability on the Claudin-16 Gene Promoter

We next examined whether Mg^{2+} availability affects human CLDN16 transcription in HEK293 cells via its promoter. To this end, a plasmid, previously constructed in our laboratory, containing 2.5 kb of the hCLDN16 promoter cloned upstream to a luciferase reporter gene in pGL3-basic vector, and termed pJ2M, was employed [9]. Having previously shown that this fragment contained a renal cell-specific promoter (that was Mg^{2+} - sensitive in proximal tubular opossum kidney (OK) cells [9]), we now investigated whether the activity of this promoter in HEK293 cells [displaying Mg^{2+} - sensitive changes in endogenous CLDN16 mRNA level (Fig. 4B)] was also Mg^{2+}

concentration-dependent. For this purpose, pJ2M was transfected into native HEK293 cells (shown by us to express endogenous claudin-16 mRNA [9]) and exposed to various concentrations of Mg^{2+} in serum-free growth media (see Methods). hCLDN16 promoter-driven luciferase activity at various Mg^{2+} concentrations was compared. As seen in Fig. 5, hCLDN16 promoter activity increased two fold when exposed to low Mg^{2+} and decreased by 30% when exposed to high Mg^{2+} compared to control Mg^{2+} conditions.

Discussion

In this study, we demonstrate that the tight junction channel, claudin-16, encoded by the gene CLDN16, plays an important role in the renal adaptive response to Mg^{2+} availability. We demonstrate that Mg^{2+} deprivation increases and Mg^{2+} load decreases claudin-16 protein (Fig. 2) and mRNA (Fig. 3) expression in the mouse kidney. Furthermore, we show that extracellular Mg^{2+} concentration influences endogenous CLDN16 mRNA level in HEK293 cells, an effect that is particularly pronounced in HEK293 cells expressing the CaSR (Fig. 4). Finally, we show that Mg^{2+} level modulates hCLDN16 promoter activity (Fig. 5) by acting on the 5'-flanking 2.5kb hCLDN16 promoter region previously characterized by us [9]. These findings suggest that paracellin-1 is subject to transcriptional regulation by Mg^{2+} availability.

The effect of dietary Mg^{2+} levels on mineral concentration in serum and urine of mice demonstrated in our study (Fig. 1) is in line with previous findings [17] showing that Mg^{2+} restriction enhanced Mg^{2+} and Ca^{2+} transport in the mouse cortical TAL, and support the notion that claudin-16, a facilitator of paracellular divalent cation transport, is responsible for Mg^{2+} as well as Ca^{2+} reabsorption in the renal tubule. Also in line with findings of previous studies [17] is the lack of compatibility between dietary Mg^{2+} content and plasma Ca^{2+} level which remained constant despite changes in urine Ca^{2+} level (Fig. 1). Maintaining constant blood Ca^{2+} levels despite variations in urine Ca^{2+} concentration may be attributable to compensatory changes in metabolic pathways participating in Ca^{2+} balance including mobilization of Ca^{2+} from bone, altered intestinal Ca^{2+} absorption and, possibly, changes in the activity of additional transporters/carriers responsible for Ca^{2+} transport in the renal tubule. The potential role of bone Ca^{2+} in preserving normal blood Ca^{2+} in states of Mg^{2+} deficiency is supported by a recent study demonstrating a decrease in bone Ca^{2+} level

in mice fed a Mg^{2+} - deficient diet [23]. Regulation of Ca^{2+} transport in the intestine and kidney is achieved by modulating the activity of the epithelial Ca^{2+} channels TRPV5 and TRPV6, expressed in the small bowel epithelium and the luminal membrane of renal DCT cells [24]. It is conceivable that changes in expression and activity of these Ca^{2+} channels in conditions of hypo- and hypermagnesemia may help maintain normal blood Ca^{2+} level, despite changes in claudin-16 activity. It is noteworthy that similar mechanisms may underly the normocalcemia typical of familial hypomagnesemia with hypercalciuria and nephrocinosis (FHHNC) syndrome caused by a hereditary defect in claudin-16 [7, 25].

A decrease in CLDN16 mRNA was observed in kidneys of hypermagnesemic mice as opposed to an increase in CLDN16 mRNA in hypomagnesemic mice (Fig. 3). No such Mg^{2+} -induced effect was observed on mRNA levels of claudin-2 and claudin-19 which served as control claudins. While claudin-2, which is expressed primarily in the tight junction of the proximal tubule [5], is not expected to be influenced by ambient Mg^{2+} , it is interesting that Mg^{2+} availability did not affect claudin-19 mRNA level as well. Like claudin-16, claudin-19 is expressed in the tight junction of the TAL [19]. Recent evidence suggests that claudin-16 and claudin-19 interact and that their association confers a tight junction with cation selectivity, the impairment of which results in aberrant Mg^{2+} and Ca^{2+} reabsorption [19, 26]. Mutations in CLDN19 result in FHHNC syndrome [27]. Nevertheless, the data presented here indicates a claudin-16 mRNA - specific response to changes in Mg^{2+} level. The absence of Mg^{2+} - induced effect on claudin 19 mRNA, as opposed to the change in claudin 16 mRNA, could be related to difference in expression levels of these two interacting tight junction proteins (with claudin 16 being possibly less abundant and therefore limiting in the Mg^{2+} - induced response), to a Mg^{2+} induced regulation of claudin 19 occurring at a level beyond transcription or to other factors. The *in vivo* findings on CLDN16 mRNA level (Fig. 3) together with the *in vitro* results in transfected renal cells (Fig. 4 and 5) provide, for the first time, evidence that claudin-16, which appears to play a major role in the renal adaptive response to changes in Mg^{2+} concentration, is regulated by Mg^{2+} availability at the level of gene transcription.

Mg^{2+} depletion markedly increased and Mg^{2+} load decreased endogenous claudin-16 mRNA levels in CaSR-transfected HEK293 cells when compared with native non CaSR - expressing HEK293 cells (Fig. 4). The CaSR, although present in several nephron segments, is most

abundant in the TAL [28], where claudin-16 expression is also maximal. Increasing evidence suggests that the CaSR, which belongs to the family of G protein-coupled receptors, plays an important role in controlling divalent cation reabsorption in the renal tubule in health and disease [22, 28, 29]. Activation of the CaSR by high concentrations of extracellular Ca^{2+} or Mg^{2+} triggers a series of intracellular signaling events that alter electrolyte transport in the TAL. This, in turn, affects the transepithelial electrical potential in this nephron segment resulting in increased urinary Mg^{2+} and Ca^{2+} excretion [28].

Two recent studies [30, 31] have provided evidence for the involvement of CaSR in claudin 16 - mediated Mg^{2+} transport in renal cells. Ikari et al [30] suggest that CaSR activation leads to PKA inhibition, decrease in phosphorylated claudin 16 protein and a resulting translocation of claudin 16 to the lysosome which, in turn, leads to inhibition of Mg^{2+} transport. Günzel and coworkers [31] postulate that activation of CaSR triggers an increase in intracellular Ca^{2+} concentration which activates Ca^{2+} - sensitive apical Cl^- channels. The authors suggest that interaction of claudin 16 with these channels modulates transcellular Cl^- secretion thereby affecting paracellular Mg^{2+} transport.

The CaSR - dependent augmentation of the ambient Mg^{2+} - induced effect on CLDN16 mRNA level demonstrated in our study (Fig. 4A) suggests that the CaSR and signaling pathways activated by this receptor may be involved in the Mg^{2+} - induced adaptive response at the transcriptional level. This CaSR- dependent effect was observed on CLDN16 mRNA but not on TRPM6 mRNA (Fig. 4B), which served as a control mRNA.

An inverse correlation between hCLDN16 promoter activity and extracellular Mg^{2+} concentration was demonstrated in our *in vitro* experiments using transfected renal HEK293 cells (Fig. 5). The effect of low Mg^{2+} levels on the hCLDN16 promoter was more pronounced than that of high Mg^{2+} , a finding which is in concert with the changes in claudin 16 mRNA levels *in vivo* (Fig. 3) and *in vitro* (Fig. 4). Nevertheless, the inhibitory effect of high Mg^{2+} on the claudin-16 gene demonstrated in our previous [9] and present (Figs. 3, 4, 5) studies, which differs from the previously reported lack of effect of Mg^{2+} load on the TRPM6 gene in the kidney [13], further underscores the important role of claudin-16 in the renal adaptive response to Mg^{2+} availability which is achieved via transcriptional regulation of CLDN16.

As indicated above, this transcriptional regulation may be mediated by the CaSR. What are the molecular

mechanisms involved in this CaSR-induced effect? Signalling through CaSR is known to affect steady state intracellular calcium concentrations, rapid changes in cytosolic calcium levels and variations in both the frequency and amplitude of oscillations in intracellular calcium concentrations [32-34]. These mechanisms have been suggested to mediate regulation of gene expression by CaSR [32] and may play a role in CaSR - induced claudin 16 gene regulation as well. Nevertheless, future studies are needed to determine the precise molecular mechanisms mediating this action of CaSR and whether this effect primarily occurs at the CLDN16 DNA or RNA level.

The ability of native HEK293 cells to show changes in CLDN16 promoter activity dependent on extracellular Mg^{2+} level (Fig. 5), despite their inherent lack of CaSR, may suggest the existence of an alternative Mg^{2+} sensing mechanism. Several hormones, including parathyroid hormone, glucagon, calcitonin, insulin, and vitamin D are known to influence Mg^{2+} reabsorption in the TAL and DCT [35] and, thus, could potentially serve as mediators in the renal adaptive response to Mg^{2+} availability. Previously [9], we showed that $1,25(OH)_2$ vitamin D modulates hCLDN16 promoter activity in OK cells. However, the observation in the present study that extracellular Mg^{2+} affects hCLDN16 promoter activity in a renal cell line (Fig. 5) in the absence of externally added hormones in the growth media indicates that Mg^{2+} availability can exert its influence on claudin-16 gene transcription and, perhaps on tubular Mg^{2+} reabsorption without mediation of hormones.

Finally, the possibility exists that Mg^{2+} availability influences intracellular Mg^{2+} concentrations, which, in turn, affect CLDN16 transcription via hypothetical Mg^{2+} response elements located on the CLDN16 promoter. The existence of such cis-acting elements and their exact mode of action warrant future research.

The data in this study demonstrate adaptive changes in claudin-16 gene activation but do not exclude the possibility of Mg^{2+} - concentration dependent modulation of claudin-16 activity at the translational or post-translational level as well. Nevertheless, the techniques applied for mRNA and protein determination in our study do not allow precise evaluation of the differential contribution of

transcription and translation of the claudin-16 gene to the observed adaptive response. Noteworthy is a recent study [36] demonstrating that extracellular Mg^{2+} - dependent tight junction localization of claudin16 in Madin-Darby canine kidney (MDCK) cells was mediated by ERK - dependent phosphorylation.

In summary, changes in Mg^{2+} availability influence Mg^{2+} transport in the renal tubule by regulating transcription of the claudin-16 gene. The possible involvement of the tubular cell membrane bound Ca^{2+}/Mg^{2+} sensing receptor or the potential role of a hypothetical Mg^{2+} response element on the CLDN16 promoter in the Mg^{2+} -induced effect on transcription of this gene remain to be explored.

Our findings provide evidence that aside from fine regulation of transcellular Mg^{2+} transport achieved via modulation of TRPM6 activity in the DCT [11, 13, 37], significant claudin-16 mediated regulation of paracellular Mg^{2+} reabsorption occurs in the TAL, where the bulk of the filtered Mg^{2+} is reclaimed, leading to marked adaptive variations in urinary Mg^{2+} excretion. Taken together, our findings show that the renal adaptive response to changes in Mg^{2+} availability occurs along the entire pathway of claudin-16 production: from changes in the gene encoding claudin-16, as evident from changes in activity of its promoter, changes at the mRNA level, and, finally, changes at the claudin-16 protein level. The relative contribution of each of these changes to the renal adaptive response to Mg^{2+} availability is a subject for future research.

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