Occludin Modulates Transepithelial Migration of Neutrophils*

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Neutrophils cross epithelial sheets to reach inflamed mucosal surfaces by migrating along the paracellular route. To avoid breakdown of the epithelial barrier, this process requires coordinated opening and closing of tight junctions, the most apical intercellular junctions in epithelia. To determine the function of epithelial tight junction proteins in this process, we analyzed neutrophil migration across monolayers formed by stably transfected epithelial cells expressing wild-type and mutant occludin, a membrane protein of tight junctions with four transmembrane domains and both termini in the cytosol. We found that expression of mutants with a modified N-terminal cytoplasmic domain up-regulated migration, whereas deletion of the C-terminal cytoplasmic domain did not have an effect. The N-terminal cytosolic domain was also found to be important for the linear arrangement of occludin within tight junctions but not for the permeability barrier. Moreover, expression of mutant occludin bearing a mutation in one of the two extracellular domains inhibited neutrophil migration. The effects of transfected occludin mutants on neutrophil migration did not correlate with their effects on selective paracellular permeability and transepithelial electrical resistance. Hence, specific domains and functional properties of occludin modulate transepithelial migration of neutrophils.

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The abbreviations used are: TJ, tight junctions; TER, transepithelial electrical resistance; MDCK, Madin-Darby canine kidney; HA, hemagglutinin.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Lines—MDCK cells expressing occludin, HA-occludin, HAoccludinCT3, and OcclID were previously generated and characterized; clones exhibiting average phenotypes in terms of TER and paracellular permeability were used (17, 34). OccludinCT3 was generated by converting the codon for serine 253 to a stop codon as described for HAoccludinCT3 (17) using polymerase chain reaction-based mutagenesis and previously described cDNA coding for chicken occludin as a template (see Fig. 1 for a schematic representation of the constructed mutants and chimeras). The OccludinCT3 cDNA was then stably expressed in MDCK cells as previously (17). For experiments testing the function of occludinCT3 on tight junction functions, the cells were plated on 12-well Transwell culture inserts with 0.4-μm pores (Costar Corp., Cambridge, MA) and, when indicated, were pretreated overnight with 7 mM sodium butyrate. For migration experiments, the
cells were cultured on inserts with 3-μm pores and were never pre-treated with sodium butyrate to avoid secondary effects on the neutrophils. Cells grown on inserts with 0.4- and 3-μm pores exhibited comparable TER and paracellular permeability values, but the establishment of monolayers with stable TER values took twice as long on the large pore as on the small pore filters (>8 days).

**Antibodies**—An antibody specific for the NH₂-terminal domain of chicken occludin was raised against a histidine-tagged fusion protein containing the entire NH₂-terminal domain and was generated with pTricHis2 (Invitrogen Corp., San Diego, CA). The purified fusion protein was emulsified with Specol (Central Veterinary Institute, Lelystad, The Netherlands) and subcutaneously injected into rabbits. The anti-COOH-terminal domain antibody were previously described and recognizes chicken and dog occludin (antibody A) (17). The mouse monoclonal antibody against the hemagglutinin (HA) epitope was kindly provided by Drs. P. van der Sluijs (University of Utrecht, The Netherlands) and I. Mellman (Yale University, New Haven, CT) (18). Drs. J. M. Anderson and M. S. Mooseker (Yale University, New Haven, CT) kindly supplied rat monoclonal antibody R40.76 specific for ZO-1 (19).

**Immunofluorescence and Microscopy**—To stain chicken occludin with Epon, and processed for transmission electron microscopy as described (17).

The samples were mounted with the ProLong anti-fade kit (Molecular Probes, Inc., Eugene, OR) and analyzed with a confocal laser scanning microscope (LSM 410 invert; Carl Zeiss, Inc.) equipped with an argon and a helium-neon laser for excitation at 488 and 543 nm and BP510–525 and LP590 emission filters.

**RESULTS**

**Modification of the N-terminal Cytoplasmic Domain of Occludin Results in Increase Transmigration**—In MDCK cells, stable transfection of chicken occludin and full-length occludin with an N-terminal HA epitope (Fig. 1, HAoccludin) results in increased TER, a measure for the general tightness of the monolayer, and small, if any, increases in selective paracellular permeability (17, 21). To test whether occludin is involved in the transmigration of neutrophils, we analyzed the previously described stably transfected MDCK cell lines expressing wild-type chicken occludin or HAoccludin with the migration assay established by Cramer et al. (11).

Wild-type and transfected MDCK cells were cultured on permeable supports with 3-μm pores for 10 days to allow the establishment of monolayers with stable TER and paracellular flux values. As in our previous experiments with cells grown on filters with 0.4-μm pores (17), monolayers formed by cells expressing chicken occludin or HAoccludin exhibited 2–3-fold increases in TER relative to wild-type MDCK cells, for which we measured values of about 70 V cm⁻². No significant differences in paracellular flux of [³H]mannitol between the different cell lines was observed. Electron microscopic observation of thin sectioned Epon-embedded cells demonstrated that all used cell lines formed morphologically normal monolayers on the large pore filters (not shown). Neutrophils were then added to one and chemotactant to the other side of the monolayers, and transmigrated neutrophils were collected and counted at the end of the incubation period. To allow comparison of different experiments, the values obtained from transfected cells...
Wild-type and transfected MDCK cells.

Wild-type MDCK (wt MDCK) and MDCK cells expressing wild-type or mutant chicken occludin were cultured on filters with pores of 3 μm diameter for 10 days; the migration of neutrophils was then assayed by loading 10^6 neutrophils and inducing migration with chemotactic. The numbers of neutrophils that crossed the monolayers were determined and normalized to wild-type MDCK monolayers during the 1-h incubation period. The values shown are mean ± 1 S.D. derived from 4–9 independent experiments performed in duplicate. The p values shown were obtained with a t test comparing the transfected with wild-type cells.

were normalized to wild-type MDCK cells because the efficiency of migration varied in different neutrophil preparations.

Fig. 2 shows that transfection of chicken occludin did not significantly affect the efficiency of neutrophil transmigration. Because transfected chicken occludin is efficiently targeted to tight junctions (17), this indicates that increased amounts of occludin in tight junctions did not interfere with the migration process. In contrast, when cells expressing HAoccludin were analyzed, a 4-fold increase was found. Modification of the N terminus of occludin thus leads to increased transmigration without affecting TER and paracellular permeability, suggesting that the N-terminal cytosolic domain is involved in the reversible opening and closing of tight junctions during neutrophil migration.

To test whether the increased rates of transmigration affected the integrity of the monolayers, we first compared the TER before and immediately after migration. Table I shows that the TER of monolayers formed by wild-type and transfected MDCK cells was not affected by the neutrophils. We next added [3H]mannitol together with the neutrophils and compared paracellular flux occurring during transmigration with parallel cultures incubated with only [3H]mannitol and chemotactant, but without neutrophils. Although this resulted in slightly increased ratios, the differences were not significant (p > 0.1).

To test whether transmigration of neutrophils affected the morphology of the monolayers, we fixed the cells immediately after the migration assay and then labeled the monolayers by indirect immunofluorescence for ZO-1, a submembrane protein at tight junctions that directly interacts with occludin (22–24), and for F-actin with fluorescent phalloidin. The confocal sections in Fig. 3 show that the distribution of ZO-1 was not different in cells that were incubated with neutrophils compared with cells that were incubated with chemotactant only. Serial sectioning of the samples did also not reveal any significant differences (not shown). Similarly, effects on the distribution of F-actin could not be detected in either single (Fig. 3) or serial (not shown) sections. Staining of the samples for occludin did also not reveal any differences caused by the neutrophils (not shown).

Table I

| TER | Paracellular flux |
|-----|------------------|
| Wild-type MDCK | 1.92 ± 0.27 (n = 8) |
| Occludin | 0.91 ± 0.08 (n = 6) |
| HAoccludin | 0.94 ± 0.13 (n = 14) |
| OccludinCT3 | 0.93 ± 0.20 (n = 6) |
| HAoccludinCT3 | 0.94 ± 0.09 (n = 5) |
| OcclID | 1.24 ± 0.27 (n = 6) |

Transepithelial Migration and Occludin

We next analyzed the morphology of the monolayers and of the junctional region by thin sectioning of Epon-embedded cells and electron microscopy. Because migration was analyzed in the apical to basal direction, we were looking for monolayer regions that still contained a trapped neutrophil in the intermembrane space and took images from the junctions connecting the two corresponding cells to ensure that we were looking at junctions between cells that had allowed the passage of neutrophils. Fig. 4 shows examples of junctional regions of wild-type (A without and B with neutrophils) and HAoccludin-expressing cells (C without and D with neutrophils). Fig. 4, E and F, shows examples of neutrophils in the intermembrane space of wild-type (E) and HAoccludin-expressing (F) cells. We could not detect morphological effects caused by neutrophil transmigration on the junctions even when HAoccludin-expressing cells were analyzed that allowed 4 times more neutrophils to migrate than wild-type cells. Thus, modification of the N-terminal cytosolic domain of occludin resulted in increased transmigration of neutrophils without detectably af-
Deletion of the C-terminal Cyttoplasmic Domain Does Not Affect Transmigration—To be able to analyze the importance of the C-terminal cytoplasmic domain for transmigration, we constructed a mutant lacking this domain (Fig. 1, occludinCT3) and stably expressed it in MDCK cells. The immunoblot in Fig. 5 shows that a protein with a corresponding molecular weight could be expressed and was recognized by an antibody raised against the N-terminal domain of chicken occludin.

To determine the effects of occludinCT3 on TER and paracellular permeability, we plated wild-type MDCK cells, cells expressing occludinCT3, and control transfectants on filters. Fig. 6A shows that expression of occludinCT3 resulted in monolayers that exhibited twice the transepithelial electrical resistance of those formed by wild-type and control MDCK. When the cells were incubated overnight with sodium butyrate to induce higher expression levels (Fig. 5), only small, if any, further increases in transepithelial electrical resistance could be detected (Fig. 6A). We next measured paracellular flux of [3H]mannitol to determine possible effects on selective paracellular permeability. Clones expressing occludinCT3 exhibited increased paracellular permeability of this low molecular weight tracer (Fig. 6B). This effect was even greater when higher expression levels were induced by preincubation with sodium butyrate. Because the expression of wild-type and mutant occludin does not affect transepithelial fluid phase transport (17), this increase must be due to increased paracellular permeability. Because we had observed the same behavior of TER and paracellular permeability in cells expressing HAoccludinCT3 (17), this indicates that modification of the N-terminal domain of occludin does not affect the properties of the paracellular diffusion barrier.

We then performed the migration assays with cell lines expressing occludinCT3 and HAoccludinCT3. If the transfected cells were grown on filters with 3-μm pores, we found that expression of occludinCT3 and HAoccludinCT3 induced 2–3-fold increases in TER and 3–4-fold increases in paracellular flux relative to wild-type cells as shown in Fig. 6 for cultures grown on small pore filters. As in case of cells transfected with cDNAs coding for full-length occludin, neutrophil migration did not cause alterations in TER or paracellular flux (Table I), nor in the morphological appearance of the monolayers (not shown).

Fig. 2 shows that expression of occludinCT3 did not affect the efficiency of neutrophil migration, suggesting that deletion of the C-terminal cytosolic domain does not affect transmigration even though it results in increased paracellular flux. Expression of the analogous mutant with the N-terminal modification, HAoccludinCT3, resulted in increased migration. Although the stimulation was a little smaller than the one by HAoccludin, this indicates that deletion of the C-terminal cytosolic domain...
The NH2-terminal domain antibody failed to stain, suggesting that the claudinCT3 (B) and transfected and endogenous occludin in cells expressing occludinCT3 (C–E) were continuously distributed along the junction, one could use occludin tagged at the truncated COOH terminus to continuously distributed along tight junctions in Xenopus embryos (26).

Similarly to transfected occludin, endogenous occludin exhibited a normal continuous junctional pattern in cells expressing occludinCT3 (Fig. 7C), as it does in wild-type MDCK cells (Fig. 7D), and it did not form patches, as in cells expressing HAoccludinCT3 (Fig. 7E). The expression levels of endogenous occludin were not affected by the expression of the two COOH-terminally truncated mutants (Ref. 17 and data not shown). Thus, both terminal cytoplasmic domains of transfected occludin have to be inactivated to induce a discontinuous distribution of endogenous and transfected occludin.

### DISCUSSION

Our experiments indicate that occludin is a regulator of the transmigration of neutrophils across epithelial sheets. The N-terminal cytosolic domain of occludin, which is not critical for TER and selective paracellular permeability, is important for this function. Thus, not only the C-terminal but also the N-terminal cytoplasmic domain of occludin is of functional relevance.

Modification of the N-terminal cytosolic domain of transfected occludin results in two phenotypes: an increased efficiency of transmigration and, if the C-terminal domain is also inactivated, a discontinuous distribution of transfected and endogenous occludin. The finding that the N-terminal cytosolic domain is sufficient to mediate a continuous distribution of occludin suggests that this domain also interacts with the submembrane cytoskeleton, similar to the C-terminal domain. Although several components of the submembrane plaque of tight junctions are known, the protein that binds to the N-terminal domain of occludin has not yet been identified.

The C-terminal cytosolic domain of occludin interacts with at least four proteins: the three peripheral membrane proteins ZO-1, ZO-2, and ZO-3 (23, 24, 27, 28) and the membrane protein VAP-33 (29). Because the ZO-1 complex also interacts with the actin-cytoskeleton, this protein may in fact serve as an anchor and therefore be important for the continuous distribution of occludin (24, 30). Although occludin is continuously distributed in junctions lacking detectable ZO-1 (31), this might be due to the presence of additional interactions mediated by the N-terminal domain, as we show here in cells expressing occludinCT3.

Interestingly, expression of mutants lacking the C-terminal cytosolic domain did not affect transmigration, suggesting either that this domain and its interactions with the submembrane cytoskeleton are not required for transmigration or, alternatively, that the connections provided by endogenous occludin are sufficient. Because removal of the C-terminal domain results in increased paracellular permeability, this finding also suggests that the amount of chemoattractant that crosses wild-type MDCK cells is sufficient to provide maximal stimulation. In contrast, reduction of paracellular flux by expression of OccL1D (a mutant that inhibits paracellular flux by more than 50%) (34) significantly reduced the number of transmigrating neutrophils. Nevertheless, it cannot yet be excluded that this inhibition is due to a function of this extracellular loop during the transmigration process.

Occludin is important for all functions of tight junctions tested thus far: the formation of a semipermeable paracellular diffusion barrier (17, 21, 26, 32, 33), the restriction of intramembrane diffusion between the apical and basolateral cell

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**Fig. 7. Subcellular distribution of occludinCT3.** In A1, A2, and B, cells expressing occludinCT3 (A1 and A2) or HAoccludinCT3 (B) were plated on coverslips for 2 days and were then fixed with the Triton X-100/methanol procedure. The cells were subsequently labeled with the anti-NH2-terminal domain antibody to detect transfected occludin (A1 and B) and a monoclonal anti-ZO-1 antibody (A2). In C–E, occludinCT3-expressing cells (C), wild-type MDCK cells (D), or HAoccludinCT3-expressing cells (E) were cultured on filters for 1 week and then fixed with the ethanol/acetone procedure. The samples were labeled with the anti-occludin COOH-terminal domain antibody that cross-reacts with dog occludin (antibody A) to visualize the distribution of endogenous occludin. Shown are confocal sections through the junctional area of the monolayers. Note the continuous distribution of endogenous occludin. Also, if grown on 3-μm-pore filters, expression of this mutant resulted in a reduction of paracellular flux of [3H]mannitol by more than 50% (34) significantly reduced the number of transmigrated neutrophils (Table I).
surface domains (17), and, as we show now, the regulation of neutrophil transmigration. Nevertheless, different structural domains of occludin are important for different functions. The C-terminal cytoplasmic domain modulates paracellular diffusion but does not appear to play a role in neutrophil transmigration. In contrast, the N-terminal cytosolic domain regulates transmigration but not paracellular permeability. On the other hand, modification of both cytoplasmic domains is required for a discontinuous distribution of occludin and disruption of the intramembrane diffusion barrier because only expression of HAoccludinCT3 (17), but not occludinCT3, results in a breakdown of the intramembrane diffusion barrier.

To summarize, this study identifies occludin as a regulator of neutrophil transmigration across epithelia. Specific domains and properties of occludin are involved in the regulation of transmigration. Although the precise role of occludin in the mechanism that allows the reversible opening of tight junctions is not clear, the effects of transfected wild-type and mutant occludin on transmigration do not correlate with their effects on TER and paracellular permeability. Our results also indicate for the first time that the N-terminal cytosolic domain of occludin is of functional relevance.

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