Familial hypomagnesemia with hypercalciuria and nephrocalcinosis: blocking endocytosis restores surface expression of a novel Claudin-16 mutant that lacks the entire C-terminal cytosolic tail

Dominik Müller1,‡, P. Jaya Kausalya2,‡, Iwan C. Meij3 and Walter Hunziker2,*

1Department of Pediatric Nephrology and Center for Cardiovascular Research, Charité, Berlin, Germany, 2Epithelial Cell Biology Laboratory, Institute of Molecular and Cell Biology, Singapore and 3Max-Delbrück-Center of Molecular Medicine, Berlin, Germany

Received November 8, 2005; Revised January 6, 2006; Accepted February 3, 2006

Mutations in the gene for Claudin-16 (CLDN16) are linked to familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC), a renal Mg2+ and Ca2+ wasting disorder that leads to progressive kidney failure. More than 20 mutations have been identified in CLDN16, which, with a single exception, affect one of two extracellular loops or one of four transmembrane domains of the encoded protein. Here, we describe a novel missense mutation, Cldn16 L203X, which deletes the entire C-terminal cytosolic domain of the protein. Surface expression of Cldn16 L203X is strongly reduced and the protein is instead found in the endoplasmic reticulum (ER) and lysosomes. ER-retained Cldn16 L203X is subject to proteasomal degradation. Cldn16 L203X present in lysosomes reaches this compartment following transport to the plasma membrane and endocytosis. Blocking clathrin-mediated endocytosis increases surface expression of Cldn16 L203X. Thus, endocytosis inhibitors may provide a novel therapeutic approach for FHHNC patients carrying particular Cldn16 mutations.

INTRODUCTION

Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) (OMIM 248250) is a progressive renal disorder characterized by excessive urinary Ca2+ and Mg2+ excretion. FHHNC leads to the progressive loss of kidney function, and in about 50% of cases, the need for renal replacement therapy arises already in the second decade of life (1,2). Mutations in the Claudin-16 gene (CLDN16), previously known as paracellin-1, have been linked to FHHNC (3,4).

Members of the claudin protein family are important constituents of the intercellular tight junction (TJ) barrier in various epithelia (5). Cldn16 spans the plasma membrane four times, with its N- and C-termini located in the cytosol (Fig. 1B). A C-terminal PDZ-binding motif interacts with PDZ domains of the ZO-1 scaffolding protein. The two extracellular loops mediate homo- and/or heterotypic interactions with claudins on neighboring cells (6). Claudins function as paracellular ion channels that either facilitate or restrict the paracellular diffusion of selective ions (7,8). Expression of Cldn16 is restricted to the kidney, in particular the thick ascending limb of the Henle’s loop (4), where the bulk of Mg2+ reabsorption occurs. The association of mutations in CLDN16 with FHHNC, its restricted spatial renal expression and its ability to increase the paracellular diffusion of Mg2+ (9) suggest that Cldn16 functions as a paracellular channel that facilitates the reabsorption of Mg2+ and possibly Ca2+.

A recent study shows that Cldn16 expression increases the Na+ permeability of TJs, indicating that Cldn16 helps to maintain the electrochemical gradient that drives Mg2+ reabsorption (10).

To date, over 20 different mutations in CLDN16 have been linked to FHHNC (1,2,4,11) (Fig. 1). With one exception, these mutations affect either one of four transmembrane...
Figure 1. Identification of the L203X mutation and its effect on the Cldn16 protein. (A) Sequencing data for the region in the CLDN16 gene carrying the L203X mutation. Sequences in the chromatogram read from 5' (left) to 3' (right) and the homozygous mutation in the FHHNC patient is indicated with an asterisk (*). The corresponding amino acid sequence is shown in the one-letter code above the chromatogram. (B) Predicted topology of Cldn16. Amino acid sequence is shown in the one-letter code, with the mutated residues identified in yellow and the arrows indicating the change introduced by the different mutations. The L203X mutation described in this study results in the deletion of the entire cytosolic tail of Cldn16 and is shown in red. X, stop codon; fs, frame shift. The peptide used to generate the anti-loop antibody is shaded in gray.
domains or one of two extracellular loops of the molecule. Only one mutation (T233R) targeting the cytosolic domain of Cldn16 (Fig. 1B) has been described to date. This mutation, associated with a self-limiting form of childhood HC, inactivates the PDZ-binding motif in Cldn16, abolishes binding of Cldn16 to ZO-1 and results in lysosomal mislocalization of the protein (11).

Here, we describe a novel homozygous mutation in the CLDN16 gene. This mutation introduces a premature stop codon that deletes the entire C-terminal cytosolic tail in the resulting mutant protein (Cldn16 L203X). When expressed in tissue culture cells, surface expression of Cldn16 L203X is strongly reduced and it is instead found in the endoplasmic reticulum (ER) and lysosomes. ER-retained Cldn16 is subject to proteasomal degradation. Cldn16 L203X found in lysosomes is first delivered to the cell surface, from where it is endocytosed and delivered to lysosomes. Blocking clathrin-mediated endocytosis restores cell surface expression of Cldn16 L203X, providing a possible therapeutic strategy for patients carrying this particular mutation, or mutations with a similar molecular phenotype.

RESULTS

Case report

The patient was the only child of healthy Iranian first-degree parents. Pregnancy and delivery were uneventful and the child was born in the 39th week of gestation with a weight of 3050 g and a height of 50 cm. Development was normal until the ninth month of life, when recidivant urinary tract infections started. Ultrasound analysis at the age of 2.5 years showed medullar nephrocalcinosis. Further biochemical analysis revealed low serum Mg2+ (0.52 mmol/l), normal serum Ca2+ (2.44 mmol/l) and acidosis (HCO3− 9.4 mmol/l). Urinary excretion of Mg2+ and Ca2+ were markedly enhanced (1.2 mmol/kg bodyweight/24 h and 1.88 mmol/kg bodyweight/24 h, respectively). Glomerular filtration rate as estimated by a 24 h creatinine clearance test was already diminished to 62 ml/min per 1.73 m2 body surface area (norm values 80–120).

Identification of mutations in the CLDN16 gene

A genetic screen using primers covering all four exons as well as 25 bp of the exon–intron boundaries of the CLDN16 gene of the patient was performed as described (11). The mutational analysis revealed a homozygous single base pair change of an adenine to a thymidine (AAA → TAA) at position 822 in the open-reading frame (Fig. 1A). Consequently, a lysine at position 203 in the protein encoded by the affected nucleotide triplet was replaced by a premature stop codon. Hence, the resulting mutant Cldn16 L203X protein lacks the entire C-terminal cytosolic domain (Fig. 1B).

Cldn16 L203X is not detected at TJ and surface expression is strongly reduced in transfected MDCK cells

The subcellular localization of Cldn16 L203X when compared with wild-type (wt) Cldn16 was characterized in renal epithelial MDCK cells. Hemagglutinin (HA)-tagged Cldn16 and the Cldn16 L203X mutant were expressed in MDCK cells and the steady-state localization of the two proteins analyzed by immunofluorescence microscopy. As previously shown (11), tagged Cldn16 was detected on the plasma membrane of MDCK cells where it was present at sites of cell–cell contact and extensively colocalized with the TJ marker ZO-1 (Fig. 2A–C). In contrast, Cldn16 L203X showed a predominant intracellular localization and did not colocalize with ZO-1 (Fig. 2D–F).

Cldn16 L203X predominantly localizes to the ER and lysosomes

To identify the cellular compartment(s) to which Cldn16 L203X localizes at steady state, we carried out colocalization experiments with markers for the ER (calreticulin) (12), the Golgi complex (GM130) (13) and lysosomes (CD63) (14) or (Lamp-2) (15). In addition to renal epithelial MDCK cells with a well-established capacity to polarize and form TJ, we used HeLa cervical carcinoma cells. HeLa cells are less polarized and only express low levels of the TJ protein ZO-1 (data not shown), known to interact with Cldn16 (11). In MDCK cells, Cldn16 was present at sites of cell–cell contact and showed little, if any, colocalization with calreticulin (Fig. 3A–C), GM130 (Table 1) or Lamp-2 (Fig. 3M–O). In contrast, Cldn16 showed a predominant intracellular localization in HeLa cells (Fig. 3G and S), suggesting that in these cells Cldn16 is not incorporated into TJs. Like in MDCK cells, Cldn16 in HeLa cells showed little colocalization with calreticulin (Fig. 3I), GM130 (Table 1) or CD63 (Fig. 3S–U), indicating that its intracellular localization in this cell line did not reflect a defect in biosynthetic transport or its delivery to lysosomes. Rather, Cldn16 showed extensive colocalization with EEA1, indicating that in HeLa cells it largely resides in an early endosomal compartment (data not shown). In contrast, Cldn16 L203X showed a predominant intracellular localization in...
both MDCK and HeLa cells, where it was present in the ER (Fig. 3D–F and J–L) and lysosomes (Fig. 3P–R and V–X) but only showed poor colocalization with GM130 (Table 1). A quantification of the extent of colocalization of Cldn16 and Cldn16 L203X with calreticulin, GM130 and CD63/Lamp-2 in HeLa and MDCK cells is shown in Table 1. Thus, at steady state, significant fractions of Cldn16 L203X are retained in the ER or delivered to lysosomes.

**ER-retained Cldn16 L203X undergoes proteasomal degradation**

We next determined whether ER-retained Cldn16 L203X is targeted for proteasomal degradation by the ER quality control machinery. MDCK cells expressing Cldn16 or Cldn16 L203X were incubated in the absence or presence of the proteasome inhibitor N-acetyl-leu-leu-norleucinal (ALLN) (16) for 10 h and then stained for Cldn16 and ubiquitin (Fig. 4A). Little ubiquitin staining was observed in cells expressing Cldn16, it did not colocalize with Cldn16 and it was not increased by the proteasome inhibitor ALLN (data not shown). In contrast, in cells expressing Cldn16 L203X, the detectable ubiquitin staining (Fig. 4Aa–c) was significantly increased in the presence of ALLN and extensively colocalized with the mutant Cldn16 protein (Fig. 4Ad–f).

This observation, suggesting that Cldn16 L203X is ubiquitinylated and degraded by the proteasome, was corroborated biochemically by testing whether inhibiting proteasomal activity results in the stabilization of Cldn16 L203X. HEK-293T cells transiently expressing wt or mutant Cldn16 were treated with cycloheximide to inhibit de novo protein synthesis (17) and the proteasome inhibitor ALLN, and Cldn16 protein levels were monitored every 2 h by western blot analysis. The turnover of Cldn16 was not greatly affected by ALLN, whereas the proteasome inhibitor led to a significant accumulation of Cldn16 L203X (Fig. 4B).

Taken together, the above results thus indicate that under normal conditions the ER-retained Cldn16 L203X undergoes proteasomal degradation.

**Cldn16 L203X is delivered to lysosomes following transport to the cell surface and endocytosis**

Lysoosomal delivery of integral membrane proteins can occur either from the Golgi complex via endosomes or following...
delivery to the plasma membrane and internalization (18). The steady-state localization of Cldn16 L203X to lysosomes therefore raised the question of whether lysosomal delivery involved transit through the plasma membrane or not.

To address this question, live MDCK or HeLa cells expressing Cldn16 or Cldn16 L203X were incubated at 37°C with antibodies against the first extracellular loop of Cldn16 (Fig. 1B). If Cldn16 is transiently exposed on the cell surface, these antibodies are expected to bind to Cldn16 and to be internalized. MDCK and HeLa cells expressing either Cldn16 (Fig. 5A–C and G–I) or Cldn16 L203X (Fig. 5D–F and J–L) internalized anti-loop antibodies. As expected, the internalized anti-loop antibodies partially colocalized with Cldn16 or Cldn16 L203X detected with anti-tag antibodies, probably representing a population of Cldn16 molecules that had been exposed on the cell surface and was able to bind anti-loop antibody present in the media. These data strongly suggest that Cldn16 L203X is transiently exposed on the cell surface prior to being delivered to lysosomes.

The internalization of anti-loop antibodies by MDCK cells expressing Cldn16 L203X was confirmed biochemically. Cells were allowed to endocytose anti-loop antibodies at 37°C for 60 min, fixed and either permeabilized or not prior to the incubation with horseradish peroxidase (HRP)-coupled secondary antibodies. Cell-associated HRP activity was then determined in permeabilized and non-permeabilized cells, allowing to estimate the fraction of the total cell associated anti-loop antibody that either remained on the cell surface or had been internalized during the 60 min incubation. As shown in Figure 6, the bulk of the anti-loop antibody remained on the surface of cells expressing Cldn16, with only a small fraction being internalized. In contrast, the fraction of anti-loop antibody associated with the surface of cells expressing Cldn16 L203X was dramatically decreased and a corresponding increase in the intracellular fraction was observed. For comparison, a slightly larger fraction of the anti-loop antibody remained on the surface of cells expressing the previously characterized Cldn16 T233R mutant (11).

To determine the nature of the vesicular compartment the anti-loop antibodies were delivered to following their internalization, we carried out colocalization experiments with markers for endosomes (EEA1) or lysosomes (CD63 or Lamp-2). Anti-loop antibodies internalized via either Cldn16 or Cldn16 L203X could be detected in EEA1-positive endosomes (data not shown), whereas only those internalized via Cldn16 L203X were found in lysosomes (Fig. 5V–X).

Taken together, these data indicate that Cldn16 L203X transits through the plasma membrane en route to lysosomes.

**Blocking clathrin-mediated endocytosis restores surface expression of Cldn16 L203X**

If Cldn16 L203X is delivered to the plasma membrane prior to being transferred to lysosomes, we predicted that blocking

---

**Table 1.** Quantification of the colocalization of Cldn16 or Cldn16 L203X with calreticulin (ER), GM130 (Golgi) or Lamp-2/CD63 (lysosomes) in HeLa and MDCK cells

<table>
<thead>
<tr>
<th></th>
<th>Colocalization pixels/ROI</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ER</td>
<td>Golgi</td>
<td>Lysosome</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cldn16</td>
<td>32.05 ± 6.25%</td>
<td>1.62 ± 0.67%</td>
<td>2.69 ± 1.3%</td>
<td></td>
</tr>
<tr>
<td>L203X</td>
<td>76.25 ± 10.35%</td>
<td>3.86 ± 0.42%</td>
<td>11.37 ± 2.55%</td>
<td></td>
</tr>
<tr>
<td>MDCK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cldn16</td>
<td>19.56 ± 1.96%</td>
<td>8.42 ± 0.67%</td>
<td>5.69 ± 1.12%</td>
<td></td>
</tr>
<tr>
<td>L203X</td>
<td>75.14 ± 6.40%</td>
<td>7.65 ± 2.43%</td>
<td>22.74 ± 5.2%</td>
<td></td>
</tr>
</tbody>
</table>

Shown are the percent colocalizing pixels in randomly selected transfected cells (n = 5–8). Individual cells were defined as ROI.
endocytosis might lead to the accumulation of the protein on the cell surface. To test this hypothesis, clathrin-mediated endocytosis in MDCK and HeLa cells expressing Cldn16 or Cldn16 L203X was inhibited using a cytosol acidification protocol (19). The cells were then incubated at 37°C in the absence of anti-loop antibody, and the subcellular localization of Cldn16 and the anti-loop antibody was determined by immunofluorescence microscopy.

In HeLa cells, inhibition of endocytosis led to a dramatic redistribution of the Cldn16 labeling from a vesicular to a plasma membrane staining pattern (Fig. 7G–I), consistent with the notion that in HeLa cells Cldn16 not retained at the cell surface is internalized. Although the plasma membrane localization of Cldn16 was not significantly altered in MDCK cells (Fig. 7A–C), inhibition of endocytosis resulted in a significant accumulation of Cldn16 L203X at the cell surface (Fig. 7D–F). Similarly, Cldn16 L203X was readily detected on the cell surface in HeLa cells upon blocking endocytosis (Fig. 7J–L).

Internalization of the anti-loop antibody was also strongly impaired in HeLa cells expressing Cldn16 (Fig. 7G–I) and in MDCK and HeLa cells expressing Cldn16 L203X (Fig. 7D–F and J–L), resulting in its accumulation on the cell surface (Fig. 7E, H and K). Increased surface accumulation of anti-loop antibodies in response to the inhibition of endocytosis in MDCK cells expressing Cldn16 L203X was furthermore confirmed using the biochemical assay described earlier (Fig. 6).

These data thus confirm that Cldn16 L203X is delivered to the cell surface before being endocytosed and transported to lysosomes and show that surface expression of the mutant can be significantly restored if endocytosis is blocked.

**DISCUSSION**

FHHNC is caused by mutations that interfere with the ability of Cldn16 to mediate the paracellular reabsorption
of Mg$^{2+}$ in the thick ascending limb of the loop of Henle. More than 20 different mutations in CLDN16 have been identified in patients suffering from this disease. With the exception of Cldn16 T233R, all the mutations target either the transmembrane domains or the extracellular loops of the protein. The molecular mechanism by which these mutations affect Cldn16 function has been of recent interest (10,11) but a genotype–phenotype correlation has not been established.

In a previous study (11), we described the first mutation (Cldn16 T233R) to affect the C-terminal cytosolic tail of Cldn16. This mutation, found in four members of two unrelated families, inactivates the PDZ-binding motif. This abolishes the interaction of Cldn16 with ZO-1 and leads to its lysosomal mislocalization. Interestingly, independent of family origin, all patients carrying the T233R mutation presented with a mild, self-limiting form of FHHNC. Here, we described and characterized a novel mutation, L203X, which results in the complete deletion of the cytosolic domain of Cldn16. At steady state, little Cldn16 L203X is detected on the cell surface and the protein instead localizes to the ER and lysosomes. ER accumulation indicates that, in the absence of the cytosolic tail, folding of Cldn16 L203X is impaired or occurs with slower kinetics when compared with either Cldn16 or Cldn16 T233R, which is not detected in the ER at steady state. Alternatively, efficient ER-exit of Cldn16 could require the binding of a cytosolic protein to the C-terminus or homo-oligomerization mediated by the cytosolic domain. The inhibition of Cldn16 L203X turnover and its increased colocalization with ubiquitin in the presence of ALLN indicate that ER-retained Cldn16 L203X is subject to degradation by the proteasome.

Interestingly, a significant fraction of Cldn16 L203X was apparently able to exit the ER and be delivered to lysosomes. Cells expressing Cldn16 L203X bound and internalized antibodies raised against the first extracellular loop, suggesting that, despite its minimal cell surface expression at steady state, Cldn16 L203X is transiently exposed on the cell surface. This finding is further supported by the observation that blocking endocytosis results in cell surface accumulation of both Cldn16 L203X and bound anti-loop antibodies. These data also indicate that Cldn16 undergoes clathrin-mediated endocytosis. In the absence of the cytosolic tail, which encodes several putative endocytosis signals, internalization of Cldn16 L203X is probably mediated by determinants in the N-terminal cytosolic domain or the intracellular loop. Indeed, the sequence YIKV in the intracellular loop is reminiscent of clathrin-mediated endocytosis signals.
In contrast to MDCK cells, only little Cldn16 was detected on the cell surface of HeLa cells, where the protein was detected in early endosomes but absent from lysosomes. In MDCK cells, Cldn16 may be efficiently retained at the plasma membrane through its interaction with ZO-1 (11) and tethering to the actin cytoskeleton. In HeLa cells, which are poorly polarized when compared with MDCK cells and only express small amounts of ZO-1 (data not shown), Cldn16 may not be retained at the plasma membrane and hence be internalized. This interpretation is consistent with the finding that the association between Cldn16 and ZO-1 increases the reabsorption of divalent cations in renal epithelial cells (9). As Cldn16 L203X and Cldn16 T233R no longer interact with ZO-1, they may be internalized more efficiently than Cldn16 and hence more likely to be diverted into the lysosomal pathway. Alternatively, the absence of the cytosolic domain could affect post-endocytic sorting, for example, by diverting protein from recycling into the lysosomal pathway. Binding of ZO-1 or another PDZ-domain protein could also prevent sorting from a post-endocytic compartment into the lysosomal pathway. Indeed, binding of ZO-1 to connexins has been correlated with a longer half-life of gap junction proteins (20,21).

Interestingly, Cldn16 T233R and Cldn16 L203X show reduced cell surface expression, they no longer interact with ZO-1 and they are delivered to lysosomes. Yet, patients carrying the L203X mutation present with classical FHHNC, whereas those with the T233R mutation suffer from a mild self-limiting form of the disease. At least two possible differences between the two mutants could account for the different clinical phenotypes observed. First, because a portion of Cldn16 L203X but not Cldn16 T233R (data not shown) is retained in the ER and degraded, a significant fraction of Cldn16 L203X molecules will either never reach the plasma membrane and/or do so with slower kinetics. Second, of those molecules that do reach the cell surface (as determined by their ability to bind anti-loop antibodies added to the media for 60 min), a larger fraction of Cldn16 T233R in relation to Cldn16 T203X is detected on the cell surface. This indicates differences in the rates of endocytosis, lysosomal transport and/or recycling between the two mutants, which may be determined by cytosolic signals that are intact in Cldn16 T233R but may be deleted in Cldn16 L203X. For example, although both mutants no longer bind ZO-1, Cldn16 T233R may be recycled to the cell surface more efficiently than Cldn16 L203X before eventually ending up in lysosomes.

A more efficient biosynthetic surface transport combined with differences in endocytic trafficking may result in more Cldn16 T233R transiting through the cell surface when compared with Cldn16 L203X. Such a difference may be sufficient to generate the different clinical phenotypes if the mutants transiently present on the cell surface can restore some degree of functionality. In addition, however, deletion of the complete C-terminal tail in Cldn16 L203X could affect the paracellular transport capacity of this mutant. The fact that a fraction of Cldn16 L203X is retained in the ER and subject to proteasomal degradation suggests that the folding kinetics and/or the structure of this mutant are affected. An alternative possibility is that binding of factors other than ZO-1 to the C-terminal tail of Cldn16 is required for paracellular transport function. Direct measurements of Mg$^{2+}$ transport for the different mutants will be required to experimentally address these issues.

The ability to correlate particular mutations with effects on Cldn16 function or trafficking and clinical phenotypes is of great relevance in terms of both disease prognosis and therapeutic intervention. The observation that inhibitors of clathrin-mediated endocytosis rescue surface expression of Cldn16 L203X suggests novel therapeutic approaches for patients suffering from FHHNC associated with particular mutations, provided these mutant proteins retain paracellular transport capacity. Our findings highlight the importance of characterizing the molecular defect(s) associated with other disease-causing mutations in CLDN16.

MATERIALS AND METHODS

Subjects and mutational analysis

Informed consent for the studies was obtained from the parents of the patient in accordance with the local guidelines. Mutational analysis of the CLDN16 gene was carried out as described (11).

Plasmids and cDNAs

The isolation of a full-length human Cldn16 cDNA and the addition of an N-terminal HA tag have been described (11). The L203X mutation was introduced by PCR using suitable overlapping primers. The cDNAs were cloned into the pcDNA3 expression vector (Invitrogen) and verified by sequencing.

Antibodies and chemicals

Rabbit antibodies to ZO-1 (Zymed), GM130 (BD Pharmingen), calreticulin (ABR), rat monoclonal antibodies to HA (Roche) and mouse monoclonal antibodies to CD63 (Developmental Studies Hybridoma Bank), Lamp-2 (22) and ubiquitin (Santa Cruz) were used. An affinity purified rabbit polyclonal anti-loop antibody to the first extracellular loop of Cldn16 (amino acids 52–66) was generated (Biogenes) and will be characterized elsewhere. Unless otherwise noted, all chemicals were from Sigma. Stock solutions of ALLN (Calbiochem, 26 mM in DMSO) and cycloheximide (2 mg/ml in H$_2$O) were stored at −20°C.

Cell culture and transfection

MDCK, HEK-293T and HeLa cells were cultured and stably (HeLa) or transiently (HeLa, HEK-293T and MDCK cells) transfected with cDNAs as described (11,23,24). Cells expressing similar levels of the different Cldn16 constructs were used for experiments 24 h after transient transfection. Similar steady-state distributions for the different Cldn16 constructs were observed for stably or transiently transfected HeLa cells, and results of transiently transfected cells are shown.
**Immunofluorescence labeling**

Cells were grown on cover slips and processed for immunofluorescence microscopy as described (11,23,24). Cells were stained with antibodies to HA (1:100), calreticulin (1:300), GM130 (1:100), CD63 (1:300), Lamp-2 (1:20) or ubiquitin (1:100) and suitable fluorescently labeled secondary antibodies (Molecular Probes) and visualized by confocal scanning microscopy (MRC1024 BioRad) using 10% laser power and 1400 and 1200 gains in PMT1 (red) and PMT2 (green), respectively. Single confocal sections are shown. For quantification, randomly selected individual cells (n = 5–8) were defined as region of interest (ROI), and the colocalization coefficient values based on Pearson’s correlation coefficient and percentage of pixel colocalization in ROI were calculated using Lasersharp 2000 software (BioRad).

**Monitoring of cell surface expression and endocytosis of Cldn16**

HeLa or MDCK cells expressing Cldn16 or Cldn16 L203X were incubated with the anti-loop antibody for 1 h at 37°C using a dilution that did not stain control MDCK cells. The cells were then washed in ice-cold phosphate-buffered saline (PBS) containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$, fixed, permeabilized and stained with labeled secondary antibodies. Pre-incubation of the anti-loop antibody with a 100-fold molar excess of the peptide used for immunization completely blocked the staining (data not shown).

**Biochemical analysis of the distribution of internalized anti-loop antibodies**

MDCK cells expressing Cldn16, Cldn16 L203X or Cldn16 T233R (11) were allowed to internalize anti-loop antibodies for 60 min at 37°C in the presence of normal or cytosol acidification media. The anti-loop antibody was used at a dilution that selectively stained transfected MDCK cells when compared with untransfected control cells. Cells were subsequently washed with ice-cold PBS containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$ and either fixed (2% PFA; 30 min) or fixed and permeabilized (0.2% TX-100; 10 min). Cells were incubated with blocking media (1% BSA in PBS) for 30 min, followed by HRP-conjugated secondary antibodies (BioRad; 1:400) for 60 min. After washing, HRP enzymatic activity was determined by adding HRP assay solution [50 mg O-phenylenediamine dihydrochloride (Sigma), 50 μM 30% H$_2$O$_2$ (Merck) in 50 ml PBS, pH 6.0] for 10 min at room temperature. The reaction was stopped by the addition of 5 μl of 6 N HCl and the absorbance measured at 490 nm using a BioRad Model 680 microplate reader.

**Inhibition of proteasomal degradation and endocytosis**

To inhibit proteasomal activity, transfected MDCK or HEK 293T cells were incubated in the presence of 100 μM ALLN for the periods indicated. In some experiments, cycloheximide (20 μg/ml) was added to block de novo protein synthesis. Cells were then processed for immunofluorescence microscopy as described above (MDCK or HeLa cells) or harvested for western blot analysis (HEK 293T cells). Clathrin-mediated internalization was blocked by incubating transiently transfected MDCK or HeLa cells for 1 h in the presence of anti-loop antibody and cytosol acidification media as described (19). Cells were then washed and processed for immunofluorescence microscopy as described above.

**Western blot analysis**

Cells were harvested, lysed and 50–100 μg of protein lysate was analyzed by SDS–PAGE and western blot as described (11,23,24) using antibodies to HA (1:1000) and actin (1:2500).

**ACKNOWLEDGEMENTS**

We thank the family and the patient for their cooperation. This work was supported by the Agency for Science, Technology and Research, Singapore, and by the EU FP6 founded Consortium EuReGene (FP6005085).

**Conflict of Interest statement.** None declared.

**REFERENCES**


