Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Barriers and Channels Formed by Tight Junction Proteins*

SUMOylation of claudin-2

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The *C*-terminal cytoplasmic tails of claudins are likely sites for interaction with proteins that regulate their function. We performed a yeast two-hybrid screen with the tail of human claudin-2 against a human kidney cDNA library and identified interactions with the PDZ3 domain of ZO-2 as well as ubiquitin-conjugating enzyme E2I (SUMO ligase-1) and E3 SUMO-protein ligase PIAS; the first is a predicted interaction, while the latter two are novel and suggest that claudin-2 is a substrate for SUMOylation. Using an *in vitro* SUMOylation assay, we identified K218 as a conjugation site on claudin-2; mutation of that lysine to arginine blocked SUMOylation. Stable expression of inducible GFP-SUMO-1 in MDCK cells resulted in decreased levels of claudin-2 protein by immunoblot and decreased claudin-2 membrane expression by immunofluorescence microscopy. We conclude that the cellular levels of claudin-2 may be modulated by SUMOylation, warranting further investigation of cellular pathways that regulate this modification *in vivo*.

Keywords: claudin; claudin-2; MDCK; SUMO-1; tight junction; yeast two-hybrid

Introduction

Claudins form a large family of tetraspanning membrane proteins that create the variable permselective barrier properties of tight junctions.[1] It is presumed that their function, including barrier properties, assembly, trafficking, and half-lives, might be regulated by posttranslational modifications or through binding other proteins to their C-terminal cytoplasmic sequences. Currently, it is known that most claudins bind to the PDZ domains of the ZO-1, -2, and -3 MAGUK proteins through PDZ binding motifs on their extreme C-termini,[2] and several claudins are known to be phosphorylated or palmitoylated with functional consequences.[3,4] Claudin-1 provides the single example of covalent modification by ubiquitin, a modification that enhances delivery to and destruction by the proteosome.[5,6] We performed yeast two-hybrid (Y2H) screening with the C-terminal tail of claudin-2 in an effort to identify novel binding proteins, which could provide further insight into the regulation of claudin function. Our results demonstrate that claudin-2 has the capacity for modification on lysine-218 by SUMO-1 (small ubiquitin-like modifier-1), a modification-like ubiquitination that in other proteins is known to regulate a range of protein functions, including protein–protein interactions, subcellular localization, and trafficking.[7–12] Like ubiquitination, the covalent attachment of SUMO proteins to lysine residues on target proteins requires a series of conjugation factors that recognize sequences surrounding the target lysine;[13–19] interaction with SUMO ligases in Y2H screen provided the initial indication that numerous proteins were SUMOylated.

Materials and methods

Y2H screening was performed as previously described in our laboratory using the L40 yeast strain as described in Niethammer *et al.*[20,21] The bait consisted of the cytoplasmic C-terminal sequence of human claudin-2 (residues 185–230) subcloned in frame with the lexA DNA-binding domain into vector pBHA5. The bait was used to screen a human kidney cDNA library constructed in pGAD5 (Clontech Laboratories, Inc., Mountain View, CA). DNA

doi: 10.1111/j.1749-6632.2012.06541.x

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from positive interacting clones, as assayed by beta-galactosidase staining, was rescreened against the claudin-2–containing vector in binary assays and autoactivators eliminated by unitary transformation assays.

For *in vitro* SUMOylation assays, the carboxyl-terminal tail of canine claudin-2 (amino acids 189–230) was amplified from MDCK II cell mRNA and cloned into pCR TOPO. The sequence was verified and the insert subcloned into pGEX4T (GE Healthcare, Port Washington, NY). For use in some studies, K218 was mutated to arginine by site-directed mutagenesis (Quik Change Site-Directed Mutagenesis Kit, Agilent Technologies, Santa Clara, CA); sequences were verified and proteins expressed in *Escherichia coli*, as described previously by our laboratory.[22] *In vitro* SUMOylation was performed using a Active Motif SUMOlink SUMO-1 kit (Active Motif, Carlsbad, CA); this kit includes a mutant SUMO-1 that lacks SUMOylation function.

GFP-SUMO-1 was constructed from a human SUMO-1 cDNA (Open Biosystems, Lafayette, CO) cloned into the pTRE vector (Clontech) that had been modified to include EGFP-human SUMO-1 was cloned downstream of the EGFP-coding region. This vector was cotransfected into MDCK II Tet-off cells (Clontech) with pSVZeo; stable cell lines were selected with 1 mg/mL Zeocin (InVivogen, San Diego, CA). Transfected cells were maintained without GFP-SUMO-1 expression by addition of 50 ng/mL doxycycline and protein induction was performed by removal of doxycycline from media. GFP-SUMO-1 expression was verified by fluorescence microscopy and immunoblot analysis. MDCK cell culture, protein induction, immunoblots, and immunofluorescence microscopy were performed as described elsewhere.[23] All antibodies were purchased from Invitrogen (Carlsbad, CA).

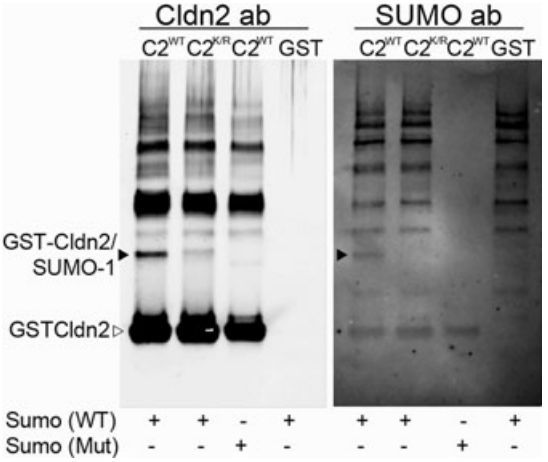

Figure 1. *In vitro* assay reveals that claudin-2 can be SUMOylated on K218. Wild-type (lanes 1 and 3) and mutant K218R (lane 2) claudin-2 tails were expressed as GST fusion proteins and purified by glutathione-affinity chromatography; GST alone was used as a nonspecific control (lane 4). GST proteins were mixed with SUMOylation reagents according to the manufacturer's directions; SUMO-1 mutant that cannot be complexed was used as a negative control (lane 3). Immunoblot with claudin-2 antibody (left immunoblot) reveals that GST-claudin-2 (≈30 kDa) and a single unique band at approximately 42 kDa representing claudin-2/SUMO-1; this band is also immunoreactive with a SUMO-1 antibody (right immunoblot). This 42 kDa band is not present in the reaction mixture containing either claudin-2 K218R or the mutant SUMO-1. Other bands are present in all lanes and thus are unlikely to represent claudin-2/-1 forms. These are the same blot probed (at the same time) with antimouse claudin-2 primary antibody and IR700 antimouse secondary antibody and SUMO-1 rabbit primary antibody and IR800 antirabbit secondary antibody. The secondary antibodies do not cross react, but the faint staining at the site of the GST-cldn2 signal with the SUMO-1 primary antibody is probably a nonspecific protein: protein interaction due to the large amount of fusion protein.

Results and discussion

Y2H screening

A human kidney cDNA library in the pGAD10 Y2H vector was screened using a sequence encoding the entire 45 residue C-terminal cytoplasmic tail sequence of human claudin-2 in pBHA5. Of 99 positive sequences, 65 encoded ubiquitin-conjugating enzyme E2I (also called SUMO-1 ligase) (Genbank NM˙003345), 17 encoded the E3 SUMO ligase protein inhibitor of activated STAT-2 (PIAS2, Genbank NM˙173206), 5 included the third PDZ domain of ZO-2, and the other 12 were out of coding frame. An interaction with the PDZ3 domain of ZO-2 has not been reported but is not unexpected, since the PDZ1 and PDZ2 domains of ZO-1 and ZO-2 are known to bind PDZ motifs of claudins;[2] however, those with proteins involved in SUMOylation was unexpected and novel. Regardless of length, all E3 SUMO ligase PIAS2 clones included sequences encoding the SP-RING domain.[8] This domain is required for binding to both the SUMO donors like UbcE2I and the acceptor recognition region on the targets for E3 SUMO ligase, suggesting that the tail of claudin-2 might be a substrate for the SUMO ligases. Further, as expected, the C-terminal

three residues of claudin-2 were required in Y2H assays for interaction with ZO-2 but not to bind UbcE2I or SUM0-ligase PIAS2. Of note, claudin-2 is the only claudin with a potential lysine acceptor residue (K218) within a SUMOylation recognition motif (ΨKXE) at VKSEFNSYSLTGYV, although it is possible that other claudins could be SUMOylated on cytoplasmic lysines. Lysine 218 is positioned 13 residues preceding the C-terminal PDZ binding motif suggesting the possibility that conjugation of a SUMO protein at K218 might sterically inhibit binding to ZO proteins; however, this was not tested.

Claudin-2 can be SUMOylated on K218

Next we tested whether the tail of claudin-2 purified as GST-fusion protein from *E. coli* was a substrate for SUMO-1 modification in a standard *in vitro* test assay and whether the predicted acceptor residue at K218 was the site of conjugation. The latter was tested by mutating K218 to arginine, which lacks the epsilon-amino conjugation nitrogen. Immunoblot analysis of purified GST-claudin-2 tail after incubation in the SUMOylation assay (including E1 activating enzyme, E2 conjugating enzyme, and SUMO-1 protein) with GST-claudin-2 tail reveals a band at the expected size for GST-claudin-2 (approximately 30 kDa, GSTCldn2) and a higher molecular weight (MW) band corresponding to a GST-claudin-2-SUMO-1 complex (approximately 42 kDa, GSTCldn2). This higher MW band is also detected with a SUMO-1 antibody (Fig. 1, left-most lane of both immunoblots), verifying that it is the conjugated GST-claudin-2 tail. This band is not present in incubation mix containing the mutated GST-claudin-2 K218R tail (Fig. 1, middle lanes) or in incubation mix containing wild-type GST-claudin-2 tail but with a mutated control SUMO-1 that cannot be conjugated (Fig. 1, third lanes). These data demonstrate that *in vitro*, K218 can be SUMOylated.

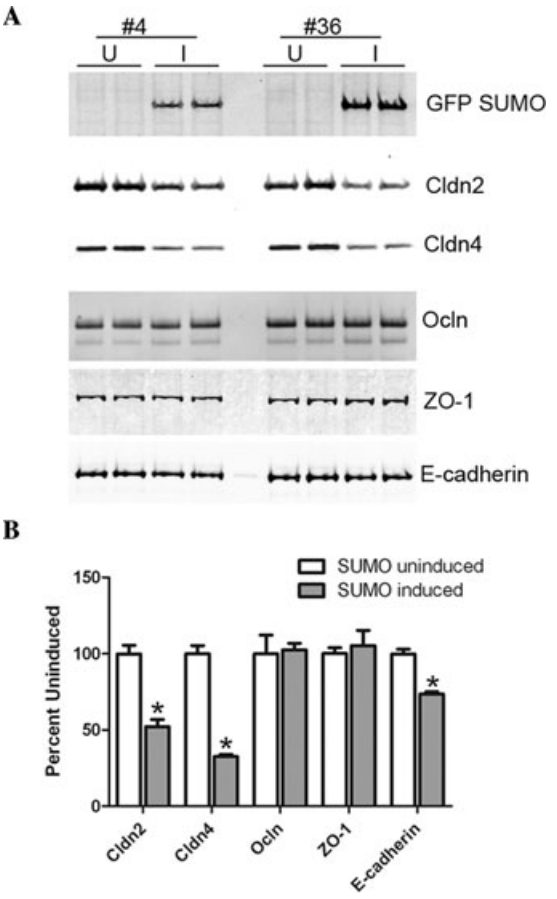

Figure 2. **Immunoblot analysis of MDCK cells stably expressing GFP-SUMO-1. Two separate MDCK cell lines were not induced (U) or induced (I) to express GFP-SUMO-1 for seven days; wells were plated in duplicate. Cells were processed for immunoblot analysis (A); both clones expressed GFP-SUMO-1 only when induced. Both claudin-2 and claudin-4 levels were decreased in cells expressing GFP-SUMO-1 compared with uninduced cells while there was no change in occludin or ZO-1 protein levels and only a small change in E-cadherin expression. The changes in expression levels (average of the two clones) are quantified in (B) (mean ± SEM), revealing a 50% decrease in protein expression for claudin-2 and -4 and a 20% decrease in E-cadherin levels, *$P < 0.05$ by untailed Student's *t*-test.**

Expression of SUMO-1 in MDCK cells reduces the level of claudin-2

We were unable to detect baseline SUMOlyation of claudin-2 in cultured MDCK epithelial monolayers by immunoblotting of cell lysates or after immunoprecipitating of claudin-2. In the absence of knowledge about how to stimulate physiologic conjugation, we overexpressed SUMO-1 to drive conjugation of all substrates and assayed for potential changes in the levels or location of claudin-2. SUMO-1 was expressed in a tet-inducible system fused to GFP so that changes in claudin-2 could be detected in the same clone before and after induction and so that the expression of SUMO-1 could be detected by fluorescence microscopy. Induction of GFP-SUMO-1 in two separate clones of MDCK II tet-off cells (immunoblot, Fig. 2A; quantified in Fig. 2B) resulted in significant decreases in the levels

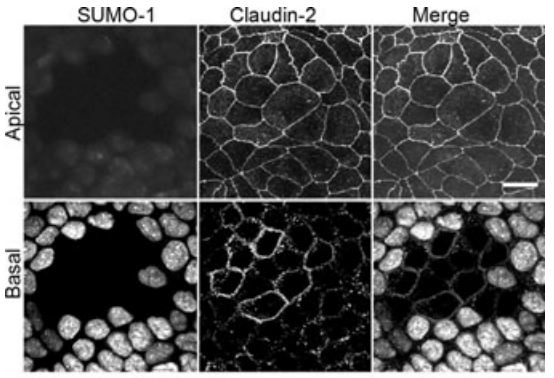

Figure 3. **Confocal microscopy analysis of a cocultured mixture of nontransfected MDCK cells and cells expressing GFP-SUMO-1 reveals that GFP-SUMO-1 is concentrated in nuclei (left panel). Cells were imaged below the TJ at the level of the mid-lateral members and revealed that claudin-2 staining is reduced in cells expressing GFP-SUMO-1, middle panel and merge, right panel. Bar = 10 μm.**

of claudin-2 (to 50%), claudin-4 (to 35%), and to a lesser extent E-cadherin, but had no effect on the levels of ZO-1, ZO-2, or occludin.

Changes in claudin-2 were also detected by immunofluorescence microscopy after induction of SUMO-1. A cocultured mixture of untransfected MDCK II cell and GFP-SUMO-1–expressing cells reveals that cells expressing GFP-SUMO-1 have reduced levels of claudin-2 on their lateral membranes (Fig. 3). The inverse correlation between induced expression of SUMO-1 and decreased lateral claudin-2, was obvious in two different GFP-SUMO-1–expressing cell lines. In contrast, immunofluorescent claudin-2 expression at the level of the tight junction was apparently identical in all cells, regardless of GFP-SUMO-1 expression (not shown). This observation leads to speculation that SUMOylation may target lateral but not tight junction claudin-2 for removal and degradation, but this conclusion would require verification.

The mechanistic relationship between expression of GFP-SUMO-1 and the changes in claudin-2 is unclear. Although claudin-2 can be SUMOylated *in vitro*, we have so far been unable to demonstrate *in vivo* SUMOylation. However, the correlation between increased GFP-SUMO-1 and decreased claudin-2 levels, demonstrated both by immunoblotting and immunofluorescence microscopy, suggests that SUMO-1 acts to regulate claudin-2 level. This could occur through altered trafficking or endocytosis and degradation, either by direct tagging of claudin-2 or by altering an indirect pathway, which alters claudin-2 levels. The observation that claudin-4 levels (and cadherin levels) are reduced in GFP-SUMO-1–expressing cells is likely an indirect effect, since claudin-4 does not contain a consensus peri-lysine sequence for SUMOylation. However, the lack of effect of GFP-SUMO-1 expression on other tight junction proteins argues that the effects on claudin levels are not due to a global increase in degradation, but may represent a specific, physiologically relevant regulatory mechanism. There is considerable interest in how cells might regulate tight junction barrier properties through differential regulation of specific claudin levels. There is considerable knowledge about how claudins are differentially regulated at a transcriptional level;[24] however, regulation at a posttranscriptional level by SUMO-1 conjugation is novel and deserves further study.

Conflicts of interest

The authors declare no conflicts of interest.

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