Journal of Cellular Biochemistry 78:85–96 (2000)

Multiple Domains of Occludin are Involved in the Regulation of Paracellular Permeability

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Abstract Tight junctions form selective paracellular diffusion barriers that regulate the diffusion of solutes across epithelia and constitute intramembrane diffusion barriers that prevent the intermixing of apical and basolateral lipids in the extracytoplasmic leaflet of the plasma membrane. In MDCK cells, previous expression experiments demonstrated that occludin, a tight junction protein with four transmembrane domains, is critically involved in both of these tight junction functions and that its COOH-terminal cytoplasmic domain is of functional importance. By expressing mutant and chimeric occludin that exert a dominant negative effect on selective paracellular diffusion, we now demonstrate that the extracytoplasmic domains and at least one of the transmembrane domains are also critically involved in selective paracellular permeability. Multiple domains of occludin are thus important for the regulation of paracellular permeability. Expression of chimeras containing at least one transmembrane domain of occludin also resulted in an enhanced intracellular accumulation of claudin-4, another transmembrane protein of tight junctions, suggesting that the two proteins may cooperate in the regulation of paracellular permeability. J. Cell. Biochem. 78:85–96, 2000. © 2000 Wiley-Liss, Inc.

Key words: occludin; paracellular permeability; claudins

Epithelia form diffusion barriers that allow the generation of compositionally distinct fluid phase compartments. High molecular weight tracers can freely diffuse along the paracellular pathway until the level of tight junctions, the most apical structure of the epithelial junctional complex [Farquhar and Palade, 1963], indicating that tight junctions seal the paracellular route [for review, see Anderson and Van Itallie, 1995; Balda and Matter, 1998; Gumbiner, 1993]. This paracellular diffusion barrier is not an absolute one but behaves as though it contains pores that allow selective paracellular diffusion [for review, see Cereijido, 1991; Madara, 1998].

In thin section electron microscopy, tight junctions appear as zones of closely apposed plasma membranes that sometimes contain very close focal contacts [Farquhar and Palade, 1963; Pinto da Silva and Kachar, 1982]. These close contacts are thought to represent the intramembrane strands observed in freeze-fracture replicas as netlike meshworks of fibrils [Staehelin, 1973; Tsukita and Furuse, 1999]. The precise biochemical composition of these strands is unknown, but they contain at least two types of transmembrane proteins, one of which is occludin [Fujimoto, 1995; Furuse et al., 1998a]. Occludin, however, does not appear to be a main structural component of these intramembrane strands [Balda et al., 1996a; Saitou et al., 1998].

Occludin is a polytopic membrane protein that appears to span the membrane four times and to expose both termini to the cytosol [Furuse et al., 1993; for review, see Matter and Balda, 1999]. Overexpression of chicken occludin in MDCK cells results in efficient integration of the transfected protein into tight junctions, increased transepithelial electrical resistance (an instantaneous measurement that primarily reflects the degree of tightness) but, at high expression levels, also increased paracellular permeability (a parameter reflecting the passive transport capacities over a period of time) [Balda et al., 1996a; McCarthy et al., 1996]. Although deletion of the COOH-terminal cytoplasmic domain has been shown

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Received 5 November 1999; Accepted 18 January 2000

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This article published online in Wiley InterScience, April 2000.

to result in increased paracellular diffusion of small molecular weight tracers without causing a decrease of transepithelial electrical resistance or affecting the ion- and size-selectivity of the paracellular route [Balda et al., 1996a], the role of occludin in paracellular permeability and the functional relevance of its other domains are not clear.

The second type of strand-associated transmembrane proteins are members of the claudin multigene family [Furuse et al., 1998a; Morita et al., 1999]. Although claudins are an important structural component of tight junction strands, little is known about their functional role [Furuse et al., 1998b]. The exception is paracellin-1, a recently discovered claudin family member required for paracellular magnesium ion resorption in the kidney, suggesting that claudins may also function in allowing selective paracellular permeability [Simon et al., 1999].

By stably expressing occludin bearing mutations in different domains as well as chimeric constructs in MDCK cells, we now show that different structural domains of occludin, including at least one of the transmembrane domains and both extracellular domains, are important for the regulation of selective paracellular permeability. Our data suggest that occludin acts together with other transmembrane components of tight junctions, perhaps claudin-4, to allow selective diffusion of hydrophilic molecules across tight junctions.

MATERIALS AND METHODS

Cell Culture

MDCK (strain II) cells and transfected cell lines were grown and maintained as described [Matter et al., 1992]. For experiments, the cells were plated on Transwell filters with a pore size of 0.4 μm in 12-well clusters (Costar Corp., Cambridge, MA) and cultured for at least 7 days. MDCK cell lines expressing chicken occludin, FcOCD (see Fig. 1 for a definition of these occludin constructs), and LDL receptor were previously described [Balda et al., 1996a; Matter and Balda, 1998; Matter et al., 1992]. When indicated, the cells were incubated with 7 mM sodium butyrate added to normal culture medium overnight to induce higher levels of expression of the transfected proteins.

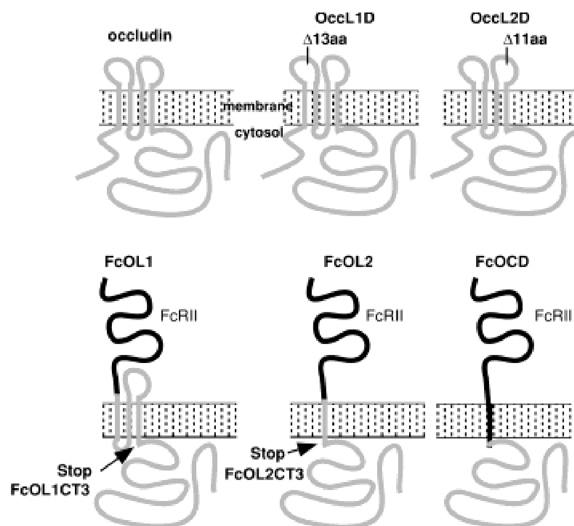

Fig. 1. Schematic view of wild-type, mutant, and chimeric occludin. Deletions in the extracytoplasmic loops are marked with dashes and the number of deleted amino acids. Occludin chimeras were constructed using the mouse Fc receptor for IgG (FcRII). The positions of STOP codons, introduced to eliminate the COOH-terminal cytoplasmic domain, are indicated.

Antibodies

The two anti-occludin antibodies raised against the COOH-terminal domain were previously described: antibody A recognizes chicken and dog occludin while antibody B is specific for chicken occludin [Balda et al., 1996a]. Occludin/Fc receptor chimeras were detected with a rabbit polyclonal antibody or monoclonal antibody 2.4G2, both recognize the mouse Fc receptor ectodomain [Green et al., 1985; Unkeless, 1979]. Drs. J. M. Anderson and M. S. Mooseker (Yale University, New Haven, CT) kindly supplied rat monoclonal antibody R40.76 specific for ZO-1 [Anderson et al., 1988] and Drs. J. Gruenberg and M. Rojo (University of Geneva, Switzerland) the rabbit polyclonal antibody against p23 [Rojo et al., 1997]. The rabbit polyclonal antibody recognizing the COOH-terminus of claudin-4 [Morita et al., 1999] was generously provided by Drs. M. Furuse and S. Tsukita (Kyoto University, Japan) and the monoclonal antibody against JAM by Dr. E. Dejana (Mario Negri, Italy).

cDNAs and Mutagenesis

All mutations were introduced by PCR-based mutagenesis using the previously described cDNA coding for chicken occludin as a template and Pfu polymerase (see Fig. 1 for a schematic representation of the constructed mutants and

chimeras). A 13 amino acid deletion in the first extracellular loop was introduced using a sense primer containing a BsiWI site, a G and a C to complete the frame, and nucleotides 211 to 222 of the coding sequence; an 11 amino acid deletion in the second extracellular loop of occludin was generated using an antisense primer containing a StuI site, a G to complete the frame, and the sequence that corresponds to nucleotides 582 to 564. To generate the occludin/Fc receptor chimeras, two sense primers were used that contained ApaI sites and then either nucleotides 361 to 372, to generate chimeras starting with the second transmembrane domain, or nucleotides 676 to 687, to synthesize chimeras starting at the fourth transmembrane domain. PCR-fragments synthesized with these primers were then cloned into the ApaI site at the end of the extracytoplasmic domain of the mouse Fc receptor for IgG.

Transfection and Selection of Clones

All cDNAs were inserted into pCB6 and MDCK cells were transfected using calcium phosphate precipitation as described [Matter et al., 1992]. Transfected cells were selected using G418 (0.6 mg/ml) and single clones (24 clones per transfection) were picked from the selection plates. The isolated clones were then grown on coverslips, and tested for expression by immunofluorescence (see below); only clones exhibiting homogenous expression ($>$ 95% expressing cells) were grown up and then tested again by immunoblot without or with a preincubation with 7 mM sodium butyrate overnight, a treatment that results in increased expression levels of the transfected cDNAs (see below). At least three independent, homogeneously expressing clones per transfection were then used for the functional analysis.

Immunofluorescence

To stain chicken occludin with the above mentioned anti-NH_2-terminal domain antibody, cells were cooled on ice, extracted for 3 min with 0.2% Triton X-100 in 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 200 mM sucrose, and 10 mM Hepes (pH 7.1) at 4°C, and were then fixed for 5 min in methanol at −20°C. To label chicken occludin with the anti-COOH antibody B or endogenous occludin with antibody A, filter-grown monolayers were permeabilized for 2 min on ice with 0.2% Triton X-100 in 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 200 mM sucrose, and 10 mM Hepes (pH 7.1) and were then immediately fixed for 30 min with 95% ethanol on ice. Alternatively, the cells were directly fixed on ice with 95% ethanol for 30 min and then incubated for 1 min at room temperature with acetone [Balda et al., 1996a]. The anti-Fc receptor antibody was used with cells that were permeabilized and fixed with the Triton X-100/ethanol procedure or that were directly fixed with 3% paraformaldehyde in PBS for 20 min and subsequently permeabilized with 0.1% saponin. For experiments with anti-claudin antibodies, the cells were fixed for 5 min in methanol at −20°C. The fixed cells were blocked and incubated with antibodies as described [Balda et al., 1996a], and the samples were mounted with the ProLong anti-fade kit (Molecular Probes, Inc., Eugene, OR), which resulted in significant improvement of sensitivity. The samples were then 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.

Endoglycosidase Digestions

For endoglycosidase digestions, transfected MDCK cells were metabolically labeled with [^{35}S]-methionine/cysteine overnight as described with the exception that the labeling medium was supplemented with 10% normal medium to avoid depletion of methionine and cysteine during the long labeling time [Matter et al., 1992]. After solubilization of the cells with extraction buffer (10 mM Hepes, pH7.4, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.2% Na-dodecylsulfate, 1% Empigen BB, and 40 μg/ml phenylmethylsulfonyl fluoride), wild-type and chimeric occludin were immunopreciptated with antibody B against occludin or the anti-Fc receptor antibody, respectively [Matter and Balda, 1998]. The washed beads were then digested with endoglycosidase H or endoglycosidase F/N-glycosidase F (Roche Molecular Biochemicals, Switzerland) as described [Matter et al., 1989].

Immunoblots, Transepithelial Electrical Resistance, Paracellular Permeability, and Electron Microscopy

Total cell extracts were separated on SDS-PAGE gradient gels, transferred to nitrocellulose, and probed with primary antibodies,

HRP-conjugated secondary antibodies, and ECL [Balda et al., 1996a]. Transepithelial electrical resistance, paracellular flux of HRP and [^{3}H]-mannitol, and freeze-fracture analysis were performed as previously described [Balda et al., 1996a].

RESULTS

Short Deletions in the Extracytoplasmic Loops of Occludin Result in Inefficient Integration Into Tight Junctions

One would expect that the extracytoplasmic domains of occludin are intimately involved in paracellular permeability, independent of whether occludin directly mediates paracellular permeability or whether it acts as a regulator. Therefore, we expressed mutants with short deletions in the extracellular loops, speculating that they would still be transported to tight junctions. We introduced these mutations into chicken occludin since it is efficiently incorporated into tight junctions of transfected MDCK cells [Balda et al., 1996a; McCarthy et al., 1996] and then used a species-specific antibody to visualize transfected chicken occludin [Balda et al., 1996a]. In the first extracellular loop, we deleted amino acids 92 to 104, a stretch of 13 amino acids containing five glycine and three tyrosine residues, to produce the mutant called OccL1D (Fig. 1). In the second extracellular loop, we removed amino acids 195 to 205 corresponding to 11 amino acids also containing three tyrosine residues, to produce a mutant called OccL2D (Fig. 1).

The two deletion mutants were transfected into MDCK cells, and stable cell lines were tested for expression by immunoblot using an antibody specific for the COOH-terminal domain of chicken occludin that does not cross-react with dog occludin ([Balda et al., 1996a]: antibody B). Figure 2A shows that both mutant proteins OccL1D and OccL2D could be stably expressed and that expression levels were further increased when the cells were pretreated with sodium butyrate. We then plated these transfected cells on filters and, after 7 days of culture, permeabilized and fixed the cells using the Triton X-100/ethanol procedure. The samples were stained and processed for immunofluorescence and confocal microscopy using the anti-chicken occludin antibody and a rat monoclonal antibody against ZO-1, a peripheral membrane protein of tight junctions. The samples were then analyzed by serial confocal xy-sections. Figure 3 shows the distribution of wild-type chicken occludin (A) in a clone of transfected MDCK cells that, based on immunoblot analysis, expresses about twice as much transfected protein as the analyzed clones expressing OccL1D (B) and OccL2D (C). As described previously, chicken occludin was efficiently integrated into tight junctions since most of the detected protein co-localized with ZO-1 [Balda et al., 1996a]. Additionally, we observed some staining of occludin at the lateral membrane, similar to what has been described for the localization of this protein in vivo [Sakakibara et al., 1997]. The visualization of previously not detected lateral staining is due to improved microscopy technique (see Materials and Methods). Confocal sections through samples of cells expressing OccL1D and OccL2D demonstrated that both deletion mutants accumulated in the lateral membrane (Fig. 3: B, OccL1D; C, OccL2D). In contrast to transfected wild-type occludin, the staining for the two mutants was not concentrated at the

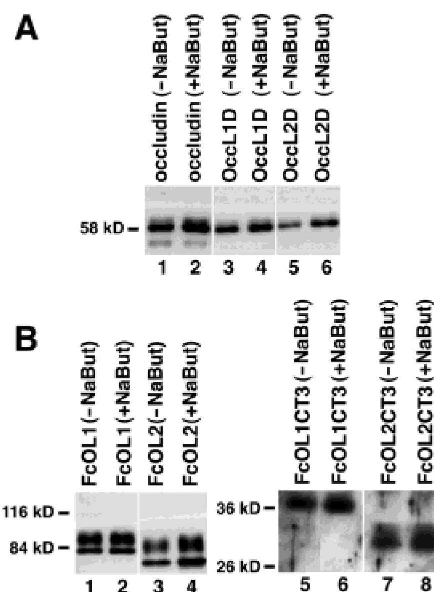

Fig. 2. Expression of mutant and chimeric occludin. Proteins of total cell extracts of non-transfected and stably transfected cells, preincubated without or with sodium butyrate, were separated by SDS-PAGE and transferred to nitrocellulose. Expression was then tested with an antibody against the COOH-terminal domain (antibody B) of chicken occludin (**A;B**: **lanes 1–4**), or an anti-Fc receptor antibody (B: lanes **5–8**). Bound primary antibodies were then detected with a HRP-conjugated secondary antibody and enhanced chemiluminescence. Note that all analyzed clones expressed the transfected protein without preincubation with sodium butyrate but that the pretreatment resulted in increased expression levels.

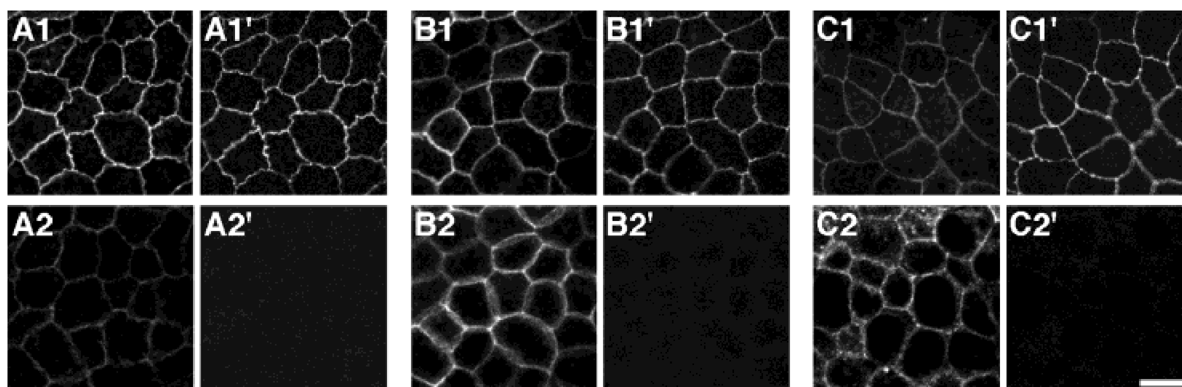

Fig. 3. Subcellular distribution of transfected occludin with deletions in the extracellular loops. Transfected MDCK cells expressing chicken occludin (**A**), OccL1D (**B**), or OccL2D (**C**) were cultured on filters and then permeabilized and fixed with the Triton X-100/ethanol procedure. The cells were then labeled with the anti-occludin antibody that is specific for the COOH-terminal domain of chicken occludin (**A1, A2, B1, B2, C1, C2**) and monoclonal antibody R40.76 specific for ZO-1 (**A1′, A2′, B1′, B2′, C1′, C2′**). Shown are two confocal xy-sections from each sample: one is derived from the junctional area of the monolayers (A1, B1, C1) and one from the middle of the lateral membrane (A2, B2, C2). Scale bar = 10 μm.

level of tight junctions, which were labeled with anti-ZO-1 antibodies. Moreover, xz-sectioning showed that the two mutants were distributed along the entire lateral membrane (not shown), suggesting that both mutant proteins were only inefficiently integrated into tight junctions. Since an antibody that only recognizes dog occludin and does not cross-react with chicken occludin is not available, we could not test whether endogenous occludin is normally distributed in these cells. As shown for the distribution of ZO-1 in Figure 3, we did also not observe morphological alterations caused by the expression of OccL1D and OccL2D when the cells were labeled with antibodies against the lateral membrane protein Na^+/K^+-ATPase or with fluorescent phalloidin (not shown). Furthermore, morphological changes were also not detected when the monolayers were embedded in Epon, thin sectioned, and observed by electron microscopy (not shown). The inefficient integration into tight junctions of mutant occludin with deletions in the extracellular loops supports the importance of these domains for occludin targeting suggested by the previous findings that integration into tight junctions is inhibited by a peptide corresponding to the second extracellular loop added to cultured epithelial monolayers [Wong and Gumbiner, 1997] and by N-linked carbohydrates added to the extracellular loops of occludin [Matter and Balda, 1998].

Inhibition of Paracellular Diffusion by Expression of Mutant Occludin With Altered Extracellular Loops

It had been speculated that the extracellular loops of occludin would form the paracellular seal of tight junctions; therefore, we measured transepithelial electrical resistance of cell lines expressing OccL1D and OccL2D. Figure 4A shows that the transepithelial electrical resistance of monolayers formed by OccL1D- and OccL2D-expressing cells was not significantly different from that of monolayers formed by wild-type cells. When higher expression levels were induced by incubating overnight with sodium butyrate, small but significant increases in transepithelial electrical resistance could be detected. Thus, expression of occludin with deletions in the extracellular domains did not prevent the formation of electrically tight monolayers.

To test whether expression of these mutants would affect selective paracellular permeability, we measured paracellular flux of two different molecular weight tracers. Paracellular flux of ^{3}H-mannitol was reduced by 30 to 40% by the expression of the two deletion mutants and flux of the higher molecular weight tracer HRP was even reduced by about 75% (Fig. 4B). This effect was only slightly enhanced by the butyrate-treatment (Fig. 4C). Thus, OccL1D and OccL2D inhibit the paracellular pathway that allows passive selective diffusion of solutes across epithelial sheets. Since occludin

possesses weak adhesive properties [Van Itallie and Anderson, 1997], the inhibition of paracellular permeability could have been due to extra-junctional intercellular interactions mediated by the extracellular domains of laterally accumulating occludin. Nevertheless, occludin appears to be only adhesive if concentrated in ZO-1-containing membrane subdomains [Van Itallie and Anderson, 1997], and we observed neither a redistribution of ZO-1 to the lateral membrane in OccL1D- and OccL2D-expressing cells (Fig. 3) nor any evident morphological effect on the intercellular space by electron microscopy of thin sections of Epon-embedded cells (not shown). Additionally, the reduction in paracellular permeability occurred without a significant increase in transepithelial electrical resistance, also arguing against a lateral enlargement of the junction.

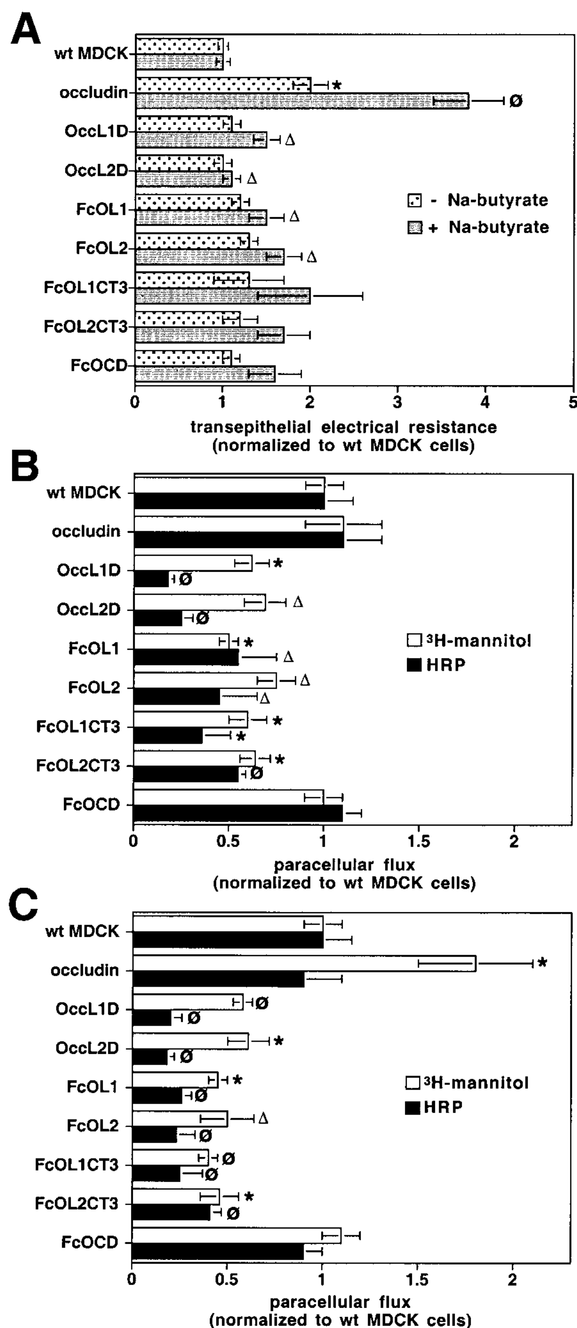

Fig. 4. Transepithelial electrical resistance and paracellular permeability of monolayers formed by cells expressing mutant and chimeric occludin. Wild-type MDCK cells, chicken occludin-expressing cells, and transfected cells expressing mutant and chimeric occludin (three independent clones for each construct) were plated on filters at confluence and were then cultured for 1 week. **A**: Transepithelial electrical resistance was measured without (dotted bars) or with (gray bars) an overnight preincubation with sodium butyrate. The values of wild-type cells were 65 ± 4 Ωcm^2 without and 67 ± 5 Ωcm^2 with sodium butyrate. The shown results for chicken occludin are derived from a previously described clone exhibiting an average phenotype that was assayed together with the new constructs as a control. The range of values obtained from clones expressing the different constructs were as follows: OccL1D, 66–75 Ωcm^2 without and 84–110 Ωcm^2 with sodium butyrate; OccL2D, 63–64 Ωcm^2 without and 75–85 Ωcm^2 with sodium butyrate; FcOL1, 73–82 Ωcm^2 without and 91–107 Ωcm^2 with sodium butyrate; FcOL2, 75–82 Ωcm^2 without and 104–124 Ωcm^2 with sodium butyrate; FcOL1CT3, 62–100 Ωcm^2 without and 89–142 Ωcm^2 with sodium butyrate; FcOL2CT3, 65–74 Ωcm^2 without and 99 and 137 Ωcm^2 with sodium butyrate; FcOCD, 65–74 Ωcm^2 without and 70 and 127 Ωcm^2. Paracellular flux of [^{3}H]mannitol and HRP was measured without (**B**) or with (**C**) a preincubation with sodium butyrate [[^{3}H]mannitol: empty bars; HRP: filled bars). The values of the three clones that were assayed for each construct were averaged and normalized to wild-type MDCK cells. Bars labeled with a symbol represent values that are significantly (Ø, $P < 0.01$; *, $P < 0.02$; Δ, $P < 0.05$) bigger than the ones obtained from wild-type MDCK cells.

Intracellular Accumulation of Occludin/Fc Receptor Chimeras

To test whether proteins lacking the adhesive extracellular loops would also be able to inhibit paracellular permeability, we constructed chimeric proteins consisting of the ectodomain of the mouse Fc receptor for IgG and occludin starting at the second (FcOL1) or fourth (FcOL2) transmembrane domain (Fig. 1). Because the COOH-terminal cytosolic domain is known to be important for the regulation of paracellular permeability in MDCK cells [Balda et al., 1996a], we constructed analogous chimeras lacking this domain (FcOL1CT3 and FcOL2CT3).

Figure 2B shows immunoblots of total extracts of cells stably expressing these chimeric

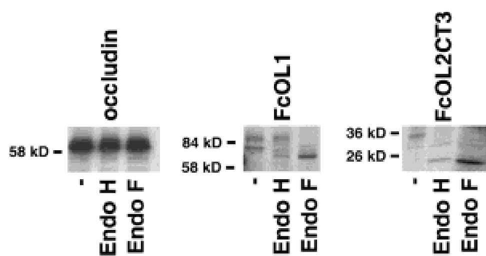

Fig. 5. Endoglycosidase digestion of occludin/Fc receptor chimeras. Transfected MDCK cells were metabolically labeled with [^{35}S]methionine/cysteine overnight and the transfected proteins were then immunopurified. The immunoprecipitates were incubated with buffer only (-), endoglycosidase H (Endo H), or endoglycosidase/peptide N-glycanase F (Endo F). The digests were then analyzed by SDS-PAGE and fluorography.

proteins. The blots were probed either with an antibody specific for the COOH-terminal domain of chicken occludin (lanes 1 to 4), to see FcOL1 and FcOL2, or with an anti-Fc receptor antibody (lanes 5 to 8), to detect FcOL1CT3 and FcOL2CT3. In lanes derived from cells expressing FcOL1, FcOL2, and FcOL2CT3, a sharp lower molecular weight band and a more diffuse higher molecular weight band could be detected, while FcOL1CT3 appeared as a single band. Since the Fc receptor ectodomain is N-glycosylated [Green et al., 1985], we tested whether the different bands represent high-mannose and complex glycosylated forms. To do this, we metabolically labeled cells expressing wild-type or chimeric occludin overnight and immunopurified the transfected proteins. We then digested the immunoprecipitates with endoglycosidase H, to remove high-mannose oligosaccharides, or with endoglycosidase F/N-glycosidase F, to cleave all N-linked glycans, and subsequently analyzed the digests by SDS-PAGE and fluorography. Figure 5 shows that occludin did not exhibit any shift after the endoglycosidase treatments, confirming the absence of N-linked glycans. In contrast, all chimeras (shown are FcOL1 and FcOL2CT3) were sensitive to the endoglycosidase treatment. In all cases, endoglycosidase H resulted in a shift of the lower molecular weight forms, indicating that these indeed represent high-mannose forms. The upper bands were not or only partially sensitive to endoglycosidase H but completely sensitive to endoglycosidase F/N-glycosidase F, suggesting that the upper bands represent chimeras at least partially converted to complex glycosylated proteins. This suggests that the chimeras are only inefficiently transported along the secretory pathway. Moreover, the presence of N-glycans indicated that the Fc receptor ectodomain was translocated into the lumen of the endoplasmic reticulum as expected since there are no potential N-linked glycosylation sites in the chicken occludin sequence. The topology of the chimeras was further supported by protease protection experiments (not shown).

We next tested the subcellular distribution of the chimeras by immunofluorescence and confocal microscopy. Figure 6A shows that most of FcOL2CT3 chimera accumulated in intracellular vesicular structures as well as some diffuse staining reminiscent of the endoplasmic reticulum. Similar structures were labeled with an antibody against p23 (Fig. 6C, p23; D, FcOL1), a membrane protein that localizes to the ER-Golgi intermediate compartment and the Golgi complex [Rojo et al., 1997]. Hence, the chimeras accumulated in the early secretory pathway. This subcellular localization matches the above described glycosylation patterns. No clear fluorescence was detected at the cell surface (addition of antibodies to non-permeabilized cells did also not result in detectable labeling; not shown). All four chimeras exhibited essentially the same distribution with the exception that a faint lateral staining could be observed when cells expressing chimeras containing the occludin COOH-terminal cytoplasmic domain were analyzed (Fig. 6E, FcOL1). The tight junction protein ZO-1 exhibited a normal distribution, indicating that it was not affected by the expression of the chimeric proteins (Fig. 6B, cells expressing FcOL2CT3; F, cells expressing FcOL1). Using the antibody that recognizes the COOH-terminal cytoplasmic domain of dog occludin, we also found that endogenous occludin was not affected by the expression of the chimeras lacking the COOH-terminal domain (Fig. 6G, endogenous occludin; H, ZO-1).

Inhibition of Paracellular Diffusion by Expression of Chimeras Containing at Least One Transmembrane Domain of Occludin

Similarly to the deletion mutants, the chimeras did not significantly affect transepithelial electrical resistance in the absence of sodium butyrate and caused modest increases in butyrate-treated cells (Fig. 4A). To test whether expression of the chimeras affected paracellular diffusion, we measured paracellular flux of ^{3}H-mannitol and HRP. Similar to OccL1D and OccL2D, all chimeras containing

at least one transmembrane domain of occludin inhibited paracellular flux of both tracers (Fig. 4B without and C with a preincubation with sodium butyrate). In contrast, the previously described basolaterally expressed chimera [Matter and Balda, 1998] containing only the COOH-terminal cytoplasmic domain and no transmembrane domain of occludin (Fig. 1: FcOCD) did not exert any significant effects on paracellular flux of ^{3}H-mannitol and HRP. Thus, expression of chimeras containing different parts of the NH_2-half of occludin decrease selective paracellular permeability. Importantly, an extracellular domain is not required for this phenotype. Since the chimeras did not visibly accumulate in tight junctions, and a single transmembrane domain was sufficient to mediate this dominant negative effect (i.e., FcOL2CT3), it is likely that these chimeras mediated the negative effect by preventing junctional integration of another transmembrane component.

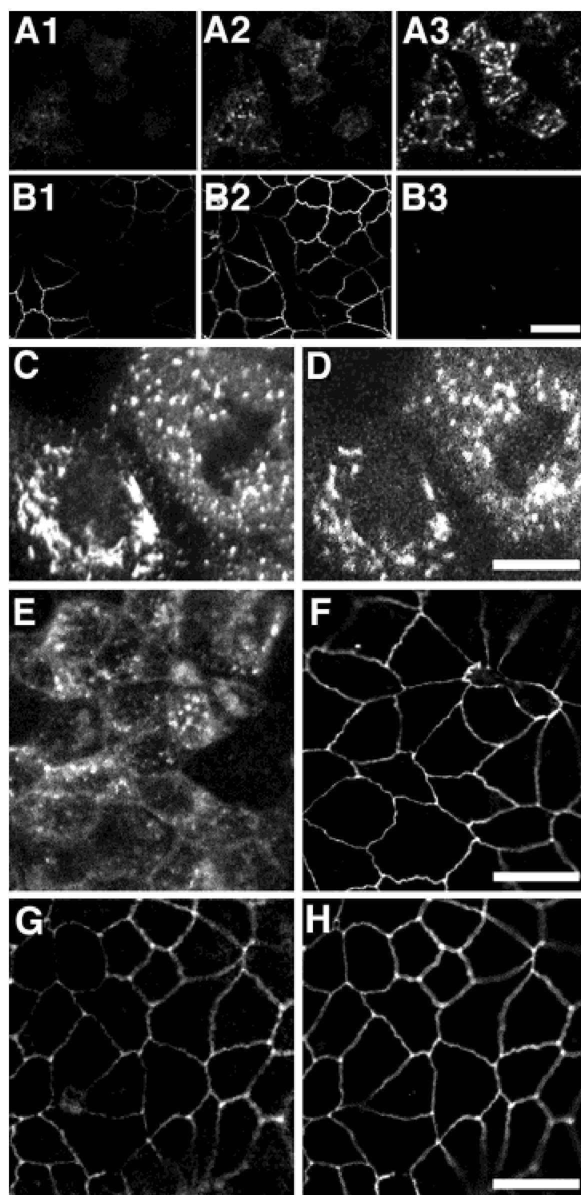

Fig. 6. Subcellular distribution of occludin/Fc receptor chimeras. Transfected MDCK cells, which were grown on filters for 7 days, were permeabilized and fixed with the Triton X-100/ethanol procedure (A,B,E–H) or were fixed with paraformaldehyde and permeabilized with saponin (C,D). **A,B**: FcOL2CT3-expressing cells were double labeled with the anti-Fc receptor antibody (A) and the anti-ZO-1 antibody R40.76 (B). Shown are three confocal sections: A1 and B1 are derived from the apical area of the monolayer, A2 and B2 from the junctional area, and A3 and B3 from approximately the middle of the cells that contains most of the labeling with the anti-Fc receptor antibody. Scale bar =, 10 μm. **C,D**: FcOL1-expressing cells were labeled with a polyclonal antibody specific for p23 (C) and the monoclonal anti-Fc receptor antibody 2.4G2 (D). Scale bar =, 3 μm. **E,F**: FcOL1-expressing cells were double labeled with the anti-chicken occludin antibody (antibody B) specific for the COOH-terminal domain (E) and the anti-ZO-1 antibody (F). Scale bar = 10 μm. **G,H**: FcOL2CT3-expressing cells were double labeled with the anti-occludin antibody A that recognizes dog occludin (G) and the anti-ZO-1 antibody (H). Scale bar = 10 μm. E–H show confocal sections taken from the junctional area of the monolayers. Note that the localization of endogenous occludin and ZO-1 is not affected by the expression of the occludin/Fc receptor chimeras.

Expression of Chimeric Occludin Causes an Increased Intracellular Accumulation of Claudin-4

To test the hypothesis that chimeric occludin constructs exert their dominant negative effect by preventing efficient cell surface transport of another tight junction component, we wanted to stain cells expressing such chimeras with antibodies recognizing other tight junction membrane proteins. The most promising membrane proteins to try were members of the claudin multigene family since several of them are known to be associated with the intramembrane strands of tight junctions as occludin [Furuse et al., 1998a,b; Morita et al., 1999]. Reverse transcription-PCR experiments demonstrated that MDCK cells express claudin-4 (not shown). Therefore, we stained wild-type and transfected MDCK cells expressing chimeric occludin with anti-claudin-4 antibodies [Morita et al., 1999].

Figure 7A shows that claudin-4 colocalizes with ZO-1 in wild-type MDCK cells. Additionally, some intracellular vesicular staining could also

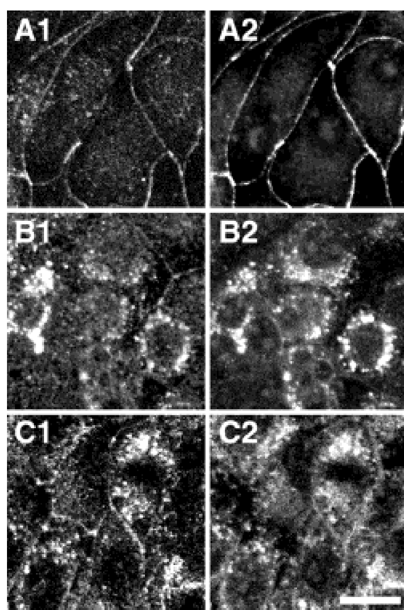

Fig. 7. Effect of occludin/Fc receptor chimeras on the subcellular distribution of claudin-4. Wild-type (**A**) and transfected MDCK cells expressing FcOL1 (**B**) or FcOL2CT3 (C) were fixed with methanol and labeled with antibodies against claudin-4 (A1, B1, C1) together with either a monoclonal antibody specific for ZO-1 (A2) or for Fc receptor (B2, C2). Note the enhanced intracellular labeling for claudin-4 in cells expressing chimeric occludin. Scale bar = 5 μm.

be observed. In cells expressing FcOL1 (Fig. 7B) or FcOL2CT3 (Fig. 7C), claudin-4 labeling resulted in only little junctional staining but in a strong intracellular vesicular staining. Intracellular claudin-4 colocalized to a large extent with the occludin chimeras. Thus, expression of chimeric occludin resulted in an enhanced intracellular accumulation of claudin-4. This suggests that the dominant negative effect of the chimeras on paracellular permeability may be due to an effect on cell surface transport of claudin-4. We also tried to stain MDCK cells with a monoclonal antibody against the junctional adhesion molecule JAM [Martin-Padura et al., 1998], but we could not obtain a signal. Therefore, we transiently transfected cDNAs coding for chimeric occludin into the human intestinal epithelial cell line Caco-2 and then stained for JAM and transfected chimeras. These experiments did not reveal any significant effect of chimeric occludin on the subcellular distribution of JAM (not shown).

Expression of Chimeric Occludin Does Not Affect the Morphology of Tight Junctions in Freeze-Fracture Replicas

Tight junctions appear in freeze-fracture replicas as networks of intramembrane stands that completely encircle the cells [Staehelin, 1973]. To test whether the inhibition of paracellular permeability is paralleled by alterations in these intramembrane strands, we processed wild-type cells and monolayers of transfected cells with reduced paracellular permeability for freeze-fracture electron microscopy. To have strongest effects, we analyzed cells expressing chimeric constructs after a preincubation with sodium butyrate.

Electron micrographs of these freeze-fracture replicas did not indicate morphological alterations caused by the expression of the chimeras (Fig. 8A, wild-type MDCK; B, FcOL2; C, FcOL1CT3). Particularly, no lateral extension of the tight junctions was observed in transfected cells. Quantification of the number of strands did also not suggest an enlargement of the strands but rather resulted in small decreases (average number of strands per segment: wild-type MDCK, 3.4 ± 1.6; FcOL2, 2.9 ± 1.3; FcOL1CT3, 3.1 ± 1.3).

DISCUSSION

Occludin is a functional component of the semipermeable paracellular diffusion barrier formed by tight junctions. Our data indicate that transmembrane domains and both external loops are important for occludin's function in paracellular permeability and that occludin cooperates with other transmembrane components to allow selective paracellular diffusion.

The paracellular diffusion barrier of low resistance epithelia like MDCK strain II cells is generally characterized by measuring transepithelial electrical resistance, an instantaneous measurement that reflects the degree of sealing at a given point in time, and paracellular flux, a measure for the selective permeability of the paracellular pathway that is determined over a period of time. In MDCK cells, both junctional parameters are affected by the stable expression of wild-type and mutant occludin [Balda et al., 1996a; McCarthy et al., 1996; this paper]. Transepithelial electrical resistance was thus far never reduced by the expression of wild-type, mutant, or chimeric occludin in MDCK cells but either remained the same or was increased. Nevertheless, depletion of occludin from the tight junctions of Xenopus A6 cells causes a large reduction of transepithelial electrical resistance [Wong and Gumbiner, 1997]. In contrast, in a murine epithelial cell line, not only the expression of mutant but also the over-expression of wild-type occludin

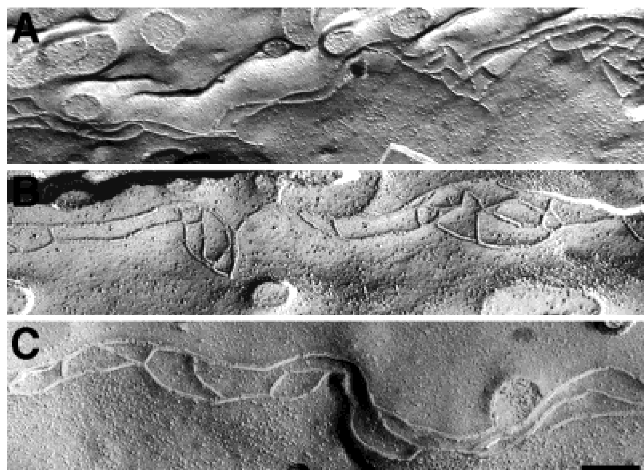

Fig. 8. Freeze-fracture electron microscopy of MDCK tight junctions. Wild-type MDCK cells (**A**) and transfected cells expressing FcOL2 (**B**) or FcOL1CT3 (**C**) were cultured in plastic flasks for 10 days after they had reached confluence. The monolayers were then incubated overnight with sodium butyrate and then fixed and processed for freeze-fracture. Replicas were observed by electron microscopy. Scale bar = 200 nm.

resulted in a decrease in transepithelial electrical resistance [Bamforth et al., 1999]. The expression level of occludin thus directly affects the strength of the junctional seal in a manner that appears to depend on the genetic background of the analyzed cells. A possible explanation of this is that occludin is a regulatory component of the tight junctional seal. This is also supported by occludin-deficient stem cells that are able to differentiate into epithelial cells with apparently closed tight junctions [Saitou et al., 1998].

In addition to playing a role in the sealing of the junction, occludin regulates selective paracellular permeability. Previously, increased expression of full length and COOH-terminally truncated occludin was demonstrated to result in electrically tighter junctions but also increased selective paracellular permeability [Balda et al., 1996a; McCarthy et al., 1996]. We now show that the extracytoplasmic loops and least one of the transmembrane domains are also critical for this function of occludin.

The mechanism by which occludin regulates or mediates selective paracellular permeability is not clear. Since the increases in paracellular permeability were observed in dog cells transfected with wild-type and mutant chicken occludin, it was proposed that they were due to rapid nonspecific opening of the junction because of imperfect extracellular interactions caused by the differences in sequence between dog and chicken occludin [Van Itallie and Anderson, 1997]. Such nonspecific opening and closing of the junction would result in a changed size- and ion-selectivity of the paracellular diffusion pathway and to lower transepithelial electrical resistance. This was not observed [Balda et al., 1996a]. Moreover, we now show that expression of mutant (mutated extracellular loops) and chimeric occludin can result in large decreases in paracellular permeability, directly confirming that occludin participates in a mechanism that mediates selective paracellular diffusion.

Tight junctions were long suspected to contain aqueous pores that allow paracellular diffusion in a size- and charge-selective manner [Reuss, 1991]. Tight junctions have been proposed to represent a series of diffusion barriers that contain fluctuating channels [Cereijido et al., 1989; Claude, 1978]. The channel hypothesis would explain the exponential relationship between the number of intramembrane strands and transepithelial electrical resistance that is found in many tissues [Claude, 1978], but there are numerous exceptions to this rule [González-Mariscal et al., 1989; Martínez-Palomo and Erlij, 1975; Stevenson et al., 1988] including the results from the occludin expression experiments. The transepithelial electrical resistance is thus not necessarily related to the number of strands and, if the strands are like resistors, the molecular characteristics of the strands must be as important as their number. A model of tight junctions in which selective permeability is mediated by a transport system that does not form a continuous conductive route would be easier to reconcile with the observation that inhibition of paracellular flux by the expression of mutant occludin did not result in large increases in transepithelial electrical resistance. Hence, selective flux across tight junctions may not be due to a simple type of fluctuating channel that is either completely open or completely closed

but that is, at a given time, only open on one side of the diffusion barrier. Such a transport system would work like a lock on a river to allow the passage of ships (and water) without forming a continuously open pathway. In this lock-model, the selectivity of the paracellular pathway is determined by the size of the lock and the charge of the internal surface.

According to these considerations, occludin could either be a subunit and/or a regulator of a transport system that allows selective diffusion across tight junctions. While the current data could be explained in either way, our results with chimeric constructs that inhibit paracellular flux without becoming integrated into tight junctions and without affecting the distribution of endogenous occludin suggest that they inhibit integration of another component into tight junctions. Since a single transmembrane domain of occludin is sufficient to cause this phenotype, the retained component is likely to be a transmembrane protein that might otherwise combine with occludin to form the junctional locks. Since the expression of such dominant negative chimeric occludin mutants causes an enhanced intracellular accumulation of claudin-4, it could be that claudin-4 (and perhaps other claudins) cooperate with occludin to mediate selective paracellular permeability. An association between occludin and claudins is also suggested by the recruitment of occludin to claudin-induced intramembrane strands in L-cells [Furuse et al., 1998b]. Interestingly, it has recently been shown that paracellin-1, a member of the claudin family, is required for the paracellular resorption of magnesium ions in the kidney [Simon et al., 1999]. Although it is not clear whether paracellulin-1 forms pores or acts as a sensor regulating paracellular magnesium ion resorption, it is tempting to speculate that occludin and claudins cooperate in the regulation of selective paracellular permeability. Since claudin-4 is part of a large multigene family whose members are expressed in a tissue-specific manner [Morita et al., 1999], it could be that the claudin-composition of tight junctions determines the capacity and the selectivity of the paracellular diffusion pathway.

ACKNOWLEDGMENTS

We thank Dr. J. Andrew Whitney for critical reading of the manuscript and Drs. J.M. Anderson, M.S. Mooseker, J. Gruenberg, M. Rojo, M. Furuse, S. Tsukita, E. Dejana, and M. Caplan for providing antibodies. K.M. is a fellow of the START (Swiss Talents in Academic Research and Teaching) program of the Swiss National Science Foundation. This research was supported by the Swiss National Science Foundation, the Canton de Genève, and the CONACYT of Mexico.

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