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Highly Conserved Cysteines Are Involved in the Oligomerization of Occludin—Redox Dependency of the Second Extracellular Loop

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Abstract

The tight junction (TJ) marker occludin is a 4-transmembrane domain (TMD) protein with unclear physiological and pathological functions, interacting with other TJ proteins. It oligomerizes and is redox sensitive. However, oligomerization sites and mechanisms are unknown. **Aims:** To identify hypoxia-sensitive binding sites, we investigated the consequences of amino-acid substitutions of highly conserved cysteines in human occludin, under normal and hypoxic incubations. **Results:** (i) The extracellular loop 2 (ECL2) showed homophilic *trans*- and *cis*-association between opposing cells and along the cell membrane, respectively, caused by a loop properly folded *via* an intraloop disulfide bridge between the shielded C216 and C237. Hypoxia and reductants prevented the associations. (ii) C82 in TMD1 directly *cis*-associated without disulfide formation. (iii) C76 in TMD1 and C148 in TMD2 limited the *trans*-interaction; C76 also limited occludin-related paracellular tightness and changed the strand morphology of claudin-1. (iv) The diminished binding strength found after substituting C82, C216, or C237 was accompanied by increased occludin mobility in the cell membrane. **Innovation:** The data enable the first experimentally proven structural model of occludin and its homophilic interaction sites, in which the ECL2, *via* intraloop disulfide formation, has a central role in occludin's hypoxia-sensitive oligomerization and to regulate the structure of TJs. **Conclusion:** Our findings support the new concept that occludin acts as a hypoxiasensor and contributes toward regulating the TJ assembly redox dependently. This is of pathogenic relevance for tissue barrier injury with reducing conditions. The ECL2 disulfide might be a model for four TMD proteins in TJs with two conserved cysteines in an ECL. *Antioxid. Redox Signal.* 20, 855–867.

Introduction

THE JUNCTIONAL COMPLEX between endothelial and epithelial cells consists of tight junctions (TJs), adherens junctions, desmosomes, and gap junctions. Cell layers with TJs separate lumina of the organisms, which is important in maintaining specific organ functions. TJs play a crucial role in cell polarity and regulation of paracellular permeation of ions and solutes. Thus, TJs provide the basis for directed transport and maintaining homeostasis in the organism. Moreover, TJs play an important role in various signaling processes, in cell cycle, proliferation, and migration (2, 15, 31).

The TJs are mainly formed by transmembranous proteins, such as claudins, occludin, tricellulin, marvelD3, and junc-

tional adhesion molecules. These proteins undergo homo- and hetero-oligomerization (9), and are scaffolded by submembranous *zonula occludens* proteins (14). Other associated proteins are involved in different cellular functions, including cingulin, vinculin, G-proteins, regulator protein of G-protein signaling 5, ahnak, β -subunit of the L-type calcium channel (1), transcription factor ZONAB (2), or Na^+/K^+ -ATPase (28).

The first identified transmembrane TJ protein was occludin (12), containing four transmembrane domains (TMDs), two extracellular loops (ECL) ~ 45 amino acids (aa's), a small intracellular loop, ~ 10 aa, and cytosolic N- and C-termini. Occludin, tricellulin, and marvelD3 belong to the TJ-associated marvel proteins. The function of occludin remains unclear. Its crystal structure is not available (3). However, it is assumed to

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Innovation

The function of occludin, marker protein of the tight junctions (TJs) in all tissue barriers, is unclear. Our results demonstrate that the occludin oligomerization regulates the function and structure of TJs. The extracellular loop 2 (ECL2) forms an intraloop disulfide bridge, which is identified as playing a central role in the homo-oligomerization and is hypoxia sensitive *via* cysteines, giving the ECL2 a specific fold, which indirectly regulates the intermolecular occludin association such as a hypoxia sensor. The innovation is the identification of oligomerization sites enabling the first experimentally supported structural model of occludin oligomers. The hypoxia sensitivity is of great relevance for diseases accompanied by hypoxic events, such as ischemia, inflammation, or tumors affecting tissue barriers.

play a regulatory role, as it has a specific region at the cytosolic C-terminal coiled coil domain (24) with multiple phosphorylation sites (3, 10). Occludin knockdown in Madin Darby canine kidney II cells (MDCK-II) alters their claudin composition, thus affecting permeability (38). In addition, occludin exhibits redox-dependent alterations in expression (7), localization (8), and oligomerization (19, 33). Upon hypoxia, occludin is the first protein in the TJs of the blood-brain barrier to be directed toward the cytosol (20). Occludin is an indicator of oxidative stress, for example, early after the onset of stroke or in other pathological conditions accompanied by changes in the redox status, including various infections, inflammation, and some cancer types, as reviewed (3).

Under reducing conditions, cysteine residues possess free sulfhydryl residues, but may form disulfide bridges under an oxidative environment. Therefore, the question was, which cysteines in occludin are hypoxia sensitive? Sequence alignment between species revealed five highly conserved cysteines, three localized within TMD1 and TMD2 and two more in the ECL2; the latter two are postulated, but not yet proved to form intramolecular disulfide bonds (3). We, therefore, studied the oligomerization of human occludin after a single exchange of the C76, C82 (TMD1), C148 (TMD2), C216, and C237 (ECL2). The studies were mainly performed in TJ-free human embryonic kidney (HEK-293) cells, in order to prevent interference from other TJ proteins. For the first time, we demonstrated that residues C216 and C237 provided an intraloop disulfide bridge and, thus, indirectly influenced *trans*- and *cis*-interactions in a redox-dependent manner. Furthermore, residue C82, without forming a disulfide bond, is a part of the binding area exposed by TMD1. C76 and C148 of TMD1 and TMD2, respectively, may prevent excessively strong oligomerization and barrier tightening.

Results

Alterations in localization, and in the trans- and cis-interactions of wild-type occludin due to hypoxia

When expressed in HEK-293 cells, yellow fluorescence protein (YFP)-occludin was found mainly in the cell membrane and enriched at cell-cell contacts between two expressing cells (Fig. 1A). In order to obtain greater insight into the

oligomerization of occludin under pathological conditions, cells were studied under hypoxic conditions, resulting in drastic changes. The membrane pool of occludin was partially shifted to the cytosol, resulting in a membrane to cytosol ratio of 0.55 ± 0.09 for the hypoxic wild type (wt) relative to the normoxic wt control (Fig. 1D, left two columns). Similar results were found for wt occludin transfected into MDCK-II cells. In this case, the membrane localization of occludin was not only higher than in HEK-293 cells but was also reduced by about 50% due to hypoxia. In HEK-293 cells, hypoxia affected occludin's *trans*-interaction and reduced contact enrichment to $60\% \pm 16\%$ relative to normoxia (Fig. 1E, left two columns). *cis* interactions of occludin—association of two molecules expressed within the same plasma membrane—were also hypoxia sensitive. The relative fluorescence resonance energy transfer (FRET) efficiency of the cotransfected cyan fluorescence protein (CFP)-occludin^{wt} and YFP-occludin^{wt} pair was significantly reduced to 0.57 ± 0.07 under hypoxia relative to normoxic incubation (Fig. 2, bar chart, left two columns).

Cysteine loss in the ECL2 abolished enrichment of occludin in the cell membrane

To show that cysteines are involved in the hypoxia sensitivity of human occludin, we substituted cysteine on positions C76, C82, C148, C216, and C237 with alanine. In normoxic HEK-293 cells, only C216A and C237A in the ECL2 reduced the occludin's membrane pool to 0.52 ± 0.04 and 0.31 ± 0.03 , respectively, relative to the wt (Fig. 1D). The difference in localization of wt and C216A was comparable when transfected into MDCK-II cells (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). In HEK cells, the ECL2 mutants were the only mutants that were unable to respond to hypoxia (Fig. 1D) after the loss of cysteine. The mutants of the TMDs reacted in a similar manner to hypoxia as the wt, with a reduction in membrane localization from 0.87 ± 0.16 to 0.39 ± 0.04 for C76A, 0.86 ± 0.08 to 0.73 ± 0.05 for C82A, and 0.87 ± 0.15 to 0.52 ± 0.06 for C148A (Fig. 1D). In addition, the phenotypic reduction of membrane localization caused by hypoxia of the wt (Fig. 1F) differed from the phenotype of membrane localization of the ECL2 cysteine substitutions under normoxic conditions (Fig. 1C). The ECL2 mutants showed smaller aggregates (Fig. 1C) than the wt under hypoxia (Fig. 1F). These aggregates were redistributed into the endoplasmic reticulum (ER) to a significant extent, although another fraction clearly reached the plasma membrane (Fig. 1C). This distribution was evaluated by cotransfecting the constructs with CFP-tagged ER marker CFP-ER (BD Biosciences) (26); almost 100% of the marker was then colocalized with the ECL2 mutants, with regard to the intracellular structures.

Alterations in localization, trans- and cis-interaction of occludin by a reducing agent are caused in the membrane and via cysteines in the ECL2 of occludin

To study another type of reducing conditions the reducing agent dithiothreitol (DTT) was applied. Similar as under hypoxia, 5 mM DTT caused about 40% reduction of the membrane localization of wt occludin 30 min after the administration (Fig. 3A, left two columns), which maintained at least 90 min. C216A and C237A did not respond to DTT (Fig. 3A, right four columns). The *trans*-interaction of wt occludin

FIG. 1. The localization of human Occl and its cysteine substitution mutants in cell-cell contacts and its *trans*-interaction between opposing cell membranes are cysteine dependent and hypoxia sensitive. Under normoxia, Occl mutants C76A, C82A, and C148A (position in a TMD) did not change membrane to cytosol ratio compared with the wt (A), but in case of C76A, the distribution of the protein was more oriented to cell-cell contacts (B). The mutants C216A (C) and C237A in extracellular loop 2 showed reduced membrane localization (D). C76A (B) and C148A exhibited higher *trans*-interactions than wt Occl, as determined in cell-cell contacts between two transfected cells (E). C216A (C) and C237A caused reduced *trans*-interactions (E). After 3 h hypoxia, membrane localization (D) and *trans*-interaction (E) for C216A (H) and C237A were kept at the level of the respective normoxic mutant. In contrast, the other mutants resulted in a reduced membrane localization and *trans*-interaction (F–H) compared with the respective normoxic mutant. The experiments were performed in HEK-293 cells that were stably transfected with constructs of YFP-Occl. For constructs with a similar morphology, a representative example is shown only: Wt pictures are also representative of C82A, C76A for C148A, and C216A for C237A. The red fluorescence of trypan blue was used to visualize the membrane. Arrows indicate cell-cell contacts between two transfected cells, and arrowheads indicate membrane outside the contacts. CRFR 1 used as negative control, dashed line indicates threshold for *trans*-interactions. Mean $\pm$ SEM; $n \geq 25$. ##, ###— $p < 0.01$ and 0.001 , respectively, compared with normoxic wt. *, **, ***— $p < 0.05$, 0.01 , and 0.001 , respectively, compared as indicated. Inset in (D): position of highly conserved cysteines in the topology of Occl. CRFR, corticotropin releasing factor receptor; HEK, human embryonic kidney; Occl, Occl, occludin; SEM, standard error of the mean; TMD, transmembrane domain; wt, wild type; YFP, yellow fluorescent protein. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

decreased to $52\% \pm 9\%$ by DTT, whereas the values for C216A were comparable: $31\% \pm 3\%$ and $32\% \pm 3\%$ without and with DTT, respectively. The FRET efficiency declined to $4.6\% \pm 2.1\%$ after DTT compared with the control (Fig. 2, left group of columns), indicating a loss of *cis* interactions. DTT did not influence the *cis* association of C216A (Fig. 2, second group of columns from the right), further supporting the assumption of reduction-sensitive cysteines in the ECL2 of occludin.

To check whether hypoxia-like reducing conditions affect occludin in the cell membrane, protein supply was blocked by brefeldin or cycloheximide, retaining proteins in the ER and inhibiting protein synthesis, respectively (11, 17) (Fig. 3). Thus, brefeldin led to intracellular accumulation of the protein, cycloheximide to its decrease in the perinuclear space. However, the plasma membrane-localized occludin was found stable even after 16 h of cycloheximide and after 1.5 h of brefeldin. After pretreatment of the blockers for 6 h and 30 min, respectively, 30 min of incubation of 5 mM DTT decreased the

membrane localization by about 40% (Fig. 3B), indicating that the occludin molecules were impaired at the cell surface.

Cysteine substitutions altered membrane mobility, trans- and cis-interactions of occludin

Trans-interaction was influenced by the C216 and C237, as the enrichment factor was reduced after substitution with alanine (Fig. 1E). The relative enrichment factor dropped to 0.46 ± 0.04 (C216A) and 0.35 ± 0.03 (C237A), respectively, relative to the normoxic wt control. C76 and C148 are a part of the wt protein, and their function seems to be the hindrance of an excess of *trans*-interaction, as the *trans*-interactions were enhanced after substitution of those cysteines with alanine. Under normoxia, both mutants showed a 3.3-fold enrichment factor (C76A, 3.29 ± 0.58 ; C148A, 3.28 ± 0.39) compared with that of the wt. C82A exhibited a relative enrichment factor of 0.82 ± 0.08 , which was not significantly reduced from that of

FIG. 2. FRET measurement between human Occ and its cysteine substitution mutants in cell-cell contacts of transfected HEK-293 cells show that homophilic *cis*-interaction is cysteine dependent, sensitive to hypoxia and reducing agents. In case of the mutants Occ^{C82A}, Occ^{C216A}, and Occ^{C237A}, a reduced FRET efficiency was detected under normoxic conditions (upper pictures). C82, C216, and C237 seem to play a role in the homo-oligomerization of Occ. A reduction of the FRET efficiency was detected 3 h after the onset of hypoxia except in case of the mutants C216A and C237A (for respective cells, see lower pictures). These two residues indicate a role in the hypoxia-sensitive oligomerization of Occ. This sensitivity was confirmed by addition of 5 mM DTT, resulting in another type of reducing condition; it diminished the FRET of Occ^{wt} with Occ^{wt} but did not with Occ^{C216A}. FRET was measured within the red frame, indicating the cell-cell contact. CFP/YFP were used as a tag of Occ. Mean ± SEM; $n \geq 25$. Blue asterisks, significance versus wt/wt control. Black asterisks, significance versus respective normoxic construct as indicated. *, and **, $p < 0.05$ and 0.001, respectively. DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescence protein; YFP, yellow fluorescence protein. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

the wt. In MDCK-II cells, the *trans*-interaction changes were much weaker than in HEK-293 cells due to endogenously expressed occludin. Nevertheless, the *trans*-interaction of the ECL2 mutant C216A tends to be weaker compared with the wt control (Supplementary Fig. S1B). As seen in the investigation of the membrane localization, hypoxia of the transfected HEK cells had no effect on C216A (0.4 ± 0.03) and C237A (0.35 ± 0.03); whereas with the other mutants and wt, the *trans*-interactions were reduced under hypoxia (0.61 ± 0.16 , wt; 2.38 ± 0.39 , C76A; 0.59 ± 0.07 , C82A; 1.43 ± 0.27 , C148A, compared with the normoxic data mentioned earlier).

Compared with the normoxic wt control ($13.0 \pm 0.8\%$), the normoxic *cis*-interactions were diminished by C82A to $8.5 \pm 0.9\%$ and by the cysteine replacements of the ECL2 to

$3.1 \pm 1.1\%$ (C216A) and $0.4 \pm 1.9\%$ (C237A). This assay showed no response for C216A and C237A when faced with hypoxia ($4.2 \pm 1.7\%$, C216A; $1.2 \pm 2.5\%$, C237A), indicating a role in the hypoxia-sensitive oligomerization of occludin. In contrast to the ECL2, the TMD-localized mutations still showed hypoxia sensitivity, as indicated by the reduction in their FRET efficiencies (from normoxic $11.5 \pm 1.0\%$ to hypoxic $4.3 \pm 1.1\%$ for C76A; $8.5 \pm 0.9\%$ to $5.1 \pm 1.3\%$ for C82A). One exception was C148A, which did not respond to hypoxia— $11.7 \pm 1.1\%$ to $11.3 \pm 1.2\%$ (Fig. 2, diagram).

The FRET results were supported by a fluorescence recovery after a photobleaching (FRAP) experiment, which was used to quantify membrane mobility. The mutants that showed reduced *cis*-interaction, and thereby less oligomerization, showed increased mobility (relative initial slope:

FIG. 3. DTT decreases membrane localization of human Occ in the absence (A, left two columns) and presence of blockers of membrane protein supply (B), but does not alter cysteine replacement in the ECL2 of Occ (A, right four columns). 1 μ g/ml brefeldin A, retaining proteins in the endoplasmic reticulum, and 50 μ g/ml of the protein synthesis inhibitor cycloheximide were preincubated in HEK-293 cells transfected with YFP-tagged Occ, for 30 min and 6 h, respectively. Arrows indicate decreased membrane signals 30 min after the incubation with 5 mM DTT. Mean $\pm$ SEM, $n > 15$; *, **, ***, $p < 0.05$, 0.01, and 0.001, respectively, compared with DTT-free wt. #, ##, $p < 0.05$ and 0.01, respectively, compared with respective DTT-free wt with a blocker.

1.32 $\pm$ 0.18, C82A; 1.89 $\pm$ 0.13, C216A; 1.92 $\pm$ 0.41, C237A) relative to the wt value, taken as 1.00. The two other TMD mutants showed no difference from the wt (0.84 $\pm$ 0.12, C76A; 0.89 $\pm$ 0.11, C148A) (Fig. 4).

The FRET efficiencies in MDCK-II cells were weaker compared with those in HEK-293 cells. Nevertheless, the FRET efficiency was significantly lower in the ECL2 mutant C216A than in the wt (Supplementary Fig. S1C).

Cysteines of the ECL2 of occludin may form an intraloop disulfide bridge

Figure 1E illustrates that the free side chain of C237 in occludin^{C216A} did not *trans*-interact with the free side chain of C237 in another occludin^{C216A}; similarly, the free C216 in occludin^{C237A} did not *trans*-interact with the free C216 in another occludin^{C237A}. To find out whether C216 *trans*-associates with C237, a mixed culture of cells expressing YFP-occludin^{C216A} and of cells expressing YFP-occludin^{C237A} was established (Fig. 5A). To distinguish between the two cell lines, the YFP-occludin^{C216A} cells were cotransfected with CFP (Fig. 5C, D). Measurements of the contact enrichment between YFP-occludin^{C216A} and YFP-occludin^{C237A}-expressing cells indicate that there were no *trans*-interactions between the C216 and C237 (Fig. 5E). This finding, along with the result of the monotransfections (no direct association between C216 and C216 or C237 and C237; Fig. 1E), indicates that C216 and C237 form an intramolecular bridge as indicated in Figure 5F.

To further establish whether the binding of the ECL2 is related to a disulfide bridge within the loop, we replaced C237 by serine, in order to avoid a covalent cysteine–cysteine bond without larger physicochemical changes of the side chain.

Figure 6 (ECL2) revealed that the reduction of *trans*- and *cis*-interaction caused by Occ^{C237A} (Fig. 6; columns 2, 5) was not rescued by Occ^{C237S} (Fig. 6; columns 3, 6), supporting the assumption that there is an intramolecular disulfide bridge within the ECL2.

FIG. 4. The mobility of human Occ is affected after replacement of the cysteines 82, 216, and 237 by alanine as measured by fluorescence recovery after photobleaching in cell–cell contacts of HEK-293 cells. Inset: YFP-Occ signal recovery after photobleaching in the contact area (arrows) of cells transfected with wt Occ. The mobility was quantified from the initial slope of the recovery curve related to that of the wt. CRFR 1 was used as a highly mobile control protein, and claudin-1 (Cld-1) was used as a low mobile control protein. Mean $\pm$ SEM; $n \geq 7$; *, and ***, $p < 0.05$ and 0.001, respectively, compared with the wt.

FIG. 5. Cysteines 216 and 237 of human Occ form an intraloop disulfide bridge within the ECL2. Mixed culture of HEK-293 cells co-transfected with YFP-Occ^{C216A} + CFP and HEK-293 cells transfected with YFP-Occ^{C237A} did not show enrichment of Occ in contacts between two differently transfected cells (A), which is quantified in (E). This means that C216 in Occ^{C237A} did not *trans*-interact with Cys237 in Occ^{C216A} intermolecularly. Trypan blue served as a membrane marker (B) to identify the membrane fluorescence of YFP; CFP was used to identify the YFP-Occ^{C216A} expressing cells (C), which is merged in (D). (F) Proposed intramolecular disulfide bond visualized at the ECL2 indicated by double arrows; dashed lines, no association detectable. CRFR 1 was used as a negative control. Mean $\pm$ SEM, $n \geq 18$; ***, $p < 0.001$ compared with the wt. ECL, extracellular loop. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 6. Serine substitution of C237 (C237S) in the ECL2 of human Occ prevents *trans*- and *cis*-interaction of Occ, whereas serine substitution of C82 (C82S) in the TMD1 does not affect the *cis*-interaction. The bar chart showed that C237S strongly reduced contact enrichment (column 3) and FRET efficiency (column 6) compared with the corresponding control (column 1 and 4, respectively). These effects indicate that C237 is essential for the *trans*- and *cis*-interaction of the ECL2. Cotransfection of Occ^{C82A} tagged with YFP and of Occ^{C82A} tagged with CFP in HEK-293 cells (column 8) revealed an additive effect of the reduction of the FRET efficiency compared with CFP-Occ^{wt}/YFP-Occ^{C82A} (column 7). C82S led to a complete recovery of the FRET efficiency (column 9) to the level of the wt/wt (column 4), indicating no disulfide formation. *Trans*-interaction was quantified as enrichment of Occ constructs in cell-cell contacts of two transfected cells (contact enrichment). *Cis*-interaction was determined as FRET between a CFP- and a YFP-fusion protein of Occ in cell-cell contacts of cotransfected cells (FRET efficiency). Mean $\pm$ SEM, $n \geq 12$; * and ***, $p < 0.05$ and 0.001 , respectively, compared with wt and wt/wt, respectively, or as indicated.

FIG. 7. Replacement of cysteine 76 in TMD1 of human Occ by alanine increases the paracellular tightness in MDCK-II cells. Measurements of the TEER using MDCK-II cells stably transfected with human FLAG-Occ. Mean $\pm$ SEM, $n > 4$; * $p < 0.05$ compared with wt. The inset exhibits immunoblots of the transfected Occ constructs determined *via* their FLAG tag, and actin served as a house-keeping protein. MDCK, Madin Darby canine kidney; TEER, transepithelial electrical resistance.

C82 in the TMD1 is involved in the oligomerization of occludin without forming a disulfide bridge

It has been established that C82 is a determinant of occludin's membrane mobility (Fig. 4) and *cis*-interaction (Fig. 2). It was, therefore, tested whether the dimerization characteristics are related to the formation of a disulfide bridge. For this

purpose, HEK-293 cells cotransfected with C82A/C82A, C82A/wt and wt/wt were compared in a FRET assay (Fig. 6, TMD1; columns 8, 7, 4). It was found that the FRET value of C82A/wt was reduced to 65% and then to 8% in C82A/C82A compared with the wt/wt control (100%). This additive effect indicates that the binding is not a disulfide bond.

To avoid substantial alterations in the structure of the TMD1, while preventing a potential cysteine-cysteine association and maintaining the physicochemical properties of the cysteine side chain, the C82S substitution was investigated. However, the serine substitution did not affect the *cis*-interaction (Fig. 6, TMD1, column 9), suggesting that the *cis*-interaction on position 82 needs a small uncharged polar side chain.

C76 in the TMD1 limits paracellular tightness and contributes toward maintaining a proper TJ strand network

Since C76A increased the *trans*-interaction of occludin in HEK-293 cells (Fig. 1E), this substitution was tested in MDCK-II cells. In this case, C76A caused an increase in transepithelial electrical resistance (TEER), from $145 \pm 23 \Omega \text{cm}^2$ for the cells transfected with FLAG-occludin^{wt} to $243 \pm 60 \Omega \text{cm}^2$ after transfection with FLAG-occludin^{C76A} (Fig. 7). This means that C76 is involved in a mechanism maintaining a specific level of paracellular permeability. Other mutants did not affect the TEER. Influence of the expression levels on TEER was excluded, as immunoblotting showed comparable bands for the different transfectants (Fig. 7, inset).

FIG. 8. The substitution C76A of human Occl transfected in HEK-293 cells modifies the tight junction strand network of claudin-1 (Cld-1) as visualized by freeze-fracture electron microscopy. (A) FLAG-Cld-1 monotransfected cells showed a P-face-associated tight junction strand network of continuous particles. (B) Cotransfection of FLAG-Cld-1 with FLAG-Occl wt reduced the mesh size of the network and increased the degree of P-face association. (C) Cotransfection of FLAG-Cld-1 and FLAG-OcclC76A considerably increased the strand density. (D) Cotransfection of FLAG-Cld-1 with FLAG-OcclC216A resulted in a network comparable to that of the cotransfection with Occl wt. Although transfection of HEK-293 cells with FLAG-Occl wt alone showed no strand formation (not shown), the presence of Occl may stabilize the network of Cld-1-based tight junctions. Scale bars, $0.2 \mu\text{m}$. P-face, protoplasmic face.

FIG. 9. Proposed binding sites for the homophilic association of human Occl from the perspective of cysteine residues. (A) Lateral view on four Occl molecules interacting in *trans* (vertical arrows) and *cis* (horizontal arrows) at the plasma membranes of two adjacent cells. Circles, cysteine residues; red, hypoxia sensitive. (B) View on the cell surface from outside the cell on two Occl molecules *cis*-interacting. Double arrows indicate interactions for C82 of TMD1, for the ECL2, and for C409 (33) at the cytosolic C-terminal coiled coil-domain. Solid lines, extracellular; dotted lines, intracellular. (C) Side view and (D) top view of a potential structure of the ECL2 of Occl¹⁹⁶⁻²⁴³ considering an intraloop sulfhydryl bridge between C216 and C237 (yellow letters) and highly conserved tyrosine residues suggested to contribute to the oligomerization of Occl (white arrows; 36). Dashed line, membrane surface; red, α -helix; blue, β -strand; yellow, cysteine. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

To analyze how C76A induced stronger *trans*-interaction and higher paracellular tightness, its influence on TJ strands formed by claudin-1 was studied by freeze fracturing. When expressed alone in HEK-293 cells, FLAG-claudin-1 formed a protoplasmic face (P-face)-associated TJ strand network of continuous ridges. The diameter of the meshes was 0.4–0.6 μ m (Fig. 8A). When cotransfected with FLAG-occludin, the strands appeared more complex and the diameter of meshes was smaller, being $\sim$ 0.2 μ m; the association of strands with the P-face was even increased (Fig. 8B). Furthermore, the shape of the meshes is more oval than that of the meshes formed by claudin-1 alone. FLAG-occludin alone did not form TJ strands in HEK-293 cells (data not shown). FLAG-occludin^{C76A} increased the density and length of the claudin-1 strands (Fig. 8C), which could be caused by stronger *trans*-interaction and would explain the higher tightness. The ECL2-localized mutant FLAG-occludin^{C216A} cotransfected with claudin-1 showed a similar strand morphology to that with claudin-1 cotransfected with occludin^{wt} (compare Fig. 8D with 8B). Thus, occludin forming no strands, if transfected alone, seemed to stabilize claudin-1-based TJs.

ECL2 and TMD1 are binding sites for occludin homo-oligomerization

Based on our findings and on literature data (33, 36), a structural scheme was drawn up for the homophilic association between occludin molecules and its ECL2. Figure 9A and B show that a disulfide bridge formed within the ECL2 is indirectly involved in *trans*- and *cis*-association. The figure also suggests direct participation of C82 in the interaction between the TMD1 of different occludin molecules. Modeling

of occludin's ECL2 (aa 196–243) led to a compact structure consisting of one α -helix and two antiparallel β -strands (Fig. 9C, D). The two cysteines are in close proximity and inaccessible from outside when forming a disulfide bridge. The resulting structure contains highly conserved aromatic aa, which are outward directed and can be proposed as potential oligomerization sites (36).

Discussion

Previous occludin studies have established a close association between changes in the redox state of tissue barriers and alterations in the assembly and function of TJs. Occludin was identified as the most sensitive TJ protein and potential TJ regulator under reducing conditions and oxidative stress (3). However, the molecular details of the oligomerization of occludin were unclear, even though cysteine residues were assumed to be involved in the interactions (19, 21, 33). Now, we demonstrate for the first time that the ECL2 is an oligomerization area of occludin. Both mutants of the ECL2 C216A and C237A altered membrane localization, TJ enrichment, FRET efficiency, and the membrane mobility of occludin. The TMD1 was identified as another new binding area, as C82A reduced occludin's FRET efficiency and raised its membrane mobility. The results obtained in HEK-293 and MDCK-II cells suggest an association scheme in which the ECL2 and TMD1 are important binding sites between the occludin molecules. The ECL2 forms a complex structure in which a hypoxia-sensitive disulfide bridge ensures folding, which indirectly enables two association modes: *trans*-interaction to an ECL2 of an opposite cell and *cis*-interaction to an ECL2 at the same cell membrane. In addition, a cysteine in the TMD1 is suggested to associate in

cis to TMD1' of another occludin molecule, without forming a disulfide bridge (Fig. 9, upper part).

Disulfide bonds, an oxidized state of sulfide residues, are well known in ECLs (25,32). They exist between extracellular cysteine residues, if localized in suitable position to each other, as the normal extracellular space is an oxidizing environment. Conversely, disulfide bonds are removed in reducing environments (34), such as hypoxia or caused by reducing agents. Oxidation of sulfhydryl groups in occludin is proposed to lead to disulfide binding within the ECL2. However, a lipophilic reductant was able to diminish occludin oligomerization (21), whereas the oligomers were resistant to a hydrophilic reductant (22). Our findings with a single substitution of C216 or C237 in the ECL2 show that *trans*- and *cis*-interaction of occludin was hindered. This favors the idea of a disulfide structure within the ECL2 as we postulated earlier (3). In each substitution tested, one cysteine residue was still present, but unable to maintain occludin-occludin interactions. Neither C216 *trans*-associates in the cell pair C237-C237A nor C237 in C216-C216A, nor C216 to C237 in C237A-C216A (Fig. 5). These results support the assumption that the cysteines in ECL2 are shielded from the extramolecular space. Consequently, the ECL2 forms an intraloop disulfide bridge, similar to that described for other membrane proteins (5), and does not form an intermolecular disulfide bond. Substitution of a single cysteine in the ECL2 with serine, a physicochemically comparable aa without the ability of disulfide bridge formation, exhibited the same effect as alanine replacement, that is, reduced *trans*- and *cis*-interaction. This further argues against the assumption of an intermolecular S-S bridge. Taken together, the results demonstrate for the first time that there is an intraloop disulfide bridge between shielded cysteines under normoxic conditions, giving a specific fold, which then indirectly contributes to the intermolecular occludin association.

On the other hand, a reducing environment, as in our hypoxia or DTT experiments, abolishes disulfide binding. Thus, *trans*- and *cis*-association and membrane localization of occludin decreases within 3 h hypoxic or 30 min DDT incubation. Interestingly, a decrease in *trans*-, *cis*-interaction, and membrane amount under either condition could not be observed with the ECL2 mutants C216A and C237A. This underlines the role of cysteines in this ECL in the response of occludin oligomerization to reducing circumstances. These data provide further evidence for the hypoxia sensitivity of the loop. Hypoxia alone resulted in reversible redistribution of occludin from the cell membrane to the cytosol in TJ-free HEK-293 and TJ-expressing MDCK-II cells. This means that redistribution is a general phenomenon. Hypoxia sensitivity of occludin has already been indicated by others, who found occludin diminished in the plasma membrane of alveolar epithelium (8), or altered localization in ileal loops (29). The molecular mechanisms for these effects have been unknown. Now, our findings provide new insights into the molecular mechanisms of the hypoxia-dependent oligomerization of occludin.

Our findings provide evidence that the population of occludin which is affected has already been present at the cell membrane before reducing conditions. Blocking of its membrane supply with brefeldin (17) or cycloheximide (11) shows a stable presence at the plasma membrane for many hours. However, incubation of DTT mimicking hypoxia-like reduc-

ing conditions, after blocking the supply, leads to reduced membrane localization within some minutes. This effect clearly demonstrates that occludin has directly been affected at the cell membrane. The loss from the membrane is mimicked by cysteine exchange in the ECL2, which, hence, cannot be intensified by reducing agents. Thus, we can argue that changes in the conformation of the ECL2 by reducing conditions are responsible for the alteration in the TJs and not incomplete folding of newly forming occludin.

There are three scenarios for hypoxia (6). First, it directly activates a variety of transcription factors regulating the expression of TJ proteins. Second, hypoxia leads to Ca^{2+} uptake and, thus, to the activation of kinases, which, in turn, causes alterations in TJs. Third, hypoxia leads to the production of reactive oxygen species and thereby activates cytokines, that is, nuclear factor kappa B, which are able to change the pattern of activated transcription factors and also alter TJ integrity. The hypoxia response of occludin discovered in this study is a new mechanism and is specific for TJs, as occludin is a unique TJ marker (3). A specific function of occludin in hypoxia is supported by Caraballo *et al.* (8), who found that occludin affected only, but all other TJ proteins analyzed remained unaffected, under the same conditions. Therefore, occludin is confirmed as being the first and most sensitive marker of hypoxic TJ injury as suggested earlier (3).

In contrast to the ECL2, C82 in TMD1 is not hypoxia sensitive. Replacement of this cysteine by alanine reduced the *cis*-association and hypoxia additionally decreased the association relative to the normoxic substitute. Replacement of C82 by serine—which has similar physicochemical properties—did not affect this interaction. These data demonstrate that a disulfide mechanism does not play a role in the *cis*-binding of C82. This is understandable, as a redox state is not defined in the lipophilic milieu of the membrane and implies that C82 is involved in the association of occludin's TMDs. Polar residues of cysteine, serine, or threonine have also been made responsible for putative hydrogen bonds between TMDs of other proteins (13). It is noted that S85 and T86 are conserved in all occludins in closest proximity to C82 within the transmembrane helix. In addition, the *cis*-interaction for occludin^{wt}-occludin^{C82A} was roughly halved and almost eliminated in occludin^{C82A}-occludin^{C82A} relative to occludin^{wt}-occludin^{wt}. The additive effect supports the assumption that C82 bridges the TMD1s of two occludin molecules, but without disulfide bridge formation. This orientation fits with our observation that the ECL2 is a *cis*-interaction site, as the TMD4 is close to aromatic residues at the C-terminal end of the ECL2, which are proposed to be involved in occludin oligomerization (36). The same authors suggest that occludin forms a four TMD bundle. Thus, TMD4 faces TMD4' of another occludin molecule and TMD1, the respective TMD1' (Fig. 9B).

Surprisingly, C76A in TMD1 and C148A in TMD2 increase the *trans*-interaction between occludin molecules of opposing cells. This effect is supported by an increased TEER of C76A-transfected MDCK-II cells compared with control transfectants. Moreover, occludin^{C76A} strengthens the claudin-1 TJ network of cotransfected HEK-293 cells to form very small meshes with many parallel strands compared with occludin^{wt} cotransfections. Occludin is known to improve the tightness of epithelial barriers (38) and TJ morphology (9). Our data indicate that transmembranous cysteine residues are

indirectly involved in the *trans*-association. For C76 and C148, no polar residues for hydrogen bond formation are available in the proximity of all TMDs of occludin. The alignment only shows highly conserved non-polar residues next to both cysteines. Therefore, it is assumed that the cysteines prevent the TMDs of occludin from becoming too close and, hence, indirectly affecting the *trans*-binding properties of the ECLs.

The majority of our findings were obtained using TJ-free HEK-293 cells. However, the cysteine substitutions cause similar alterations in membrane localization, *trans*- and *cis*-associations in MDCK-II cells. Thus, the conclusions drawn are also relevant for cells with endogenous TJs. Moreover, the same occludin transfectants showed the same intracellular redistribution in hypoxic HEK-293 as in hypoxic MDCK-II cells. The hypoxia-related redistribution has been found for endogenous occludin in further cell types by other authors (8, 29).

On the basis of our result that the ECL2 of occludin plays a key role in its oligomerization between and along the cell membranes, we have developed a preliminary molecular model of this loop. In doing so, we considered our identification of an intraloop disulfide bridge which is inward oriented within the ECL and the report of Yaffe *et al.* (36) that two tyrosine residues at the C-terminal end of the ECL2 are outward oriented. The secondary and tertiary structure model of the ECL2 obtained, which fulfilled the criteria mentioned earlier, is shown in Figure 9 (lower panel). The figure visualizes a complex structure in which the binding between C216 and C237 forms a compact fold. This fold contains two anti-parallel β -strands and one α -helix, which are tightly connected between C237 from the C-terminal β -strand and C216 from the α -helix. Thus, the disulfide bridge is shielded, inaccessible from the outer space, and causes a β -sheet stabilized by the α -helix. The C-terminal β -strand carries two outward-directed tyrosines that are considered involved in occludin oligomerization (36).

In summary, our investigation provides new insights into the molecular mechanisms of occludin's oligomerization. It identifies cysteine-related oligomerization sites and mechanisms causing *trans*- and *cis*-interaction in the oligomerization of occludin, its membrane localization and mobility. The ECL2 is a principle and hypoxia-sensitive interaction site and a preliminary molecular model, including an intramolecular disulfide bridge, is suggested for this loop. Since two cysteines are conserved in one ECL of all four-transmembrane TJ proteins (3, 18) and as they may interact heterophilically (9), the ECL2 of occludin has a model character for such TJ proteins. We draw conclusions about the structural and functional regulation of occludin under hypoxic conditions and its sensor function for regulating the TJ assembly.

Materials and Methods

Plasmids

Plasmids containing YFP- and CFP-occludin were generated by polymerase chain reaction using the primers 5'GATCTCGAGCTCAGATGTCATCCAGGCCTCTTG3' and 5'GAGCACGGTACCCTATGTTTTCTGTCTATCATAG3' and cloning the gene into the vectors pEYFP-C1 and pECFP-C1 (Clontech) by using the restriction sites *SacI* and *KpnI* (Fermentas). Claudin-1-YFP, claudin-5-YFP and -CFP, corti-

cotropin-releasing factor receptor (CRFR)-1-YFP, and -CFP were used as described (26). FLAG-occludin was provided by Otmar Huber (Jena) and FLAG-claudin-1 as reported (9). The position of CFP, YFP, and FLAG before or after the protein name describes N- or C-terminal position. Human proteins were used except murine claudin-5. To generate occludin substitutions, the following primers were ordered (BioTez): C76A fw, TATGCTCATTATTGTGATGGCCATTGCCATC TTTGCCTG, rev, ACAGGCAAAGATGGCAATGGCCATC ACAATAATGAGCAT; C82A fw, CATTGCCATCTTTGCC GCGTGGCCTCCACGCTTG, rev, CAAGCGTGGAGGCC ACGGCGGCAAAGATGGCAATG; C82S fw, ATTGCCATC TTTGCCTCAGTGGCCTCCACGCTTG, rev, GCAAGCGT GGAGGCCACTGAGGCAAAGATGGCAAT; C148A fw, CCATGGCTGCCTTTGCCTTCATTGCCGCGTTG, rev, CAA CGCGCAATGAAGGCAAAGGCAGCCATGG; C216A fw, CACAAATATATGCCCTCGCCAAACCAATTTATACACC, rev, GGTGTATAAAATTGGTTGGCGAGGCATATATTTGTG; C237A fw, CAGTATTGTATCACTACGCCGTTGTGGATCCC CAGGAGG, rev, CCTCCTGGGGATCCACAACGGCGTAGTG ATACAAATACTG; 237S fw, GTATTTGTATCACTACTCTGTT GTGGATCCCCAGG, rev, CCTGGGGATCCACAACAGAGTA GTGATACAAATAC.

Cell culture, hypoxia, reductants, and transfection

HEK-293 and MDCK-II cells were cultured with Dulbecco's-modified Eagle medium (Invitrogen), enriched with 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin (Biochrom), and 1% L-alanyl-L-glutamine (Invitrogen) under air and 5% CO₂ at 37°C (26), termed normoxia. For hypoxia, cells were transferred into an anaerobiosis workstation Concept Plus (IUL Instruments/Ruskin Technology Ltd.) with 0.6% O₂ at 37°C (16) for 3 h. To induce reducing conditions by an independent mechanism, cells were treated with 5 mM DTT (Roth) if not stated otherwise for 30 min (23); for approximately at least 6 h, no cellular alterations were detectable. For the transfections, polyethylenimine (Polysciences) was used; cells were seeded on 10 cm² wells of six-well plates and transfection followed the next day according to the manufacturer's instructions at 85%–90% confluence. After growing overnight, cells were split 1:2 on 30 mm coverslips coated with poly-L-lysine (Sigma-Aldrich), 25% in water. Analyses followed the next day. For generation of stable lines, cells were selected with 1 mg/ml G418 (Calbiochem).

Live cell imaging

Transfected cells transferred to 1 ml Hanks' balanced salt solution (HBSS; Invitrogen) pH 7.5 were analyzed by a laser scanning microscope LSM 510 META equipped with He/Ne and Ar laser, a Ti:Sapphire near-IR multiphotonic laser, and a spectral detector, Plan-Neofluar 100 $\times$ oil immersion objective NA1.3 (Zeiss). Excitation of CFP was performed with Ar laser line 488 nm (50% power, 8%–15% transmission), that of YFP at 514 nm (50% power, 1%–7% transmission). Detection wavelength for CFP was 463–495 nm and for YFP, 527–634 nm. Trypan blue was detected at 543 nm with the He/Ne laser line, emission >590 nm. Thickness of optical sections was 0.9 μ m. Scanning was unidirectional with a speed of $\sim$ 7 μ s per pixel; detection was averaged from eight sequential linear scans to filter out background noise and the images were stored in a 12-bit format.

Quantitation of membranous occludin

Image J (version 1.43U; NIH) was used to compare the amount of YFP-occludin in the plasma membrane (membrane pool) *versus* the intracellular amount (intracellular pool). Trypan blue was used as a membrane marker (0.5%, 75 μ l/1000 μ l plating medium; Sigma). YFP-occludin colocalizing with trypan blue was considered membranous even if internalized. The trypan blue channel was used as α -channel to perform a logical negation on the whole YFP-occludin signal. The resulting image contained exclusively the cytosolic pool of YFP-occludin; this image was used to perform a second logical negation of the whole YFP-occludin signal, resulting in an image showing the membranous pool. The images were binarized, and the surface occupied by all the YFP-signal was measured. The membrane to intracellular ratio was calculated as membrane pool/intracellular pool. For blocking protein supply to the plasma membrane, 1 μ g/ml brefeldin A (Merck Millipore) or 50 μ g/ml cycloheximide (Sigma) were incubated.

Fluorescence profiling and enrichment factors in cell-cell contacts (cell scan)

For quantification of *trans*-interactions between opposing cells, fluorescence intensity profiles through two neighboring cells were performed *via* a vectorial path on a microscopical image (27). The membrane marker trypan blue enabled the identification of contacts between two cells where TJs are formed. Calculation of the enrichment factor was = cell-cell contact intensity/sum of two non-contact intensities, at which no contact intensities were measured in plasma membranes neighbored to no cell or to a nontransfected cell (Supplementary Fig. S2). The fluorescence intensity at each point along the vectorial path was plotted using Zeiss LSM evaluation software. In a real *trans*-interaction, the ratio/factor was > 1.

Fluorescence resonance energy transfer

To analyze *cis*-interactions along the cell membrane, HEK-293 cells were cotransfected with CFP-Occ^{wt} and YFP-constructs of Occ^{wt} or Occ-mutants. CRFR1-YFP was the negative control (4), which colocalized but did not interact with occludin. The pair claudin-5-CFP/-YFP was the positive control. FRET acceptor photobleaching was performed 48 h after transfection in HBSS+/- (with Ca²⁺, Mg²⁺) pH 7.5. CFP and YFP were excited at 458 (8% transmission) and 514 nm (3% transmission) and detected from 463–495 to 527–634 nm, respectively. Photobleaching of YFP at cell-cell contacts was performed by using 30–40 pulses of 514 nm Ar laser line at 100% intensity. FRET efficiency was calculated as reported (27). Relative FRET efficiencies were calculated as %-value of a distinct CFP-Occ/YFP-Occ mutant or CFP-Occ/YFP-CRFR1 compared with CFP-Occ^{wt}/YFP-Occ^{wt} as an internal standard (4). Areas with similar CFP and YFP fluorescence intensities were analyzed.

FRAP experiment

For membrane mobility, HEK-293 cells were seeded on 10 cm²-glass coverslips (Menzel) and imaged in HBSS. After bleaching, images were collected every 2 s until steady-state fluorescence was reached. Raw data were aligned, and mean fluorescence of background, whole-cell, and bleached bicellular TJ regions were quantified over time (Microsoft Excel

2007). Data correction and mobile fraction were determined as described (37; Excel). Relative recovery velocity was calculated by determining the initial slope of the recovery curve detected in the bleached area. The measurements were performed in cell-cell contacts with similar YFP fluorescence intensities.

Immunoblotting

Monoclonal antibodies against actin were from Calbiochem, polyclonal anti-FLAG-tag antibodies were from Sigma, and secondary antibodies goat anti-rabbit and anti-mouse labeled with Alexa fluor-555 and Alexa fluor-647, respectively, were from Invitrogen. Cells were lysed in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and complete protease inhibitor cocktail (Roche). Before loading, lysate was homogenized with a 25 Gauge needle, centrifuged at (4°C, 12 min, 12,000 g). The supernatant was diluted 1:2 with loading buffer (1% SDS, 20% glycerol, 125 mM Tris HCl pH 6.8, and orange G) and loaded on 4%–20% ready-to-use polyacrylamide gels (BioRad), run at 120 V for 1.5 h in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and pH 8.3). Separated proteins were transferred on a nitrocellulose membrane (BioRad) at 20 V for 45 min in transfer buffer (25 mM Tris/HCl, pH 8.3; 190 mM glycine, and 20% methanol) and probed with the antibodies.

Freeze-fracture electron microscopy

HEK-293 cells expressing claudin-1 and/or occludin were cultured in 25 cm² flasks (Greiner). At least 48 h after transfection, cells were washed twice with phosphate-buffered saline (PBS), probed with 5 ml fixation solution (1 ml 25% glutaraldehyde, 1 ml 10 $\times$ PBS in 8 ml water), and incubated for 2 h. Afterward, cells were washed twice, and the flasks were filled completely with PBS to be processed for freeze fracturing as described (35).

Transepithelial electrical resistance

0.6 cm² HA filters (Millipore) were placed into cellZscope (NanoAnalytics) that was equilibrated with the medium described earlier for 1 h at 37°C. About 300,000 stably transfected MDCK-II cells/cm² were seeded on the filters. TEER was measured every 2 h, and the maximum values were taken to compare the different occludin constructs. TEER of nontransfected cells was 124 $\pm$ 14 Ω cm² and increased to 135 $\pm$ 12 Ω cm² after transfection of FLAG-occludin^{wt}, n = 17, p > 0.05, paired t -test.

Modeling

Molecular modeling was performed *via* iterative threading-assembly-refinement algorithm (30, 39; <http://zhanglab.cmb.med.umich.edu/I-TASSER/>; University Michigan). For selection of suitable models, the experimental results were considered, such as a disulfide bridge within the ECL2, shielded localization of C216 and C237, and proposed oligomerization potential of highly conserved aromatic aa (36).

Statistics

Data are means $\pm$ standard error of the mean. If not stated otherwise, Mann-Whitney test was used to identify

significant differences between experimental groups, with $p < 0.05$ considered significant.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

aa = amino acid
AU = arbitrary unit
C = cell–cell contact between two YFP-Occ expressing cells
CEF = contact enrichment factor
CFP = cyan fluorescence protein
CRFR = corticotropin releasing factor receptor
DTT = dithiothreitol
ECL = extracellular loop
ER = endoplasmic reticulum
F_C = fluorescence at cell–cell contact
F_{NK1/2} = fluorescence at non-contact
FRAP = fluorescence recovery after photobleaching
FRET = fluorescence resonance energy transfer
HBSS = Hanks' balanced salt solution
HEK = human embryonic kidney
MDCK = Madin Darby canine kidney
NK = non-contact or contact between a transfected and a nontransfected cell
Occ, Occl = occludin
PBS = phosphate-buffered saline
P-face = protoplasmic face
SDS = sodium dodecyl sulfate
SEM = standard error of the mean.
TEER = transepithelial electrical resistance
TJ = tight junction
TMD = transmembrane domain
wt = wild type
YFP = yellow fluorescence protein