# Palmitoylation of claudins is required for efficient tight-junction localization

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*Accepted 18 January 2005*
*Journal of Cell Science 118, 1427-1436 Published by The Company of Biologists 2005*
*doi:10.1242/jcs.01735*

## Summary

**Palmitoylation of integral membrane proteins can affect intracellular trafficking, protein-protein interactions and protein stability. The goal of the present study was to determine whether claudins, transmembrane-barrier-forming proteins of the tight junction, are palmitoylated and whether this modification has functional implications for the tight-junction barrier. Claudin-14, like other members of the claudin family, contains membrane-proximal cysteines following both the second and the fourth transmembrane domains, which we speculated could be modified by *S*-acylation with palmitic acid. We observed that [$^3$H]-palmitic acid was incorporated into claudin-14 expressed by transfection in both cultured epithelial cells and fibroblasts. Mutation of cysteines to serines following either the second or the fourth transmembrane segments decreased the incorporation of [$^3$H]-palmitic acid, and mutation of all four cysteines eliminated palmitoylation. We previously reported that expression of claudin-14 in epithelial monolayers results in a fivefold increase in electrical resistance. By contrast, expression of the mutant claudin-14 resulted in smaller increases in resistance. The mutants localized less well to tight junctions and were also found in lysosomes, suggesting an alteration in trafficking or stability. However, we observed no change in protein half-life and only a small shift in fractionation out of caveolin-enriched detergent-resistant membranes. Although less well localized to the tight junction, palmitoylation-deficient claudin-14 was still concentrated at sites of cell-cell contact and was competent to assemble into freeze-fracture strands when expressed in fibroblasts. These results demonstrate that palmitoylation of claudin-14 is required for efficient localization into tight junctions but not stability or strand assembly. Decreased ability of the mutants to alter resistance is probably the result of their less efficient localization into the barrier.**

Key words: Claudin, Claudins, Palmitoylation, Tight junctions

## Introduction

Claudins are critical structural and functional elements of the epithelial tight junction barrier. These small (20-23 kDa) tetraspan membrane proteins are the major determinants of transepithelial electrical resistance (Amasheh et al., 2002; Furuse et al., 2001; McCarthy et al., 2000; Van Itallie et al., 2001; Yu et al., 2003), paracellular ionic charge selectivity (Amasheh et al., 2002; Colegio et al., 2002; Van Itallie et al., 2001; Van Itallie et al., 2003; Yu et al., 2003) and possibly the size of molecules allowed to pass between cells (Nitta et al., 2003). Because of their importance in epithelial physiology, there is great interest in defining factors that regulate the ability of claudins to assemble into barriers and control their pore-like properties.

The physical site of the tight-junction barrier corresponds to continuous polymerized rows of claudins (Furuse et al., 1998), which pair in adhesive contacts (Kubota et al., 1999) with partner rows on adjacent cells. This characteristic organization can be visualized in freeze-fracture electron micrographic images as a network of branching strands crossing the lipid bilayer (Furuse et al., 1998). Surprisingly, a large proportion of some claudins is not in the strands but in intracellular vesicles or an unassembled state on the lateral or even basal surface (Rahner et al., 2001). Claudin-7, for example, localizes primarily to the basolateral surface in nephron segments (Li et al., 2004). Others are lateral in some instances, like claudin-4 in the colon, yet focused at the apical tight junction in other cell types, as is claudin-4 in pancreatic acinar cells (Rahner et al., 2001). These observations raise the important question of mechanisms controlling assembly into a barrier. Other identified transmembrane proteins of the tight-junction complex, such as occludin (Furuse et al., 1998) and junctional adhesion molecules (JAMs), are probably constituents of the strands but do not form strands without claudin.

Post-translational modifications, including phosphorylation or lipid modification are potential mechanisms for regulating function or assembly of claudins. There are reports implicating claudin phosphorylation state in functional changes in the paracellular barrier (Ishizaki et al., 2003; Yamauchi et al., 2004). Lipid modifications of claudins, however, have not yet been reported. Because all of the >24 claudin family members have conserved cysteines near the cytoplasmic ends of the

second and fourth transmembrane domains, we speculated that claudins might be targets for acylation with palmitic acid. Corresponding cysteines at these sites are modified with palmitic acid in other integral membrane proteins, including many G-protein-coupled receptors and tetraspanin proteins. The latter share a similar topology with the claudins (Stipp et al., 2003).

The role of palmitoylation in integral membrane proteins has variously been reported to influence trafficking (Berditchevski et al., 2002), protein-protein interactions (Berditchevski et al., 2002), internalization (Kawate and Menon, 1994) and degradation (Percherancier et al., 2001). In the case of the tetraspanin CD81 (Berditchevski et al., 2002), inhibition of palmitoylation by mutation of membrane-proximal cysteines leads to decreased association with other tetraspanins, inhibiting assembly of the so-called tetraspanin web. By analogy, it was conceivable that palmitoylation of claudins is required to assemble tight-junction strands. Tight junctions are claimed to exist in the specialized lipid environment of detergent-resistance domains (Nusrat et al., 2000), adding to speculation that lipid acylation could significantly influence their localization and function.

In this study, we provide the first demonstration that claudins are palmitoylated at two sets of membrane-proximal cysteines following the second and fourth transmembrane segments. Furthermore, we find that, in the case of claudin-14, inhibition of palmitoylation decreases its localization efficiency, decreases its association with detergent-resistant membranes and impairs its ability to form a functional paracellular barrier. However, preventing palmitoylation by removal of acylation sites does not affect protein half-life or the ability to form freeze-fracture fibrils in transfected fibroblasts.

## Materials and Methods

### Plasmid constructs and cell lines

Wild-type (WT) human claudin-14 in the pTRE vector (BD Biosciences) has been described previously (Ben Yosef et al., 2003). Claudins with mutations following either the second or the fourth transmembrane domains (claudin-14$^{CTM2S}$ and claudin-14$^{CTM4S}$, respectively) were generated using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) (Fig. 1). Mutation of the membrane-proximal cysteines at both transmembrane sites [Claudin-14$^{4S}$ (4S)] was done by digestion and ligation of the claudin-14$^{CTM2S}$ and claudin-14$^{CTM4S}$ mutants. For labeling experiments with [$^3$H]-palmitic acid, claudin-14 WT and mutants were made with an 11-amino-acid-terminal vesicular stomatitis virus glycoprotein (VSV-G) tag, as previously described (Medina et al., 2000). All constructs were verified by DNA sequencing in both directions. Clonal lines of Tet-off Madin-Darby canine kidney (MDCK) II cells (Clontech Laboratories, Palo Alto, CA) and Tet-off rat-1 cells (ATCC) were derived using standard transfection and selection techniques; regulated expression of the transgenes was carried out by varying the concentration of doxycycline in the medium as described elsewhere (Van Itallie et al., 2001). At least three to six clonal MDCK or rat-1 cell lines were generated for each construct. For electrophysiological, immunofluorescence and immunoblot analysis, cells were plated onto polycarbonate membrane filters (Costar® Snapwell®, Corning, NY), as described previously (Colegio et al., 2002).

### Metabolic labeling with [$^3$H]-palmitate and immunoprecipitation

Stably transfected Tet-off MDCK or rat-1 cells were plated into six-well dishes and induced by the removal of doxycycline for 48 hours; uninduced cells cultured in the presence of doxycycline or, in the case of rat-1 cells, untransfected cells were used as controls. Cells were placed in serum-free Dulbecco's modified Eagle's medium (DMEM) for 2 hours and then incubated with DMEM supplemented with 5% dialysed fetal bovine serum and [$^3$H]-palmitic acid (0.2 mCi ml$^{-1}$, Amersham Biosciences) for 3 hours. Cells were then washed three times with PBS, extracted in immunoprecipitation buffer (1.0% Triton X-100, 0.05% SDS, 0.2% deoxycholate, 25 mM Hepes, 140 mM NaCl, 1.5 mM $MgCl_2$, 1.0 mM EGTA, pH 7.4, plus protease inhibitors) for 20 minutes on ice. The extract was centrifuged at 10,000 ***g*** for 10 minutes and the supernatant was incubated for 60 minutes with a monoclonal antibody against VSV-G coupled to cyanogen-bromide-activated Sepharose. Bound material was washed four times with immunoprecipitation buffer, once with 25 mM Hepes, 140 mM NaCl, pH 7.4, and dissolved in SDS sample buffer. Samples were electrophoresed in paired gels and one was transferred to nitrocellulose and immunoblotted with antibody against either VSV-G or claudin-14. The second gel was fixed and prepared for fluorography by soaking in Amplify (Amersham Biosciences) according to the manufacturer's recommendations and subsequently dried and exposed to Hyperfilm MP (Amersham Biosciences) at –80°C for 3-4 weeks.

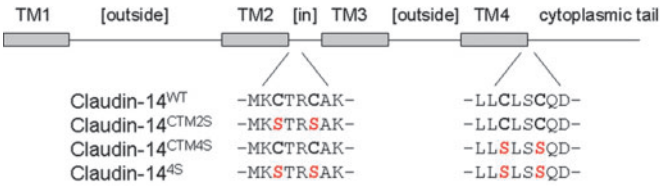


**Fig. 1.** Schematic diagram of claudin-14 to illustrate its four transmembrane domains (TM1-TM4), two extracellular loops, short intracellular domain and cytoplasmic tail. Sequences around the membrane-proximal cysteines are shown, with the pairs of cysteines indicated in bold. The cysteine-to-serine mutations are shown below the wild-type sequence. All sequences were cloned into a tetracycline-regulated expression vector; in some cases, wild-type and mutant claudin-14 were tagged at the C-terminus with an 11-amino-acid VSV-G tag.

### Immunoblots and immunofluorescence

Antibodies against claudins 14, 2 and 4, and against ZO-1 and occludin were all from Zymed Laboratories (South San Francisco, CA). Antibody against cadherin was purchased from Sigma, antibody against γ-tubulin was from Calbiochem (CA). Antibody against LAMP-2 was kindly supplied by the Rodriguez-Boulan laboratory (Weill Medical College, Cornell University, New York, NY). Antibodies against caveolin, GM130 and EEA-1 were from BD Transduction Laboratories. The monoclonal antibody against VSV-G was originally a gift from T. Kreis (deceased; University of Geneva, Switzerland) (Medina et al., 2000) and was used for immunoblots at a dilution of 1:1000. Immunoblots and immunofluorescence were performed as previously described, except that the secondary antibodies for immunoblots were goat anti-rabbit IRDye 800 (Rockland) and goat anti-mouse Alexa680 (Molecular Probes). Signals were detected and quantified using an Odyssey Infrared Imaging System (Li-Cor).

### Protein half-life studies

Claudin-14 WT- and 4S-expressing MDCK cells were cultured in 12-well dishes and induced in the absence of doxycycline for 3 days. Doxycycline was added to duplicate wells at 24 hours, 12 hours, 8

hours, 4 hours and 0 hours before collection. At 0 hours, cells were washed twice with PBS and dissolved in SDS sample buffer for immunoblot analysis. Signals were detected and quantified as described above using the Li-Cor Odyssey imaging system.

### Freeze-fracture electron microscopy

Freeze-fracture electron microscopy was carried out using established protocols (Coyne et al., 2003).

### Electrophysiology

Electrophysiological measurements were performed as previously described (Colegio et al., 2002).

### Detergent-resistant membranes

Claudin-14 WT- and 4S-expressing MDCK cells were cultured in 100 mm dishes and induced in the absence of doxycycline for 3-6 days. Detergent-resistant membranes (DRMs) were prepared as described elsewhere (Melkonian et al., 1999) with a few modifications. Briefly, cells were lysed for 30 minutes at 4°C in 0.5-1 ml TNE (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) supplemented with 1% Triton X-100 and protease inhibitor cocktail (Sigma Chemical, St Louis, MO) and 1 mM phenylmethylsulfonyl fluoride. The lysate was adjusted to 40% sucrose with an equal volume of 80% sucrose in TNE and overlaid with 6 ml 38% sucrose in TNE followed by 3 ml of 5% sucrose in TNE and centrifuged in an SW41 rotor (Beckman Instruments) at 110,000 ***g*** for 16 hours. The 5%/38% interface was collected and membranes pelleted at 120,000 ***g*** for 60 minutes, resuspended in SDS sample buffer and analysed by SDS-PAGE and immunoblotting. In some cases, the sucrose step gradient was scaled down to a final volume of 2.2 ml and centrifuged at 166,000 ***g*** for 3 hours in a Beckman TLS-55 rotor. In this case, 0.3 ml fractions were removed and fractions 2-7 were analysed by SDS-PAGE. Material in the pellet was not analysed. In all cases, after transfer to nitrocellulose, samples were analysed for expression of claudin-14 and caveolin by immunoblot as described above. Quantification was performed using the Li-Cor Odyssey system as described previously.

## Results

### Claudins are palmitoylated

All claudins have membrane-proximal cysteines in similar locations near the ends of the second and fourth transmembrane domains (Fig. 1). By analogy to comparable sequences in the tetraspanins (Stipp et al., 2003) and some other integral membrane proteins (Bijlmakers and Marsh, 2003; Kalinina and Fricker, 2003), we reasoned that these cysteines might be modified by fatty-acid acylation with palmitic acid. To test this possibility, we first used [$^3$H]-palmitic acid to label endogenous claudins in MDCK cells. Immunoprecipitation followed by immunoblotting and fluorography demonstrated incorporation of [$^3$H]-palmitic acid into claudin-2 (Fig. 2A) and claudin-4 (data not shown). To determine whether the membrane-proximal cysteines were the sites of palmitoylation, we transfected Tet-off MDCK II cells with wild-type claudin-14 tagged at the C-terminus with an 11-amino-acid VSV-G epitope and with similarly tagged claudin-14 containing three different cysteine-to-serine mutations, as indicated in Fig. 1. Multiple clones of stably expressing MDCK cells were isolated for each construct, and examples expressing about the same level of transgene were used to determine the level of incorporation of [$^3$H]-palmitic acid. As

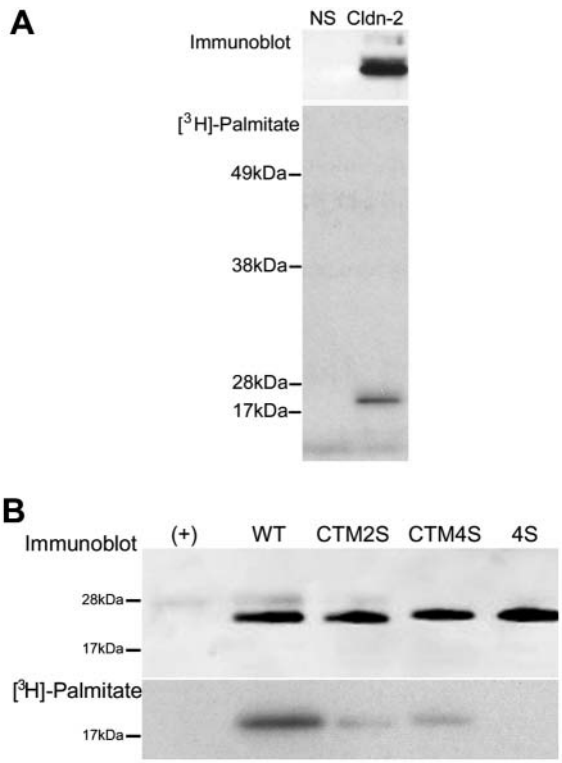


**Fig. 2.** (A) Incorporation of [$^3$H]-palmitate into endogenous claudin-2 in MDCK cells. MDCK cells were labeled with [$^3$H]-palmitic acid, extracted and immunoprecipitated. Samples were divided in half and electrophoresed on SDS-PAGE gels in parallel; one gel was transferred to nitrocellulose and immunoblotted for claudin-2 and the second was treated with Amplify fluorographic reagent (Amersham), dried and exposed to Hyperfilm MP for 3 weeks. (top) Immunoblot for claudin-2. NS refers to a nonspecific immunoprecipitate. (bottom) Signal from $^3$H-palmitate incorporation into claudin-2. Incorporation of $^3$H-palmitate into claudin-14 and C→S mutants. MDCK cells were stably transfected with wild-type and mutant claudin-14 with the VSV-G tag and transgene expression induced by removal of doxycycline. After 48 hours of induction, cells were labeled, extracted and immunoprecipitated as described above, but with a VSV-G monoclonal antibody. Lanes: (+), with doxycycline (not induced); WT, wild-type claudin-14; CTM2S, Cys→Ser mutation after the second transmembrane domain; CTM4S, Cys→Ser mutation after the fourth transmembrane domain; 4S, Cys→Ser mutation after the second and fourth transmembrane domains. (top) Immunoblot for VSV-G. (bottom) Signal from the incorporation of $^3$H-palmitic acid into the immunoprecipitated protein; this experiment was repeated with similar results. The faint band in the immunoblot above the claudin/VSV-G band is nonspecific signal from immunoglobulin light chain.

can be seen in Fig. 2B, 3 hours of incubation in the presence of [$^3$H]-palmitate resulted in label incorporation into wild-type claudin-14 expressed in MDCK cells, whereas the same labeling paradigm resulted in less incorporation into the cysteine-to-serine mutants at either of the two transmembrane sites. Mutation of the membrane-proximal cysteines at both of the transmembrane sites (4S mutant) eliminated any detectable incorporation of [$^3$H]-palmitic acid. We conclude that claudin-14 is palmitoylated following both the second and the fourth transmembrane segments.

### Induction of wild-type claudin-14 and all C→S mutants have similar effects on the levels of other tight-junction proteins

Claudin-14 and the mutants described above, but lacking VSV-G tags, were stably expressed in Tet-off MDCK cells under control of a tightly regulated doxycycline-repressible promoter. Multiple clones expressing each transgene were isolated and clones expressing similar transgene levels were used for immunoblot analysis. Expression of wild-type claudin-14 and mutant proteins did not affect endogenous levels of claudin-2, ZO-1 or cadherin (Fig. 3). However, expression of all transgenes resulted in a small decrease in claudin-4 expression and a dramatic decrease in occludin levels. The implications of the decreased levels of these proteins are not clear, but do not include disruption of the tight junction, because overexpression of wild-type claudin-14 results in increased transepithelial electrical resistance (TER; see below). Because the decrease in occludin levels was seen in cell lines expressing both WT and mutant claudin-14, it was not further analysed. Yu et al. (Yu et al., 2003) demonstrated that, when claudin-8 was expressed in MDCK cells, it replaced endogenous claudin-2, suggesting that induction of claudin-14 in these MDCK cells might replace claudin-4 and occludin.

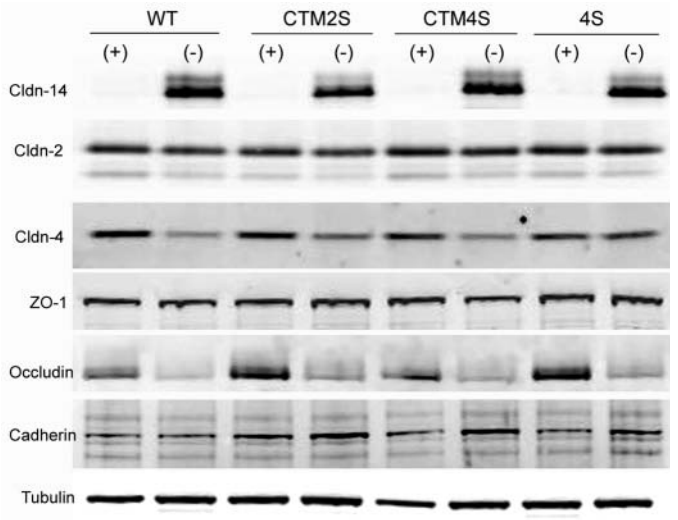


**Fig. 3.** Expression of wild-type and mutant claudin-14 and other tight-junction proteins in MDCK cells. Stably transfected Tet-off MDCK cells were plated on filters and induced or not for 4 days; filters were subjected to electrophysiological measurements and then protein was extracted by incubating filters directly into SDS sample buffer. Samples were electrophoresed and immunoblotted. Lanes: (+), with doxycycline (noninduced); (–), without doxycycline (induced). No endogenous claudin-14 could be detected in MDCK cells using either anti-human- or anti-mouse-claudin-14 antibodies. Transgene expression is tightly regulated by doxycycline. Expression of claudin-2, ZO-1, cadherin and tubulin were unaffected by claudin-14 expression, whereas the levels of both claudin-4 and occludin were downregulated by transgene induction. This immunoblot represents results from a single clone of the wild type and each mutant, but induction of transgenes were verified in all clones used in physiological experiments (three to six for the wild type and each mutant).

### Increased TER induced by wild-type claudin-14 is diminished by mutation of the palmitoylation sites

In a previous study, we demonstrated that inducing expression of claudin-14 increased TER in MDCK cell monolayers (Ben Yosef et al., 2003). Using the same technique, we compared TER in MDCK cells expressing wild-type and mutant claudin-14 (Fig. 4A). Wild-type claudin-14 expression resulted in a large increase in TER as described previously, whereas mutations following either the second (CTM2S) or the fourth (CTM4S) transmembrane domain increased TER but less than the wild-type protein. Claudin-14 mutated at both transmembrane sites (4S) showed only a small change in TER relative to baseline, uninduced levels. Although all induced

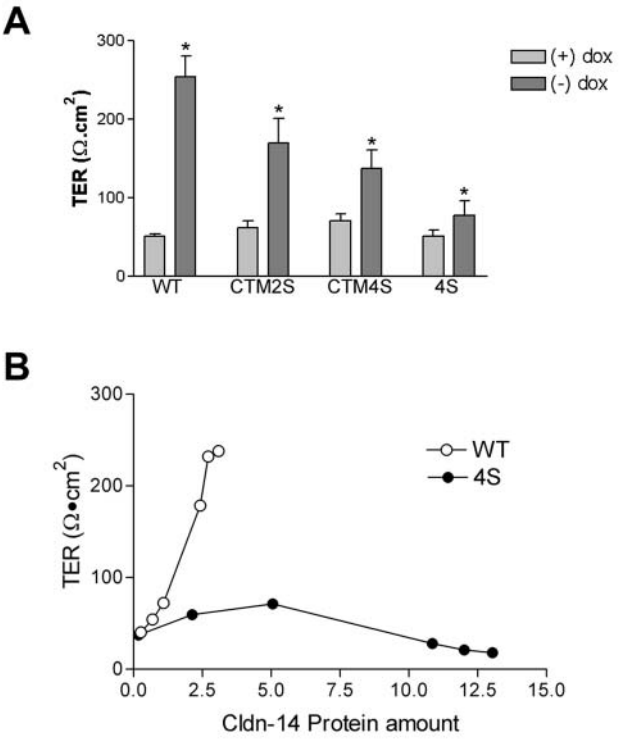


**Fig. 4.** (A) TER of MDCK cells expressing wild-type and mutant claudin-14. Tet-off MDCK cells were cultured on filters and induced, or not, to express wild-type and palmitoylation-deficient claudin-14. Light gray bars indicate uninduced MDCK cells; dark gray bars indicate induced MDCK cells. Values are means ± s.e.m. of three to six separately derived clonal cell lines for wild-type claudin-14 and each mutant. Expression of wild-type claudin-14 in MDCK cells results in a fivefold increase in TER. Mutation of either of the palmitoylation sites singly or in combination decreases the ability of claudin-14 to raise the TER. *, $P<0.05$ compared with wild-type as determined by ANOVA followed by Dunnett's test. (B) Effects on TER of graded induction of wild-type claudin-14 (open circles) and 4S mutant protein (closed circles). Tet-off MDCK cells stably expressing the transgenes of claudin 14 and mutants were variably induced to express transgenes by using different doses of doxycycline. Because the same antibody was used to detect both wild-type and mutant claudin-14, protein levels could be directly compared after quantification using the Li-Cor Odyssey detection system (*x*-axis marked with arbitrary units). Although the levels of the 4S mutant could be induced to more than four-times-higher levels than in wild-type claudin-14, there was no significant increase in TER in MDCK cells expressing the mutant protein.

mutant clones showed decreased TER relative to the behavior of wild-type claudin-14, there was more variability among clones than we have seen in other experiments expressing claudins 2, 4 or 11 (Van Itallie et al., 2003).

To test whether the difference in TER were simply a result of differences in the expression levels of wild-type and mutant claudin-14, MDCK cells expressing either wild-type claudin-14 or the 4S mutant were cultured in graded concentrations of doxycycline to produce variable levels of transgene expression. As can be seen in Fig. 4B, TER increases steeply with increasing wild-type claudin-14, whereas there is little increase in TER in 4S-expressing cells, even though the expression level for the mutant protein was up to six times higher than that of the WT protein.

## Claudin-14 mutants localize to the plasma membrane less efficiently than WT claudin-14

One possible explanation for the diminished ability of the palmitoylation-deficient mutant to increase TER relative to wild-type was mislocalization of the proteins, and so we next performed immunofluorescent analysis of the induced transgenes (Fig. 5). Wild-type claudin-14 was not detectable in the absence of induction and, when cells were induced, the transgene was mostly colocalized with ZO-1 to the apical-junctional complex. Along with the junction-associated claudin-14, there was also a minor amount of intracellular wild-type claudin-14 in induced MDCK cells. The nature of these small puncta containing both ZO-1 and claudin-4 is unknown; they might be small vesicles. This pattern has also been reported following expression of other claudins in MDCK cells (McCarthy et al., 2000).

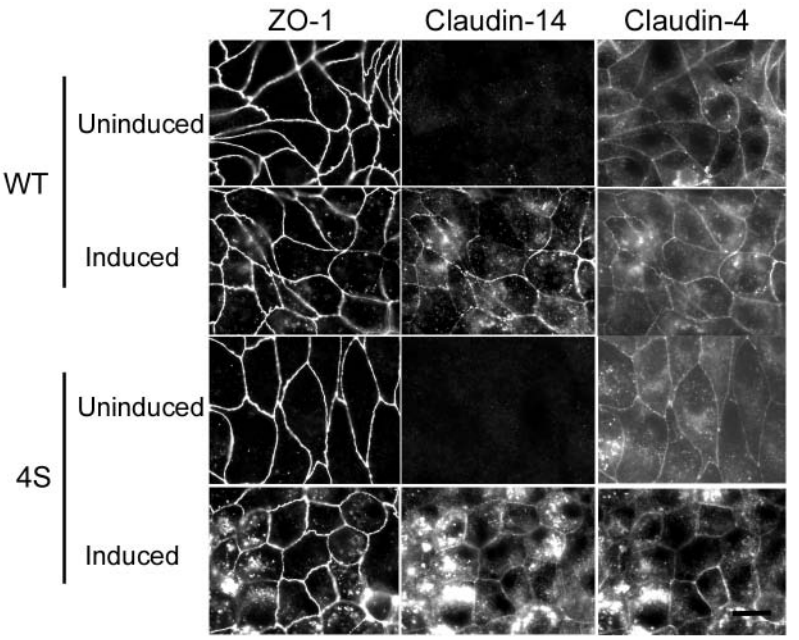


**Fig. 5.** Localization of wild-type claudin-14 and 4S mutant. Wild-type claudin-14 colocalizes with ZO-1 to the tight junction, whereas the palmitoylation-deficient mutant 4S is found both at the tight junction and in large intracellular aggregates. Stable cell lines were induced or not for 4 days and processed for immunofluorescent analysis of tight-junction proteins. Neither wild-type nor mutant claudin-14 is detectable in uninduced Tet-off MDCK cells (WT and 4S, uninduced); ZO-1 immunofluorescence (left) is concentrated at the apical junctional complex, whereas endogenous claudin-4 (right) is found both at the cell membrane and in some intracellular puncta. Wild-type claudin-14 localizes mostly to tight junctions with ZO-1 (WT, induced) but does mediate some intracellular accumulation of both ZO-1 (left) and claudin-4 (right). The 4S mutant is found both at the tight junction and in large intracellular aggregates (4S, induced), where it colocalizes with ZO-1 and claudin-4. Scale bar, 10 μm.

Immunofluorescent localization of the 4S mutant (as well as the CTM2S and CTM4S, not shown) demonstrated its colocalization with ZO-1 to the membrane, but much of the staining was in large intracellular aggregates that appeared to be vesicular (Fig. 6). These intracellular aggregates also contained occludin and claudin-2 (not shown). Colocalization with LAMP-2 (Nabi et al., 1991) identified these structures as lysosomes (Fig. 6); there was no colocalization with Golgi (GM130) or early endosome (EEA1) markers (not shown). Some ZO-1, claudin-4 and occludin (data not shown for occludin and claudin-4) were also localized to lysosomes in cells expressing mutant claudin-14, suggesting that interactions of these proteins with the mutant claudin-14 were preserved. It is not clear whether the lysosomal localization of the mutant claudin-14 and the other tight-junction proteins was due to a defect in the synthetic trafficking pathway, which would suggest that this complex is assembled before it reaches the tight junction. More likely, the mutant claudin-14 is retrieved from the membrane with associated protein and sequestered into lysosomes. To distinguish between these possibilities and because so much of the mutant claudin appeared to be intracellular, we attempted to determine the amount of surface-expressed wild-type and mutant claudin-14 by labeling with membrane-impermeant biotin compounds. Unfortunately, although biotin labeled the apical and basolateral membranes, it was excluded from the tight junction, as confirmed by confocal *z*-axis imaging (data not shown).

In some (Melkonian et al., 1999) but not all (Yang et al., 2002; Kalinina and Fricker, 2003) cases, palmitoylation of proteins enhances their association with detergent-resistant lipid rafts. To test whether palmitoylation of claudin-14 influenced its partitioning into lipid rafts, we compared the relative amounts of wild-type and mutant claudin-14 in DRMs (Fig. 7A,B). As previously described, caveolin is concentrated at the interface of the 5%/38% sucrose (fraction 2), identifying the biochemically defined DRMs (Kurzchalia and Parton, 1999). There was no difference between the caveolin distribution in cells induced to express wild-type claudin-14 and that in those expressing the mutant protein. However, although wild-type claudin-14 was also concentrated at the 5%/38% sucrose interface, the mutant claudin-14 was equally distributed at this interface and throughout 38-40% fractions from the gradient. However, neither the distribution of occludin nor claudin-3, which were only partially sequestered with mutant claudin-14 in lysosomes, differed between cells expressing wild-type claudin-14 or the 4S mutant (not shown). The altered association of the mutant claudin-14 with rafts was not unexpected, given the dramatically different subcellular

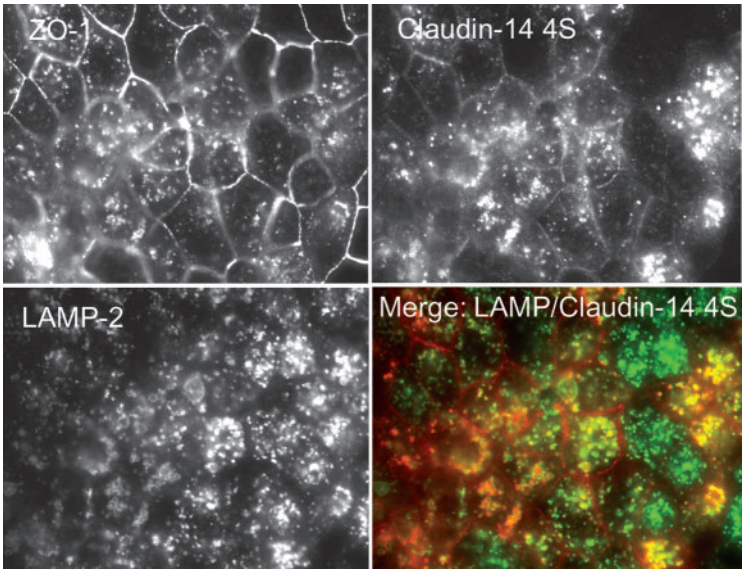


**Fig. 6.** Colocalization of the palmitoylation-deficient mutants of claudin-14 with the lysosomal membrane protein LAMP-2. Tet-off MDCK cells transfected with the palmitoylation-deficient mutant of claudin-14 were plated on filters and induced to express the transgene. As demonstrated in Fig. 5, the 4S mutant of claudin-14 was found at cell borders and in large intracellular aggregates (top right). ZO-1 (top left) was concentrated at the apical cell membrane, but was also found in large intracellular aggregates. LAMP-2-positive structures (bottom left) did not appear to be affected by induction of mutant protein (not shown). Immunofluorescent analysis revealed colocalization (bottom right) of the intracellular fraction of claudin-14 4S mutant (red) with LAMP-2 (green). Staining with antibodies against other intracellular compartments (early endosomes, Golgi) were negative for colocalization with mutant claudin-14. Scale bar, 10 μm.

distribution of this protein compared with wild-type in immunofluorescent analysis.

## Palmitoylation does not affect the half-life of claudin-14

Palmitoylation has been reported to be a requirement for the stability of some (Percherancier et al., 2001) but not all (Kalinina and Fricker, 2003) membrane proteins. This finding, together with the localization of the mutant claudins to lysosomes, prompted us to determine the half-life of induced wild-type claudin-14 and the 4S mutant. Induction of the transgenes, followed by addition of doxycycline to repress transgene expression revealed identical half-lives for wild-type and mutant claudins (Fig. 8), suggesting that differences in localization do not imply major differences in protein stability.

## Both junction targeting of mutant claudin-14 and its ability to raise TER increase with extended induction time

The main effect of preventing palmitoylation that we could document was a decreased efficiency of targeting claudin-14 to the tight junction. The studies presented above focus on cells induced to express the transgene proteins for 4 days; we next asked whether extending the period of induction influenced efficiency of targeting. Unexpectedly, by 8 days and at 12 days in culture, the localization of the mutant claudin-14 4S was progressively enhanced and approximated that of wild-type claudin-14 (Fig. 9A). Coincident with improved targeting, there was an increased impact on TER, which approached levels induced by expressing wild-type protein (Fig. 9B). Although the mechanism responsible for this progressive normalization of localization is unclear, it plainly demonstrates that, when non-

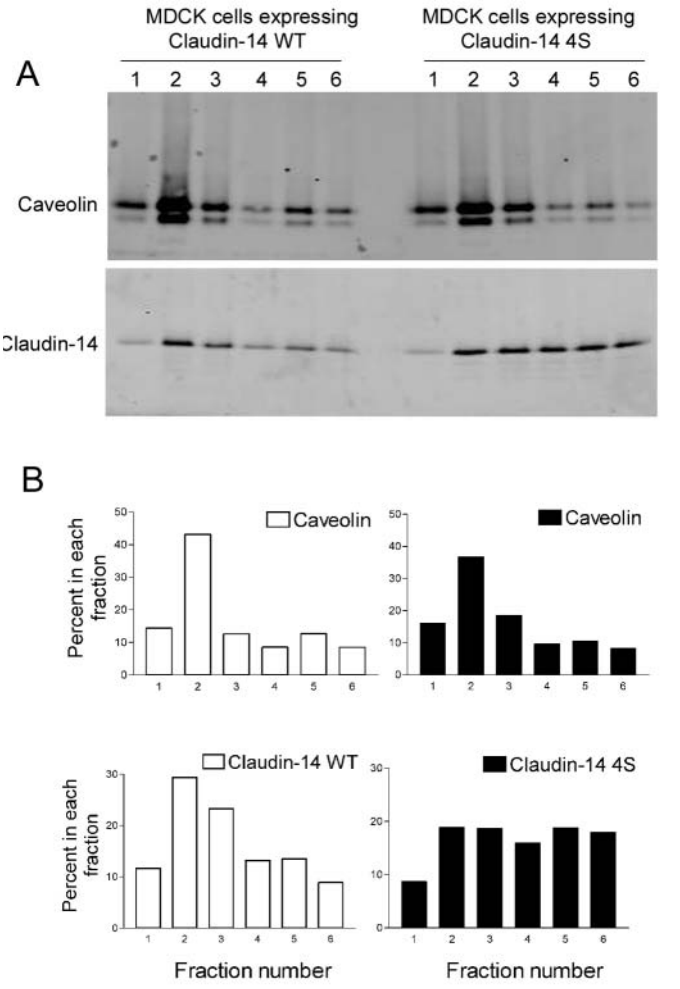


**Fig. 7.** Association of wild-type and claudin-14 with DRMs. MDCK cells were induced to express either wild-type (left) or mutant (right) claudin-14. DRMs were prepared as described and fractions of sucrose gradients were electrophoresed, transferred to nitrocellulose and immunoblotted as previously described. Caveolin (A,B, top) was used an indicator of for DRMs and was concentrated at the 5-38% interface (fraction 2). Wild-type claudin-14 was concentrated in the same fraction, whereas the 4S mutant was found equally at this interface and throughout the rest of the gradient (A,B, bottom). B represents quantification (white bars, caveolin and claudin-14 from cells expressing wild-type claudin-14; black bars, caveolin and claudin-14 from cells expressing 4S mutant) of the immunoblot shown in A; replicate experiments gave similar results.

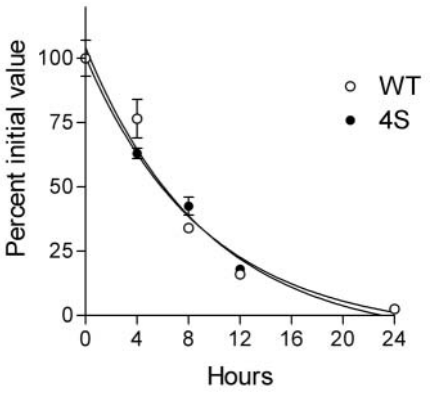


**Fig. 8.** Half-lives of wild-type claudin-14 and 4S mutant. Tet-off MDCK cells stably transfected with wild-type claudin-14 or the 4S mutant were induced to express the transgenes for 4 days. At day 4, doxycyline was added to repress transgene induction and cells were collected at the indicated times and processed for immunoblot analysis. The amount of starting protein was normalized to 100% so as to be able better to compare half-lifes. Open circles, wild-type claudin-14; filled circles, 4S mutant. Points are the means and ranges of duplicate samples. There was no difference in protein half-life in this experiment or in a replicate experiment performed on different clones expressing the same proteins; calculated half-lifes of both proteins were close to 6 hours.

palmitoylated protein becomes junction associated, it is capable of controlling the electrical barrier.

### Palmitoylation state does not affect the ability to form freeze-fracture strands in fibroblasts

As shown above, the mutant claudins had diminished TER and apparently also showed less localization to the tight junction in epithelial cells. Although the time-course experiment demonstrated the functionality of the non-palmitoylated mutant protein, it was possible that palmitoylation altered the ability of claudin assembly into tight-junction strands. To test this hypothesis, we expressed wild-type claudin-14 and the 4S mutant in Tet-off rat-1 fibroblasts, and examined their ability to form tight-junction strands. Rat-1 cells, like MDCK cells, are capable of palmitoylating the wild-type claudin-14 but not the 4S mutant (Fig. 10A). Immunofluorescent analysis of wild-type and 4S-mutant-expressing fibroblasts demonstrates that both transgenes can concentrate at sites of cell-cell contact, suggesting that they might be adhesive (Fig. 10B). When prepared for freeze-fracture and examined by electron microscopy (Fig. 10C), rat-1 cells expressing the wild-type claudin-14 were seen to express patches of tight-junction strands, as has been seen in other fibroblasts that have been transfected with different claudins (Furuse et al., 1998). The claudin-14 4S mutant also formed similar patches of freeze-fracture strands, indicating that this modification is not required for organization of claudins into typical tight-junction strands.

## Discussion

In this study, we demonstrate that claudins are multiply palmitoylated on membrane-proximal cysteines. Although expression of wild-type claudin-14 results in fivefold increases in TER in MDCK cells, inhibition of its palmitoylation impairs the ability to raise TER in MDCK cells, apparently by decreasing the efficiency of intracellular trafficking of claudin-14. Because the inhibition of claudin-14 palmitoylation was achieved by mutating the cysteine acceptor sites to serines, it is possible that alteration of these sequences disrupts normal protein folding or interactions with other proteins unrelated to palmitoylation state. In spite of this caveat, it is clear that

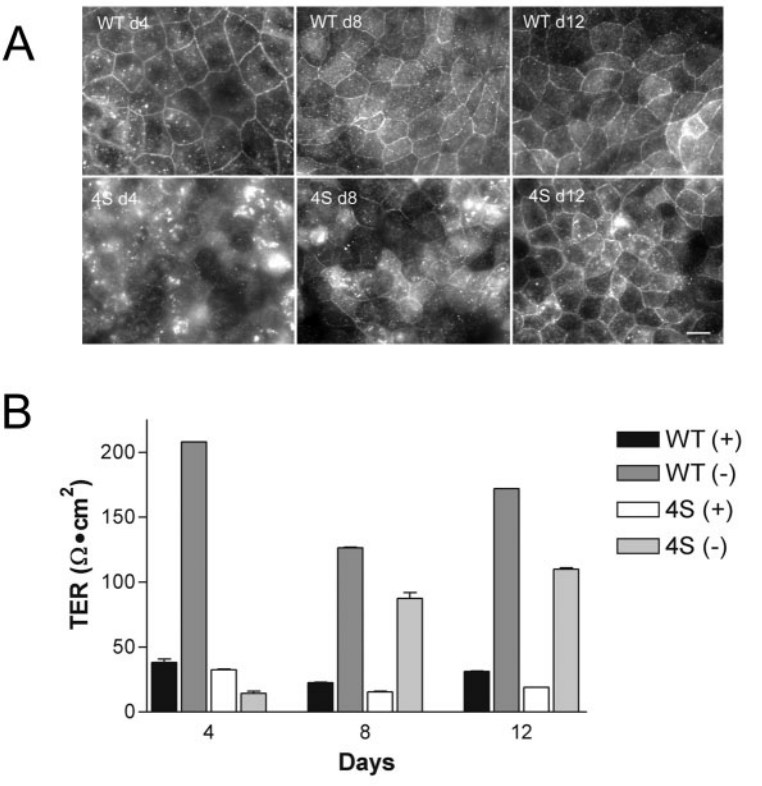


**Fig. 9.** (A) Localization of wild-type and the 4S mutant of claudin-14 at 4 days, 8 days and 12 days in culture. Filters used for determination of TER (below) were processed for immunofluorescent analysis. As shown in Fig. 5, wild-type claudin-14 localizes to cell borders and small intracellular puncta after 4 days of induction, and this localization is unaltered after 8 days or 12 days in culture. The 4S mutant of claudin-14 is found in large intracellular aggregates at day 4 but, at days 8 and 12, this same claudin-14 mutant is predominantly localized to cell borders, similar to the wild-type distribution. (B) TER of MDCK cells expressing wild-type and the 4S mutant of claudin-14 after extended periods of time in culture. Tet-off MDCK cells were cultured on filters as described previously and induced or not to express wild-type (uninduced, black bars; induced, dark gray bars) and the 4S mutant of claudin-14 (uninduced, white bars, induced, light gray bars); values are means ± range for duplicate wells. TER was determined in replicate filters at 4 days, 8 days and 12 days. At 4 days, expression of wild-type claudin-14 resulted in increased TER similar to that shown in Fig. 4, whereas expression of this 4S mutant resulted in decreased TER relative to uninduced control. After 8 days and 12 days of induction, TER was increased three to five times in both the wild-type and 4S-expressing cells.

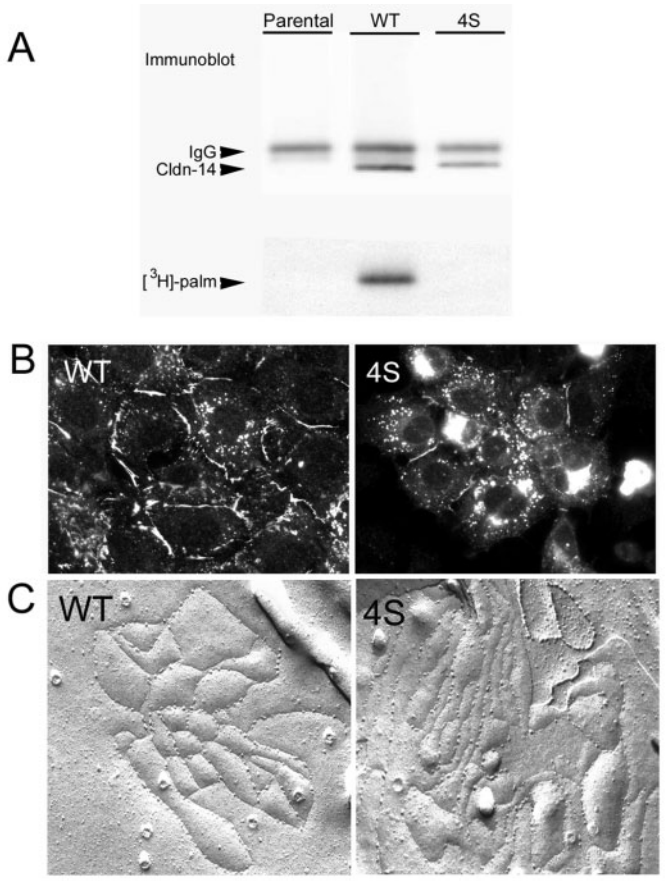


**Fig. 10.** (A) Claudin-14 but not the 4S mutant is palmitoylated in rat-1 cells. Tet-off rat-1 cells were stably transfected with VSV-G-tagged claudin-14 and 4S mutant, and expression of the transgene was induced by removal of doxycycline. Cells were labeled with $^{3}$H-palmitic acid; immunoblot analysis (top) confirmed expression of both wild-type and mutant claudin-14, and the replicate fluorograph (bottom) demonstrated palmitoylation of wild-type but not mutant claudin-14. (B) Immunofluorescent analysis of rat-1 fibroblasts induced to express wild-type claudin-14 (left) and the 4S mutant (right). Both wild-type and 4S mutant could concentrate at sites of cell-cell contact. Scale bar, 10 μm. (C) Freeze-fracture analysis revealed that both wild-type and mutant claudin-14 form similar patches of tight-junction strands. Claudin-14 and 4S mutant were cultured on glass coverslips, induced for 4 days, fixed with glutaraldehyde and prepared for freeze fracture by a conventional protocol. Electron microscopic freeze-fracture analysis of wild-type (left) and the mutant claudin (right) revealed similar appearing tight-junction strands.

palmitoylation of claudin-14 is not absolutely required for cell surface localization, regulation of TER or tight-junction strand formation, and that the claudin-14 half-life is not affected by palmitoylation.

The finding that juxtamembrane sequences of claudins are multiply palmitoylated was not unexpected, considering the presence of both conserved cysteines and the precedent of palmitoylation of similar sequences in other integral membrane proteins (Bijlmakers and Marsh, 2003). The specific sequence requirements for palmitoylation are not fully defined but, in G-protein-coupled receptors, the presence of nearby hydrophobic and basic residues are associated with palmitoylation (Bijlmakers and Marsh, 2003). Both these features are found around the claudin-14 palmitoylation sites. However, because this sequence is associated with membrane-proximal domains, the nearby presence of both hydrophobic and charged residues is unsurprising. Multiple palmitoylation like that seen in claudin-14 has been described for other integral membrane proteins, including the tetraspanin proteins, in which sites near all four transmembrane domains are reported to be *S*-acylated (Stipp et al., 2003). For example, CD151 is palmitoylated on six cysteines and simultaneous mutation of all six is required to eliminate palmitoylation (Berditchevski et al., 2002). However, results from the tetraspanins demonstrate that, although multiple members of this family are palmitoylated, not all membrane-proximal cysteines are modified in all tetraspanins (Berditchevski et al., 2002). Although the sites that we modified in claudin-14 are the most conserved among the claudins, there are other potential palmitoylation sites in some members of this family. Most notably, some claudins, including claudin-14, have additional cysteines within the second transmembrane domain. Most claudins, but not claudin-14, have one or two additional cysteines in the membrane proximal region associated with the fourth transmembrane domain. The sites of the extra cysteines in claudin-14 are not conserved among the claudins and lie deeper within the second transmembrane domain than the sites shown in Fig. 1, but their presence means that even the 4S mutant of claudin-14 might be palmitoylated. However, long exposures of [$^{3}$H]-labeled proteins did not reveal any incorporation of palmitic acid in the 4S mutant, consistent with the *S*-acylation being restricted to more conserved sites.

Although the function of palmitoylation of integral membrane proteins is not fully defined, mutations that eliminate or decrease palmitoylation on various transmembrane proteins result in various types of trafficking defects, including inefficient plasma-membrane expression and decreased endocytosis. In the cases of several receptors, however, the decreased proportion of receptor that is properly localized remains functional. For example, the chemokine and HIV receptor CCR5 is palmitoylated on cysteines in a region near the end of the seventh transmembrane domain. When this sequence cannot be palmitoylated, the protein accumulates in intracellular stores, surface expression is greatly decreased and the receptor half-life is profoundly decreased. However, although membrane expression is decreased, nonpalmitoylated plasma-membrane receptor is still able to interact normally with ligands (Percherancier et al., 2001). Similarly palmitoylation of the human thyrotropin receptor (Tanaka et al., 1998) and the $V_2$ vasopressin receptor (Sadeghi et al., 1997) are required for efficient transit to the plasma membrane but do not affect the functioning of appropriately localized receptors. Similarly, our results are consistent with a requirement for palmitoylation for efficient targeting but not for the ability to regulate the junction barrier when protein is properly targeted. Both the findings that the claudin 4S mutant did not increase TER as effectively as the wild-type protein and that the immunofluorescent distribution of the mutant proteins are consistent with a decrease in the efficiency of targeting to the tight junction. However, unlike membrane receptors, claudin functionality can only be assessed when these proteins are assembled into tight junctions, so that decreased surface expression would by itself be expected to result in decreased performance.

For some proteins (Wong and Schlichter, 2004), one important function of palmitoylation is to enhance localization to specialized membrane domains known as lipid rafts. The T-cell transmembrane adaptor protein LAT associates with lipid rafts only when palmitoylated; mutation of palmitoylation sites prevents both this association and T-cell activation (Zhang et al., 1998). However, for other proteins, the presence or absence of palmitoylation has little influence on association with lipid rafts. Although tetraspanins are sometimes associated with lipid rafts, the buoyant density of tetraspanins that could not be palmitoylated was no different to that of wild-type proteins, suggesting that targeting to detergent-resistant lipid rafts is not the main function of palmitoylation of these proteins (Stipp et al., 2003). Palmitoylation of carboxypeptidase D also has no effect on lipid-raft localization (Kalinina and Fricker, 2003). In the present study, we find that transfected wild-type claudin-14 is enriched in the fractions containing caveolin relative to the mutant protein. This result is consistent with the inability of the mutant protein to be either efficiently targeted or maintained at the tight junctions. The other identified post-translational modification of claudins (phosphorylation) appears to influence claudin expression (Ishizaki et al., 2003) and function (Yamauchi et al., 2004) but not to be specifically important in targeting.

The organization of some higher-order protein-protein interactions has been demonstrated to be palmitoylation dependent. In the case of transmembrane proteins, one example is the requirement of palmitoylation for the formation of a functional tetraspanin web (Charrin et al., 2002). By contrast, some interactions of tetraspanins and associated proteins are unaffected by the absence of palmitoylation (Berditchevski et al., 2002). Both wild-type claudin-14 and at least some of the 4S mutant were concentrated at sites of cell-cell contact in rat-1 fibroblasts, suggesting that both constructs are capable of cell-cell adhesion, as has been described for other claudins (Kubota et al., 1999). Additionally, freeze-fracture analysis of wild-type claudin-14 and the 4S mutant reveal that both proteins were able to form normal-appearing strands in rat-1 fibroblasts. Although these observations are not quantitative, they suggest that at least some normal side-to-side and cell-to-cell interactions were preserved in the palmitoylation-deficient mutants.

Palmitoylation contributes to diverse functions in different integral membrane proteins. Even within the G-protein-coupled receptor family, although palmitoylation is conserved across different family members, the functional implications of this modification are surprisingly divergent (Tanaka et al., 1998). However, overall, one of the most common outcomes of inhibition of palmitoylation is a decreased efficiency in protein trafficking. This is consistent with our findings with claudin-14, in which mutation of palmitoylation sites alters intracellular localization, representing either a decrease in the efficiency of delivery of claudin-14 to the plasma membrane or a change in the membrane recycling patterns. However, the demonstration that palmitoylation-deficient claudin-14 is competent to form strands suggests that, like other examples of transmembrane proteins, palmitoylation might decrease the quantity of membrane-associated protein but not its final behavior at this site.

We acknowledge helpful discussions with A. Fanning (UNC) and M. Caplan (Yale University) and the technical support of L. Britton (Wellesley College). This research was supported by NIH DK 034134 and the State of North Carolina.

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