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The Ubiquitin–Proteasome Pathway Regulates Claudin 5 Degradation

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ABSTRACT

The tight junctions (TJs) form continuous intracellular contacts, which help create selective barriers in epithelial and endothelial cell layers. The structures created by the TJs are very dynamic and can be rapidly remodeled in response to physiological and pathological signals. Claudin 5 is a membranal TJ protein which plays a critical role in determining the permeability of endothelial barriers. We describe the regulation of claudin 5 degradation by the ubiquitin–proteasome system (UPS). Our results indicate that claudin 5 has a relatively short half-life and can be polyubiquitinated on lysine 199. This ubiquitination appears to trigger the proteasome-dependent degradation of claudin 5. Other mechanisms also seem to be involved in the post-translational regulation of claudin 5, including a ubiquitin-independent and probably indirect lysosomal-dependent pathway. These findings provide evidence for the involvement of the UPS in the regulation of claudin 5 levels, and set the stage for further research to determine the involvement of this pathway in the modulation of the properties of TJs and cell-layer barriers. J. Cell. Biochem. 113: 2415–2423, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: CLAUDIN; TIGHT JUNCTIONS; PROTEASOME; UBIQUITIN

The tight junction (TJ) complex controls the permeability and polarity of specialized endothelial and epithelial cell layers. TJs between adjacent cells form a continuous network of strands that serve as barriers to the paracellular transport of ions, solutes, and cells [Chiba et al., 2008] and coordinate a variety of signaling and trafficking molecules that regulate cell differentiation, proliferation, and polarity [Matter et al., 2005]. TJs are composed of transmembrane and cytoplasmic proteins linked to the cytoskeleton [Chiba et al., 2008]. They include the membranal TJ proteins (TJPs) occludin, claudins, and the junctional adhesion molecules (JAMs), as well as various scaffold proteins and cytoplasmic proteins such as the zonula occludin (ZO) proteins [Guillemot et al., 2008]. The unique composition of the TJ complexes in various endothelial and epithelial cells confers the properties of the cellular layers they compose [Angelow et al., 2008]. Accordingly, TJs between the brain endothelial cells (ECs) contribute to the unique properties of the blood brain barrier (BBB), a physical and metabolic barrier between the central nervous system and the circulation system, enabling regulation and protection of the brain microenvironment [Zlokovic, 2008].

One of the major TJP in endothelial cells, and specifically in the cells comprising the BBB, is Claudin 5 [Krause et al., 2008]. Knockout experiments demonstrated its key role in determining the size selectivity of the TJs in brain endothelia and viability [Nitta et al., 2003]. Despite its strict structural organization, the TJ complex is highly dynamic and can be rapidly disassembled in response to various extracellular stimuli in order to allow remodeling of intercellular junctions [Matsuda et al., 2004]. Claudin 5 levels and cellular localization were shown to be regulated through phosphorylation [Gonzalez-Mariscal et al., 2008], matrix metalloproteinases proteolysis [Yang et al., 2007], and by various cell-signaling factors such as VEGF [Argaw et al., 2009], TGF-β [Ronaldson et al., 2009], TNF-α, and glucocorticoids [Felinski et al., 2008; Forster et al., 2008; Mandel et al., 2011]. Claudin 5 is also expressed by white blood cells, however, its role in such non layer-forming cells remains ambiguous [Mandel et al., 2011]. The expression levels of claudin 5 were altered

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in the relapse state of multiple sclerosis, an immune-mediated disease, and modulated by glucocorticoid immune-suppressive therapy. Claudin 5 expression levels were recently shown to be regulated also through NF κB signaling, [Aslam et al., 2012] adding to evidence for its modulation by the immune system's activity. Some of the cell signaling factors affecting claudin 5 are known to exert their effects through ubiquitin/proteasome system (UPS)-mediated pathways [Bianchi and Meier, 2009; Murakami et al., 2009; Schakman et al., 2009]. The UPS marks proteins for degradation by attachment of ubiquitin to their lysine residues. Ubiquitin is conjugated in a series of reactions that involves the E1 activating enzymes, the E2 conjugating enzymes, and the E3 ubiquitin ligases, in a manner that is highly regulated and substrate specific, which can be reiterated to create chains of polyubiquitin attached to the target protein [Weissman et al., 2011]. Modification of the UPS activity can lead to changes in the cell cycle and apoptosis, and cytokines such as interferon α have been shown to exert their effects through modulation of expression levels of proteins involved in the UPS activity [Caraglia et al., 2005]. The UPS has been shown to regulate the fate of various membranal proteins. This regulation through mono or polyubiquitination can lead to protein endocytosis and degradation via the lysosome and/or the proteasome [Staub and Rotin, 2006; Weissman et al., 2011].

The reports that prompted this study on the role of the UPS in the regulation of claudin 5 levels included the demonstration that the TJP occludin can undergo ubiquitin-mediated proteasomal degradation [Traweger et al., 2002], and that ubiquitination of the TJPs claudin 1, claudin 2 and claudin 4 leads to lysosomal targeting [Takahashi et al., 2009]. Furthermore, the half-life of different claudin family members appears to be dependent on their cytoplasmic carboxyl-terminal domains [Van Itallie et al., 2004], and although the mechanism involved has not yet been elucidated, the conserved lysines in the C terminus of claudin 5 could serve as a target for ubiquitination and thus enable regulation by the UPS [Traweger et al., 2002].

Therefore, the present study aimed to evaluate the role of the UPS in the regulation of claudin 5. We demonstrate that claudin 5 has a short half-life and accumulates mainly in the intracellular compartment following proteasome inhibition. We also show that claudin 5 can be directly polyubiquitinated, primarily through lysine 199. Together, these results suggest a role for the UPS in the regulation of claudin 5.

MATERIALS AND METHODS

PLASMID CONSTRUCTS AND SITE DIRECTED MUTAGENESIS

CLDN5 c-DNA was amplified from human peripheral leukocytes mRNA, using a high fidelity Taq polymerase (Phusion; Finnzymes, Vantaa, Finland) and the primers 5′-cac cat ggg gtc cgc agc gtt g-3′ and 5′-cta gac gta gtt ctt ctt gtc gta g-3′. Amplified fragments were purified (PCR clean up system; Qiagen, Chatworth, CA) and inserted into pcDNA expression vectors using the pCDNA 3.1 Directional TOPO expression kit (Invitrogen, Grand Island, NY). Site directed mutations in *CLDN5*, in which lysines at amino acid positions 114, 199, 214 and 215 were substituted with arginine, were generated by the QuickChange PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, with the primers 5′-ccg ggc cct gcc agg gcg cgt gt-3′, 5′-aca cgc gcc ctg gca ggg ccc gg-3′, 5′-agc ttc ccc gtg agg tac tca gcg ccg-3′, 5′-cgg cgc tga gta cct cac ggg gaa gct-3′, 5′-ccg gcg act acg aca gga aga act acg gtc t-3′, 5′-aga ccg tag ttc ttc ctg tcg tag tcg ccg g-3′, 5′-ccg gcg act acg aca aga gga act acg gtc t-3′ and 5′-aga ccg tag ttc ctc ttg tcg tag tcg ccg g-3′. The triple mutation of K199R, K214R, and K215R was created by first creating a plasmid with both the K214R and the K215R mutations, using the primers 5′-ccg gcg act acg aca gga gga act acg gtc t-3′ and 5′-aga ccg tag ttc ctc ctg tcg tag tcg ccg g-3′, and then adding the K199R site. All plasmid constructs were sequenced for verification (Applied Biosystems, Foster City, CA).

CELL CULTURE AND TRANSFECTIONS

HeLa cells were cultured in DMEM medium containing 10% FCS, penicillin–streptomycin (100 U/ml) and L-glutamine (2 mM; all from Biological Industries, Beit HaEmek, Israel). Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as previously described and stored in liquid nitrogen till use [Ben-Yosef et al., 2005]. HUVEC were cultured in M199 medium containing 15% FCS, penicillin–streptomycin (100 U/ml), L-glutamine (4 mM; all from Biological Industries), endothelial cells growth supplement (ECGS; 30 μg/ml; Zotal, Tel Aviv, Israel) and Heparin (5 U/ml; Sigma-Aldrich, St. Louis, MO). Peripheral blood mononuclear cells (PBMCs) obtained from healthy volunteers were isolated from heparinized blood on a Ficoll–Hypaque gradient and cultured in RPMI-1640 medium containing 10% FCS, penicillin–streptomycin (100 U/ml) and L-glutamine (2 mM; all from Biological Industries). The study was approved by the Helsinki committee of Carmel Medical Center and all study participants signed an informed consent.

Plasmids containing the wild-type sequence of *CLDN5*, or the various lysine to arginine mutations, were purified by a midiprep kit (Qiagen) and transiently transfected into HeLa cells using X-tremeGENE siRNA Transfection Reagent (Roche, Basel, Switzerland) or JetPEI (Polyplus Transfection, Bioparc, France) according to the manufacturers' instructions. Following 24 h of transfection, the cells were detached using trypsin, pooled, and re-plated before receiving the appropriate treatment according to the experimental conditions. Transfection efficiency was 50–70% as determined by parallel control transfections of a pCDNA vector expressing Lac-Z (Invitrogen), and staining with X-gal and IPTG (USB Corporation, Cleveland, OH).

WESTERN BLOT ANALYSIS

Whole cell protein extracts were prepared by lysis in a HCMF buffer that contained 137 mM NaCl, 5.7 mM KCl, 0.34 mM Na_2PO_4, 5.6 mM glucose (all from Sigma-Aldrich), 10 mM HEPES (Biological Industries), and protease inhibitors ("complete" protease inhibitor tablets; Roche). Crude membrane fractions were prepared by homogenizing cell pellets in Tris acetate 10 mM pH 7.3 buffer containing protease inhibitors ("complete" protease inhibitor tablets; Roche). Following removal of the cell nuclei by sedimentation at 800*g* 10 min, the supernatant and membranal pellet were separated by centrifugation at 17,860*g* for 45 min. The supernatant

was collected as the "intracellular fraction," and the membranal pellet was homogenized in Tris acetate buffer.

Equal protein amounts from whole cell lysates, membranal or intracellular fractions (30 μg per lane, determined by Bradford protein quantification; BioRad Laboratories), or immunoprecipitation products were separated by 12% SDS–PAGE and transferred to nitrocellulose membranes for immunodetection. The membranes were subsequently incubated with the appropriate primary antibodies (mouse anti-claudin 5 1:500; Invitrogen, mouse anti-ubiquitin P4D1 1:250; Santa Cruz Biotechnologies, Santa Cruz, CA; mouse anti-polyubiquitinated conjugates FK1 1:200; Enzo Life Sciences International, Inc., Plymouth Meeting, PA) and the peroxidase-conjugated secondary antibody goat anti-mouse 1:2,500 (Jackson Immunoresearch, West Grove, PA), followed by ECL detection, according to standard protocols (Biological Industries). Quantification of relative expression levels was performed by densitometry analysis using TINA 2.09 software (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany). Tubulin (using mouse anti-α-tubulin 1:2,500; Sigma) served as an internal reference for protein loading.

IMMUNOPRECIPITATION ASSAYS

Antibodies (8 μg of rabbit anti-claudin 5; Invitrogen) were added to cell lysates that contained equal protein amounts (1 mg) and rotated for 2 h at 4°C, after which protein G agarose beads (Santa Cruz Biotechnologies) were added for further rotation at 4°C overnight. The beads were subsequently washed twice with IMS buffer containing 2% Triton X-100, 0.5% deoxycholate, 1% BSA (all from Sigma-Aldrich), and twice with PBS 0.1× at 6,600*g* for 2 min. Immune complexes were eluted from the beads by boiling in SDS sample buffer, and analyzed by Western blot analysis.

IMMUNOFLUORESCENCE ASSAYS

HeLa cells were grown on cover slips, fixed in 3.7% formaldehyde in PBS for 15 min and permeabilized by in 0.1% Triton X-100 for 5 min at room temperature. Cells were incubated in a blocking buffer that contained 1% BSA, 4% normal donkey serum in PBS, followed by incubation with mouse anti-claudin 5 (1:100 Invitrogen), rinsing with PBS, and incubation with rhodamine-conjugated donkey anti-mouse IgG (1:100; Jackson Immunoresearch). To detect intracellular localization of claudin 5 with endosomal markers, a double staining procedure was used with the addition of one of the following primary rabbit antibodies: anti-RAB5B and RAB7 (1:75, Santa Cruz Biotechnologies). Rabbit antibodies were detected by a Cy2-conjugated donkey anti-rabbit IgG (1:100, Jackson Immunoresearch). For labeling of nuclei TO-PRO-3 (Invitrogen) stains were employed. Cover slips were then mounted on slides with mounting media (Dako, Glostrup, Denmark) and visualized by a Bio-Rad Radiance 2000 confocal set-up hooked to an upright fluorescent microscope (Nikon E600). Colocalization coefficients for the immunostained proteins RAB5 and RAB7 with claudin 5 were calculated using the Zeiss LSM 510 META software, where a colocalization of 0 means no colocalization and 1 means that all pixels colocalized. The mean values were averaged from at least five independent images.

IN VIVO UBIQUITINATION ASSAYS

pCDNA-claudin 5 transfected HeLa cells were incubated in the presence or absence of the proteasome inhibitor MG-132 (20 μM, Calbiochem, San Diego, CA, dissolved in DMSO) for 6 h. The cells were subsequently lysed under strongly denaturing conditions that inhibit isopeptidases. Briefly, the cells were lysed after three rinses in PBS, in a HLB buffer containing 1% SDS, 1 mM EDTA and "complete" protease inhibitor tablets (Roche) in PBS, which was preheated to 37°C. The lysates were then boiled for 5 min, sheared by a syringe equipped with a 25-gauge needle, boiled again for 3 min, and centrifuged for 5 min at room temperature at 20,000*g*. The supernatant was diluted twofold in IMS buffer and "complete" protease inhibitor tablets in PBS. Claudin 5 was immunoprecipitated from the lysates as described above and immunoprecipitation products were analyzed by Western blot with anti-ubiquitin and anti-claudin 5 antibodies.

PROTEASOME AND LYSOSOME INHIBITION ASSAYS

pCDNA-claudin 5 transfected HeLa cells, HUVEC, or PBMC were treated with 20 μM of MG-132 for up to 6 h. For immunofluorescence assays, claudin 5 transfected HeLa cells were treated with MG-132 (20 μM) for 4.5 h prior to the addition of cycloheximide (100 μg/ml, Sigma) for 1.5 h. For lysosome inhibition assays, HeLa cells were treated with 100 μM of chloroquine or 100 μg/ml of leupeptin (Sigma) for up to 8 h. In both cases, the cells were harvested at 2-h time intervals for lysate preparation.

DETERMINATION OF PROTEIN HALF-LIFE

In order to determine protein half-life, cycloheximide (100 μg/ml, Sigma) was added to claudin 5 transfected HeLa cells or HUVEC with or without previous treatment of the cells with the proteasome inhibitor MG-132 (20 μM) for 6 h, followed by incubation for 2–3 h. The cells were harvested at 30-min time intervals.

STATISTICAL ANALYSIS

Statistical analysis was performed by using SPSS v.14 software. The paired *t*-test was used in order to compare protein fold levels of claudin 5 in the various cell types following different treatments. The Spearman rank correlation test was used to measure the strength and direction of the relationship between the time of the different treatments and the protein levels (time-dependent response). A General Linear Model (GLM) repeated measures test was employed for analysis of half-life experiments. In all the statistic analyses performed, significant differences were inferred when $P < 0.05$.

RESULTS

CLAUDIN 5: A PROTEIN WITH A SHORT HALF-LIFE REGULATED BY THE PROTEASOME

The stability of the claudin 5 protein was evaluated by determining its half-life in pCDNA-claudin 5 transiently transfected HeLa cells and HUVEC. As demonstrated in Figure 1A, claudin 5 had a relatively short half-life of ~90 min in transfected HeLa cells and ~70 min in HUVEC, which express claudin 5 endogenously. Pre-treatment of the cells with the proteasome inhibitor MG-132 led to a significant stabilization of claudin 5 levels in both cell types

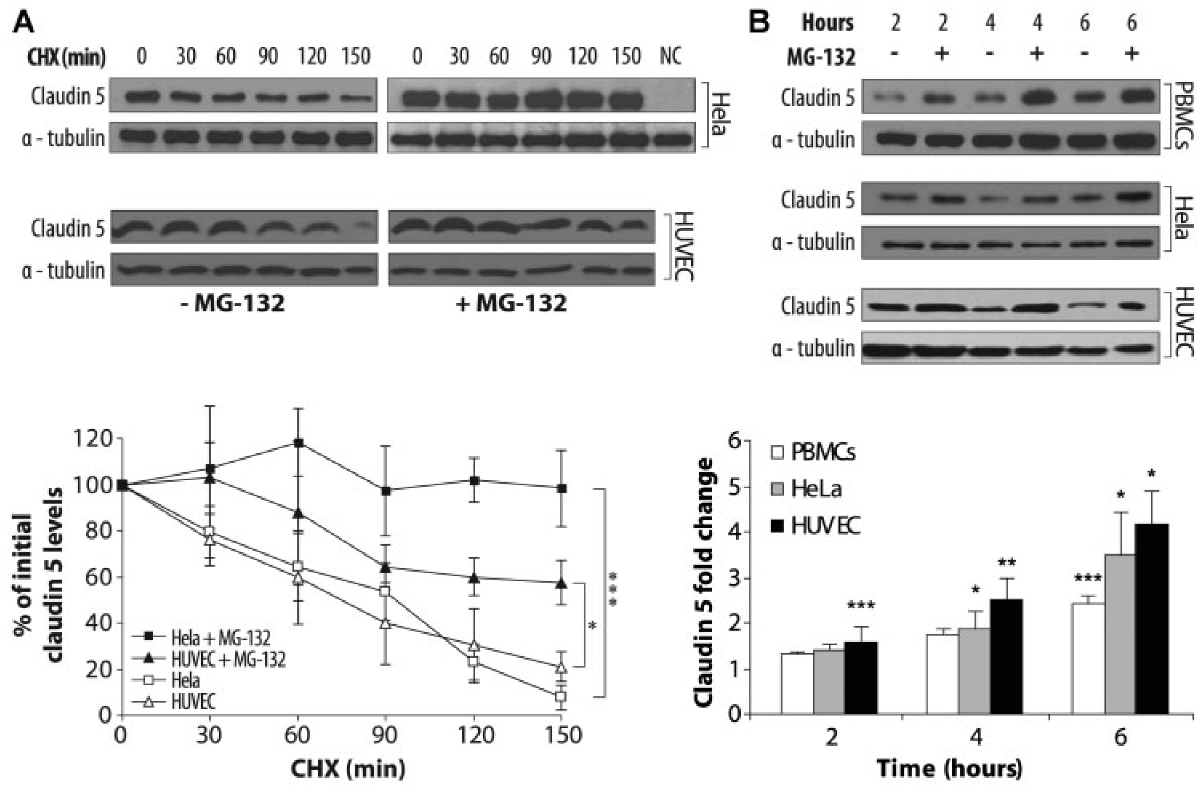

Fig. 1. The half-life and levels of claudin 5 are extended by inhibition of proteasome activity. A: HeLa cells transfected with pCDNA-claudin 5 and HUVEC were pretreated with MG-132 or DMSO as control for 6 h, followed by cycloheximide (CHX) treatment for the indicated times and Western blot analysis of whole cell lysates. Representative results of the Western blots are shown in the top panel. NC: negative control of non-transfected HeLa cells. Relative claudin 5 protein levels in HeLa cells or HUVEC, in percentages compared to the initial levels prior to addition of cycloheximide are presented in the bottom panel as a function of time of exposure to cycloheximide. All graphs depict the mean ± SD of four independent experiments. B: HeLa cells transfected with pCDNA-claudin 5, HUVEC, and PBMC treated with MG-132 were harvested after 2, 4, and 6 h, followed by whole cell lysis and Western blot analysis. Representative blots for each cell type are shown in the top panels. The fold change of claudin 5/α-tubulin compared to the relative intensity without MG-132 treatment is presented in the bar graph in the bottom panel for different times of exposure to MG-132. $^{*}P < 0.05$, $^{**}P < 0.02$, $^{***}P \leq 0.01$ (repeated measures analysis using a mixed model for A, Student's *t*-test for B).

(Fig. 1A, $P = 0.0003$ for HeLA cells, $P = 0.04$ for HUVEC, repeated measures analysis using a mixed model), suggesting that the degradation of claudin 5 is proteasome-dependent.

Indeed, MG-132 treatment led to a time-dependent increase in claudin 5 levels in both cell types, as well as in PBMC (a cell population that expresses claudin 5 endogenously [Mandel et al., 2011]), which was evident starting from 2 h after treatment (Fig. 1B) ($r = 0.97$, $P = 2 \times 10^{-9}$ for HeLa; $r = 0.98$, $P = 4 \times 10^{-8}$ for HUVEC; $r = 0.86$, $P = 4 \times 10^{-4}$ for PBMC according to Spearman's correlation test).

PROTEASOME INHIBITION LEADS TO INTRACELLULAR ACCUMULATION OF CLAUDIN 5

Following proteasome inhibition, claudin 5 was significantly elevated in the intracellular fraction ($P = 0.001$), but not in the membranal fraction of pCDNA-claudin 5 transfected HeLa cells (Fig. 2A). Immunofluorescence studies confirmed the intracellular accumulation of claudin 5 following proteasome inhibition (Fig. 2B). Intracellular accumulation of a membranal protein in an in vitro, overexpression system can also be the result of spillover of excessively synthesized proteins; however, when both protein synthesis and proteasome activity were blocked, the same intracellular localization pattern of claudin 5 protein was observed (results not shown), suggesting the intracellular accumulation is not the result of excess protein synthesized. Immunofluorescence studies to determine the subcellular location of claudin 5 upon proteasome inhibition demonstrated a colocalization with RAB5, a marker for the early endosome, but not with RAB7 a marker for the late endosome (Fig. 2C).

CLAUDIN 5 IS POLYUBIQUITINATED

To evaluate whether direct ubiquitination is involved in the process of claudin 5 degradation, we performed in vivo ubiquitination assays in pCDNA-claudin 5 transfected HeLa cells. Claudin 5 immunoprecipitated from lysates of cells treated with the proteasome inhibitor displayed high molecular weight forms, which represent claudin 5-ubiquitin conjugated complexes (Fig. 3A). To distinguish between monoubiquitination and polyubiquitination, which are known to target proteins to different processing pathways [Miranda and Sorkin, 2007], Western blot analysis of the immunoprecipitated proteins was performed with the anti-ubiquitin antibodies, FK1, which recognizes only polyubiquitin

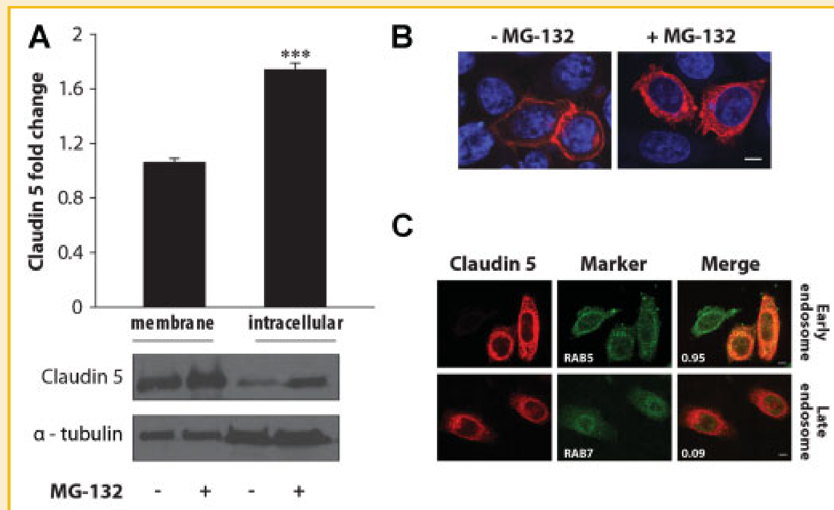

Fig. 2. The effect of proteasome inhibition on the cellular localization of claudin 5. A: Western blot analysis of membrane and intracellular fractions of HeLa cells transfected with pCDNA-claudin 5, following treatment with MG-132 for 6 h. The graph presents the mean ± SD of the claudin 5/α-tubulin levels relative to the levels in untreated cells for each cellular fraction (n = 3). $^{***}P = 0.001$ Student's *t*-test. Representative Western blot results are shown in the bottom panel. B: Immunofluorescence staining of claudin 5-transfected HeLa cells for claudin 5 (anti-claudin 5 antibody—red) and cell nuclei (TO-PRO 3—blue) treated (right) or not (left) with MG-132; scale bar: 5 μm. C: Immunofluorescence staining of claudin 5-transfected HeLa cells treated with MG-132 for claudin 5 (red) and endosomal markers (green) for early (RAB5) and late (RAB7) endosomes. Numbers in the merged panel are the colocalization coefficients averaged over five different images (standard deviation values RAB5: ±0.02; RAB7: ±0.03).

chains, and P4D1 which recognizes both mono- and polyubiquitin [Haglund et al., 2003]. As demonstrated in Figure 3B, the antibodies recognized the same pattern of bands, suggesting that claudin 5 undergoes polyubiquitination rather than monoubiquitination. Together, these results indicate that the levels of claudin 5 are regulated through polyubiquitination-mediated proteasomal degradation.

THE INVOLVEMENT OF THE LYSOSOME IN CLAUDIN 5 TURNOVER IN A UBIQUITIN-INDEPENDENT MANNER

Lysosome regulation has been recently implicated in the ubiquitin-mediated turnover of claudin 1, claudin 2, and claudin 4 [Takahashi et al., 2009]. As portrayed in Figure 4A, a slight but consistent increase in claudin 5 levels was observed after 4 h of treatment with the lysosome inhibitor chloroquine (100 μM), which continued in a time dependent manner ($r = 0.783$, $P = 0.003$ according to the Spearman correlation test), although the difference at each of the time points tested was not statistically significant. Similar results were obtained with the more specific lysosome inhibitor, leupeptin ($n = 3$, results not shown). Figure 4B shows that treating claudin 5 transfected HeLa cells with chloroquine did not lead to accumulation of ubiquitin conjugates. These results suggest the lysosome is also involved in the regulation of claudin 5 levels; however, ubiquitination does not appear to be part of the lysosome-dependent degradation process.

LYSINE 199 IS THE MAJOR SITE INVOLVED IN CLAUDIN 5 UBIQUITINATION

Claudin 5 harbors several lysine residues that are located in the predicted intracellular loop and are potential targets for ubiquitination (Fig. 5A). To determine which of these lysine residues is ubiquitinated, we generated single point mutations substituting lysines at positions 114, 199, 214, and 215 with arginine, a double mutation K214R–K215R, and a triple mutation K199R–K214R–K215R. The point mutation K199R reduced the accumulation of claudin 5 upon proteasome inhibition, from 3.9-fold elevation in the wild type to 1.6-fold in the mutant ($P = 0.02$) (Fig. 5B). Other point mutations in lysines 114, 214, 215, and a double mutation of lysine 214 and 215 had no effect on claudin 5 accumulation following proteasome inhibition with similar fold change values as the wild type. Conversely, the addition of a mutation in lysine 199 to the double mutation of lysines 214 and 215 in the triple mutation (K199R–K214R–K215R) completely eradicated claudin 5 accumulation following proteasome inhibition (1.1-fold, $P = 0.0002$). In concordance with the Western blot analysis data, claudin 5-ubiquitin conjugated forms did not accumulate in in vivo ubiquitination assays performed in HeLa cells transfected with the K199R mutant or the triple mutation (Fig. 5C). Surprisingly, the triple mutation had only a slightly longer half-life (~110 min) in comparison with the WT (~90 min), yet proteasome inhibition failed to increase the protein stability, indicating that indeed the triple mutant had lost the ability to be processed though the UPS (Fig. 5D). Furthermore, the cellular localization of the triple mutant was similar to that of the wild type, and responded similarly to proteasomal inhibition by displaying intracellular accumulation (Fig. 5E).

DISCUSSION

Claudin 5 is an important member of the TJP family, which is involved in determining the size selectivity of the BBB [Nitta et al., 2003]. TJ disruption is involved in processes such as development, cell division, tissue repair, invasion and metastasis of tumor cells

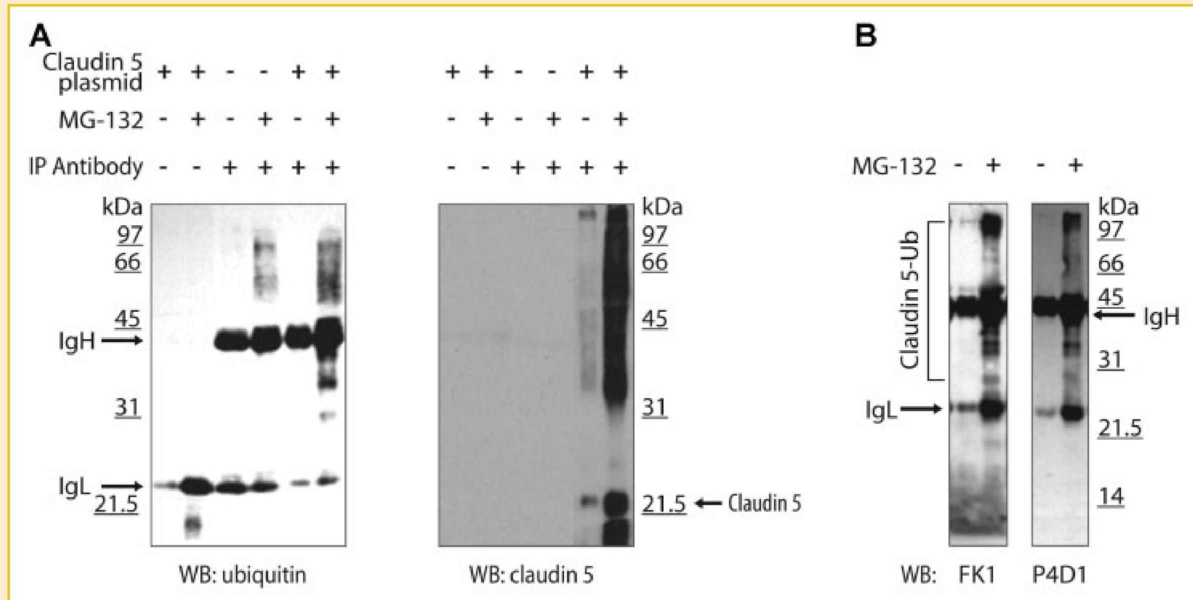

Fig. 3. Accumulation of polyubiquitinated forms of claudin 5 upon proteasome inhibition. HeLa cells transfected with pCDNA-claudin 5 or untransfected were treated with the proteasome inhibitor MG-132 or DMSO as a control for 6 h. The cells were lysed and subjected to immunoprecipitation (IP) using anti-claudin 5 polyclonal antibodies followed by (A) Western blot (WB) analysis with anti-ubiquitin antibodies (left panel). The membrane was then stripped and re-probed with monoclonal anti-claudin 5 antibodies (right panel). All IP experiments were performed with a polyclonal anti-claudin 5 antibody with experiments omitting the IP antibody as controls for immunoprecipitation specificity. B: Western blot of anti-claudin 5 IP experiments, using FK1 (specific for polyubiquitin chains) and P4D1 (recognizing both mono and polyubiquitin) anti-ubiquitin antibodies. Representative results of three independent experiments are shown. IgH and IgL denote the heavy and the light chains of the IP antibody, which are visible in A but not evident in B due to differences in exposure times of the blots. Claudin 5-Ub indicates the position of ubiquitinated forms of claudin 5.

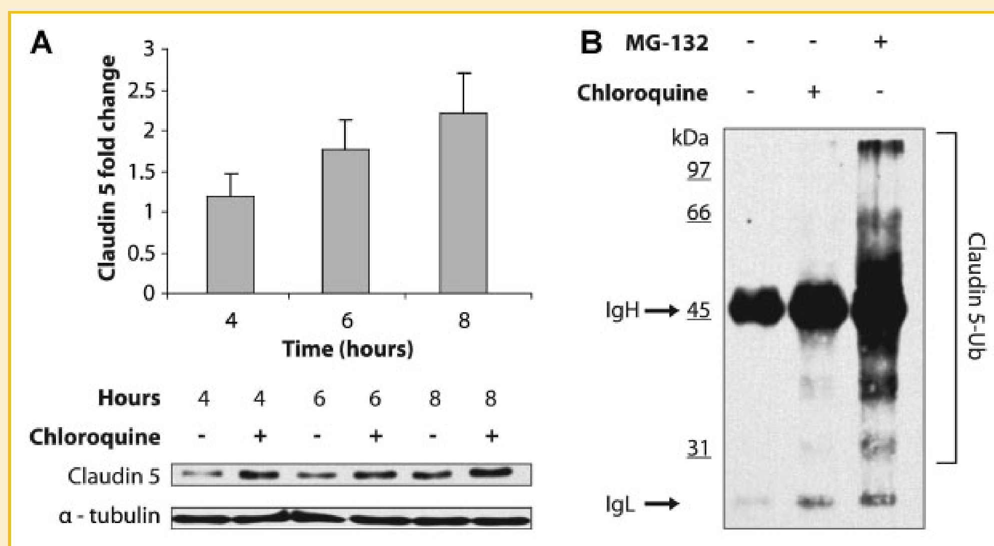

Fig. 4. The involvement of the lysosome in claudin 5 turnover. A: Western blot analysis for claudin 5 levels in cell lysates of HeLa cells transfected with pCDNA-claudin 5 and treated with chloroquine for 4, 6, and 8 h, as indicated. The fold change of claudin 5/α-tubulin compared to the relative levels without chloroquine treatment is presented in the graph in the top panel; the data points represent the mean $\pm$ SD of three independent experiments. A representative Western blot from one experiment is displayed in the bottom panel. B: pCDNA-claudin 5 transfected HeLa cells were treated with or without chloroquine for 8 h or MG-132 for 6 h as a positive control for ubiquitination of claudin 5, followed by IP using anti-claudin 5 polyclonal antibodies and Western blot analysis with anti-ubiquitin antibodies. A representative Western blot of three experiments is shown. IgH and IgL denote the heavy and the light chains of the IP antibody. Claudin 5-Ub represents ubiquitinated forms of claudin 5.

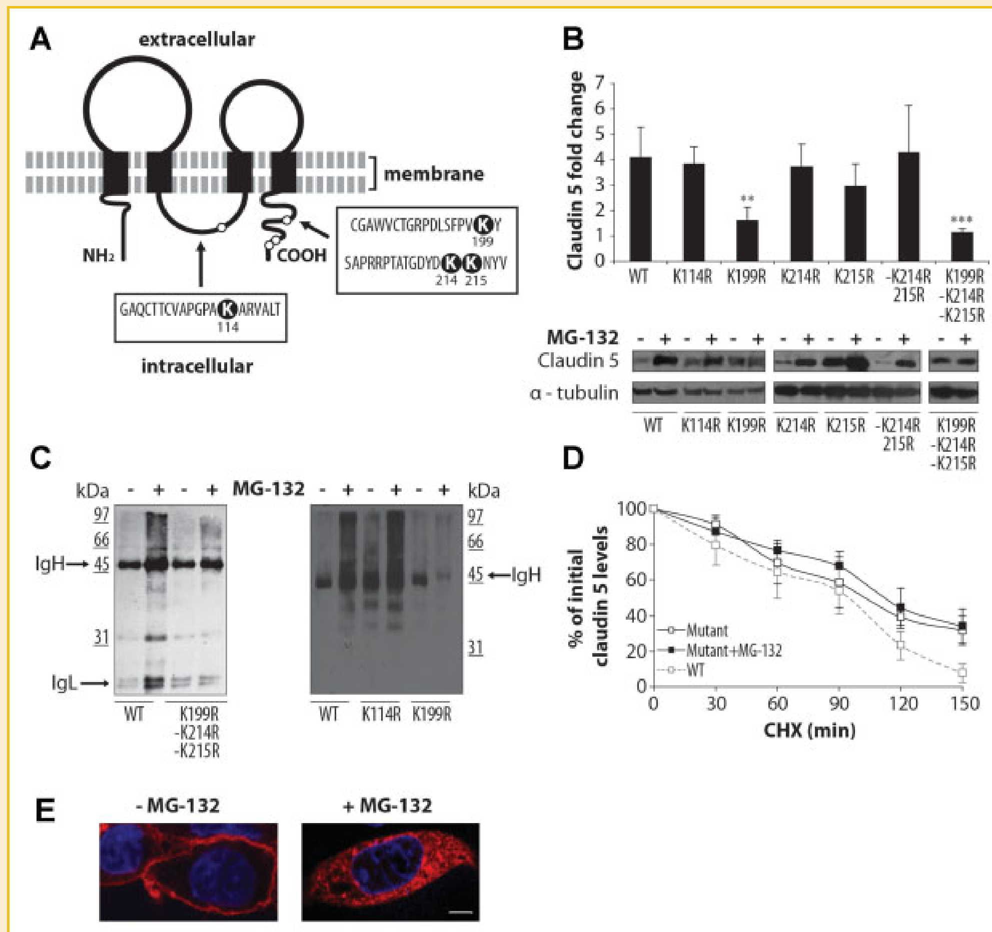

Fig. 5. Site directed mutagenesis analysis of claudin 5 potential ubiquitination sites. A: A schematic view of the predicted structure of claudin 5 according to Kyte–Doolittle hydrophobicity plot, SOUSUI software and published literature [Ruffer and Gerke, 2004]. The sequence of the intracellular domains of claudin 5 is depicted, with the position of the potential lysines for ubiquitination marked on the intracellular loops of the protein, and numbered and circled in the sequence. B: HeLa cells were transfected with plasmids encoding wild-type (WT) claudin 5, or the indicated point mutants. The cells were treated with MG-132 for 6 h followed by cell lysis and Western blot analysis. The mean fold change of claudin 5/α-tubulin levels relative to the levels without MG-132 treatment $\pm$ SD is presented in the graph in the top panel ($n \geq 3$ for each mutation). The bottom panel shows representative Western blot results. $^{*}P = 0.01$, $^{**}P = 0.0002$ (Student's *t*-test). C: immunoprecipitation (IP) using anti-claudin 5 polyclonal antibodies followed by Western blot with anti-ubiquitin antibodies. Representative results of three independent experiments are shown. IgH and IgL denote the heavy and the light chains of the IP antibody. Claudin 5-Ub represents ubiquitinated forms of claudin 5. D: HeLa cells transfected with the K199R–K214R–K215R mutant (denoted "mutant" in the graph legend) were pretreated with MG-132 or DMSO as control for 6 h, followed by cycloheximide treatment for the indicated times and Western blot analysis of whole cell lysates. Relative percentages compared to the relative intensity of claudin 5/α-tubulin at time 0 are presented. The graph depicts the mean $\pm$ SD of three independent experiments. The curve for wild type claudin 5 is shown for reference in a gray dashed line. E: Immunofluorescence for claudin 5 (red) and cell nuclei (TO-PRO 3—blue) in HeLa cells transfected with the K199R–K214R–K215R mutant treated or untreated with MG-132 and cycloheximide. Scale bar: 5 μm.

[Matter et al., 2005], as well as the migration of leukocytes through endothelial barriers [Persidsky et al., 2006]. Therefore, understanding mechanisms that control TJP expression is of utmost importance. Here we demonstrate that the UPS is involved in the regulation of claudin 5 levels, based on several lines of evidence: Firstly, we show that claudin 5 has a relatively short half-life, especially when compared to other membranal proteins [Herve et al., 2007], implicating the involvement of a rapid turnover mechanism such as the UPS in the regulation of this protein. Secondly, a proteasome inhibitor increased claudin 5 levels and prolonged the half-life of claudin 5, in both transfected, over expressing HeLa cells and endogenously expressing HUVEC and PBMC. Thirdly, the in vivo ubiquitination experiments demonstrated the presence of claudin 5-ubiquitin conjugated forms, which most likely represent poly-ubiquitinated forms. Lastly, we were able to demonstrate that the K199R in claudin 5 diminished the effects of proteasome inhibition on claudin 5 levels. The fact that these effects were more pronounced in the triple mutant (K199R–K214R–K215R) yet not evident in the double mutant (K214R–K215R), suggests that there is a redundancy in the ubiquitination process, and lack of the major lysine is compensated for by ubiquitination of other lysines.

When the proteasome was inhibited, claudin 5 seemed to accumulate mostly in the intracellular compartment. However, the triple mutation, which completely eradicated the ability of claudin 5 to undergo ubiquitination and degradation by the proteasome, also retained the intracellular localization pattern following proteasome

inhibition, in a manner similar to wild type claudin 5. This suggests that similarly to other membranal proteins regulated by the UPS [Rocca et al., 2001; Dupre et al., 2003], ubiquitination itself is not the trigger for claudin 5 internalization. In addition, there might be endocytotic mechanisms responsible for the internalization of claudin 5, that are affected by the proteasome inhibition, but do not require claudin 5 ubiquitination. Interestingly, it has recently been demonstrated that proteasome inhibition can lead to a similar accumulation of the TJP claudin 1 in the cytoplasm which is independent of its ubiquitination [Asaka et al., 2011].

Additional mechanisms besides the UPS may be involved in regulation of claudin 5 levels, as our results suggest. One would expect that since the triple lysine mutant cannot be properly targeted to the proteasome, its half-life would be significantly prolonged, however, its half-life in transfected HeLa cells was only slightly stabilized compared to that of wild-type claudin 5. The proteasome inhibitor MG-132 is also an inhibitor of some of the lysosomal proteases [Elliott et al., 2003] and therefore the involvement of the lysosomal pathway and its effects on claudin 5 levels should be considered. Indeed, we have also observed the ubiquitin-independent up regulation of claudin 5 following lysosome inhibition, although to a lesser extent than with MG-132, suggesting the contribution of the lysosomal pathway to claudin 5 turnover regulation, as also reported for other TJPs [Gonzalez-Mariscal et al., 2008; Takahashi et al., 2009]. We note, however, that the effect of MG-132 clearly involves ubiquitination of claudin 5, whereas the chloroquine effect does not. In the immunofluorescence studies of claudin 5 localization upon proteasome inhibition we performed, claudin 5 was found in early endosomes, but did not colocalize within the late endosomes. Thus, ubiquitinated claudin 5 does not appear to be targeted to the lysosome, although it is internalized apparently via the endocytic pathway, and eventually degraded most probably by the proteasome. A proteasome dependent degradation pathway for plasma membrane proteins which involves transport through the early endosomes and the activity of the Derlin 1 protein has been demonstrated for the LDL receptor [Schaheen et al., 2009] and can explain the findings shown herein for claudin 5. The fact that claudin 5 seemed to be polyubiquitinated, which is mostly associated with proteasomal degradation, rather than monoubiquitinated, which is more associated with lysosomal degradation, also supports this notion, although in some cases polyubiquitination can also target to the lysosome, such as has been reported for occludin [Murakami et al., 2009], claudin 1, claudin 2, and claudin 4 [Takahashi et al., 2009]. The effect of the lysosome inhibitors on claudin 5 accumulation observed herein can be indirect, possibly through other intermediary proteins that are regulated by lysosomal enzymes. Examples for such proteins include members of the matrix metalloproteinases, which are targeted to the lysosome in certain conditions [Takino et al., 2003; Sbai et al., 2010] and are known to interact and affect claudin 5 levels [Yang et al., 2007]. It is also possible that some of the proteins that were previously shown to be involved in regulation of claudin 5 levels, including transcription regulators and cytokines, may be subjected to regulation by the UPS and therefore the proteasome inhibition assays would have displayed a secondary effect on claudin 5 levels.

The effect of proteasome inhibition on the half-life of claudin 5 was more profound in the transfected HeLa cells than in the HUVEC, which suggests the involvement of cell specific mechanisms in the regulation of claudin 5. Further support to the presence of cell-specific mechanisms of regulation of claudin 5 levels comes from a report on VEGF induced selective degradation of occludin, but not claudin 5, which is mediated by the UPS in primary bovine retinal endothelial cells (BRECs). The half-life of claudin 5 in BRECs (over 3 h) was much longer than that recorded herein in both HeLa cells and HUVEC, and MG-132 did not affect claudin 5 stability therein [Murakami et al., 2009]. There is also evidence for the differential degradation rates and involvement of the UPS or ubiquitination in regulation of the levels of occludin and claudin 1 in various cell types [Traweger et al., 2002; Takahashi et al., 2009; Asaka et al., 2011].

This cell-specific regulation of TJPs and differential response to stimuli, can lead to changes in the composition of the TJ complex, which can eventually lead to changes in barrier properties in cellular layers. The differential regulation of the claudins and occludin by ubiquitination can be achieved by the presence of specific E3 ubiquitin ligases for each protein. It remains to be determined if the E3 ubiquitin ligase LNX1p80 implicated in the regulation of claudin1, claudin 2, and claudin 4 turnover [Takahashi et al., 2009] is also involved in the regulation of claudin 5. The known E3 ubiquitin ligase of occludin, itch [Traweger et al., 2002; Murakami et al., 2009] is not likely to be the E3 ubiquitin ligase responsible for claudin 5 ubiquitination since claudin 5 does not contain WW domains and proline rich sequences required for itch interaction [Kay et al., 2000].

In conclusion, we have demonstrated that claudin 5 is a protein with a short half-life that can undergo polyubiquitination mainly on lysine 199, which apparently leads to its subsequent degradation. The results presented indicate that direct ubiquitination is not involved in the endocytosis of claudin 5, but rather in its targeting to degradation in a UPS-dependent manner. Other mechanisms regulating the protein levels and localization of claudin 5 are likely to be in effect, allowing the differential modulation of the TJ complex properties by external signals in different cell types. Further dissection of the regulatory mechanisms controlling the TJ complex composition in the future is of importance for comprehension of endothelial layer properties, and the means to affect its permeability.

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