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# Occludin Localization at the Tight Junction Requires the Second Extracellular Loop

**R. Medina**[1], **C. Rahner**[1], **L.L. Mitic**[2], **J.M. Anderson**[1,2], **C.M. Van Itallie**[1]
[1]Departments of Internal Medicine and [2]Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

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**Abstract.** Occludin is a transmembrane protein of the tight junction with two extracellular loops. Our previous demonstration that the extracellular loops are adhesive suggested the possibility that they contribute to localizing occludin at the tight junction. To address this question, truncated forms of occludin were generated in which one or both of the extracellular loops were deleted. These constructs were expressed in both occludin-null Rat-1 fibroblasts and in MDCK epithelial cells. The patterns of sensitivity to proteinase K suggested all constructs were present on the plasma membrane and retained the normal topology. In fibroblasts, all truncated forms of occludin colocalized with ZO-1 at regions of cell-cell contact, demonstrating that even in the absence of tight junctions cytoplasmic interactions with ZOs is sufficient to cluster occludin. In MDCK cell monolayers, both full-length and occludin lacking the first extracellular loop colocalized with ZO-1 at the tight junction. In contrast, constructs lacking the second, or both, extracellular loops were absent from tight junctions and were found only on the basolateral cell surface. By freeze-fracture electron microscopic analysis, overexpression of full length occludin induced side-to-side aggregation of fibrils within the junction, while excess occludin on the lateral membrane did not form fibrils. These results suggest that the second extracellular domain is required for stable assembly of occludin in the tight junction and that occludin influences the structural organization of the paracellular barrier.

**Key words:** Tight junction — Occludin — ZO-1 — Claudin

## Introduction

Tight junctions are specialized regions of cell-cell contact at the apical end of the lateral membranes of epithelial cells. They regulate the movement of solutes through the paracellular pathway (Reuss, 1991; Anderson & Van Itallie, 1995) and restrict the intramembrane diffusion of lipids and proteins between the biochemically distinct apical and basolateral plasma membranes (Dragsten, Blumenthal & Handler, 1981; van Meer & Simons, 1986). The structure of the tight junction is best observed in freeze-fracture electron microscopic images, where it appears as a complex belt of fibrils that encircle each cell, with cell–cell contacts occurring between fibrils from adjacent cells. It remains poorly understood how proteins are assembled into the tight junction fibrils. This information is required to explain the molecular basis for the paracellular barrier and how it is regulated.

Two protein components of the tight junction fibrils have been identified; they are occludin (Furuse et al., 1995) and members of the claudin multi-gene family (Furuse et al., 1998). On the basis of their amino acid sequence, both proteins are predicted to contain four membrane-spanning regions, with two extracellular domains and cytosolic amino and carboxy termini. The contribution of each of these proteins to the tight junction seal is not fully defined, but it appears that claudins are the main structural elements of the fibrils (Saitou et al., 1998). When either claudin-1 or 2 are expressed in fibroblasts they are capable of forming elaborate freeze-fracture fibrils reminiscent of those in epithelial cells (Furuse et al., 1998). Occludin expressed under similar conditions forms only short discontinuous strands, but when co-expressed with claudin, occludin is recruited into the more extensive claudin-based fibrils (Furuse et al., 1998). In addition, embryonic stem cells from which occludin was deleted by homologous recombination still form fibrils and a paracellular barrier (Saitou et al., 1998). Thus, the structural contribution of occludin to the fibrils remains undefined.

Although occludin is not required for fibril formation, it does appear to be a functional component of tight junctions. Overexpression of occludin in MDCK cells increases the electrical resistance of the tight junction

*Correspondence to:* C.M. Van Itallie

barrier (McCarthy et al., 1996; Balda et al., 1996). The influence of occludin on the intercellular barrier may depend on adhesive interactions of its extracellular domains, since peptides encoding these domains can interrupt both cell-cell adhesion in occludin-transfected fibroblasts (Van Itallie & Anderson, 1997) and transepithelial electrical resistance in MDCK monolayers (Wong & Gumbiner, 1997). The interactions of the extracellular domains are likely homophilic in nature (Furuse et al., 1998).

Associated with the transmembrane proteins of the tight junction are a large number of cytosolic plaque proteins (for review, *see* Balda & Matter, 1998). Three of these proteins, ZO-1, ZO-2 and ZO-3 (ZOs), bind directly to both occludin (Itoh et al., 1997; Fanning et al., 1998; Wittchen, Haskins & Stevenson, 1999) and claudins (Itoh et al., 1999), as well as to actin (Fanning et al., 1998; Wittchen et al., 1999). ZO-1, ZO-2 and ZO-3 are members of the MAGUK family of proteins, whose members have been shown to function in clustering transmembrane proteins into specific membrane locations and in cross-linking these proteins to cortical cytoskeleton.

The importance of the interaction with ZOs in localizing occludin was the subject of a recent study from our laboratory (Mitic et al., 1999). In this work, chimeras between occludin and the gap junction protein connexin were constructed containing the cytoplasmic ZO-binding domain of occludin fused to the transmembrane and extracellular domains of connexin. These were able to localize precisely within the tight junction fibrils. These same chimeras localized at ZO-1-based cell contacts in occludin- and claudin-null fibroblasts. This suggested that binding to ZOs was sufficient to cluster occludin and that an interaction with the transmembrane proteins occludin and/or claudin was not required. However, other studies suggest the interaction with ZOs is not sufficient or necessary for occludin to localize to tight junctions. For example, both Balda et al. (1996) and Chen et al. (1997) demonstrated that occludin lacking the cytoplasmic ZO binding region could still localize at tight junctions in MDCK and *Xenopus* embryo epithelial cells, respectively, and could be coprecipitated with endogenous full-length occludin. In this case, as suggested by Chen et al. (1997) localization of truncated occludin may occur because it interacts with full-length occludin enroute for or already localized to the junction. An insufficient role for the interaction with ZO-1 was also suggested by studies of Matter & Balda (1998) showing that occludin chimeras containing the ZO-1 binding domain fused with the transmembrane and ectodomain of the Fc receptor were not found at the tight junction but localized to the lateral membrane of MDCK cells. In light of these results, it is possible that the special ability of the connexin/occludin chimeras to incorporate into tight junction fibrils results from both their ability to bind ZOs and their inherent ability to form cell-to-cell and lateral interactions with themselves, a property presumably shared by occludin.

Taken together, these results suggest that proper localization of occludin to the junction may require several protein interactions. Targeting of occludin to the lateral membrane and assembly into the tight junction fibrils likely occurs through interactions with both cytoplasmic scaffolding proteins such as ZO-1 and previously assembled occludin and claudin molecules. The occludin–occludin interactions might occur within the transmembrane sequences or through the extracellular loops within the same cell or between adjacent cells.

To test the possibility that the extracellular sequences of occludin are required for junctional localization, we created stable lines of MDCK and Rat-1 fibroblasts expressing several truncated forms of occludin. First, the C-terminal cytoplasmic domain of occludin was fused to the transmembrane and extracellular sequences of glycophorin C to produce a protein that might bind ZOs but lacks extracellular adhesive properties. Second, truncations of occludin were produced in which most of the first, second or both extracellular loops were deleted. Here we report that the presence of the second extracellular loop is required for the localization of occludin in the tight junction. Together with published studies our results support the involvement of multiple interactions in the localization of occludin at the junction. We also demonstrate that overexpression of occludin induces side-to-side aggregation of tight junction fibrils, supporting its adhesive ability and influence on the structural organization of the barrier-forming fibrils.

## Materials and Methods

### Plasmid cDNA Constructs

Glycophorin C: a plasmid containing full-length human glycophorin C (a gift of Dr. Narla Mohandas, University of California, Berkeley) was used as a template in polymerase chain reactions (PCRs). The glycophorin/occludin sequence was constructed by amplifying the N-terminus of glycophorin C using primer 32188 (5′GCGGTACCCCCAGGAATGTGGCGGACGAGA-3′) and 32190 (5′-GGCGGCCGCCAGCATGACGAAGAGGAGGGA-3′). The PCR product was digested with Not I and ligated to the ZO-binding domain of the occludin tail (residues 373–522) which was amplified and VSV-G-tagged using primers 19986 (5′-CGCGGCCGCGAAAACTCGAAGAAAGATGGAC-3′) and 21063 (5′-CGCGGCCGCGAACTTTGAGACAGGTTGAAAAACA-3′) and also digested with Not I.

A plasmid (Bluescript SK+, Stratagene) containing the complete human occludin cDNA was used as a template for polymerase chain reaction (PCR) to generate cDNAs encoding deletion constructs. The VSV-G epitope tagged human occludin cDNA has been described previously (Van Itallie & Anderson, 1997). The construct containing a truncated form of the first extracellular loop, Occ(1−), was constructed

by digesting and ligating two PCR products. The first PCR product was amplified using a primer to the T7 promoter and primer 27859 (5′-CCAAGCTTGCCTCTGTCCCAGGCAAG). The resulting PCR product encoded the N-terminal end of occludin including the first transmembrane domain and the first three amino acids of the first extracellular loop followed by an engineered Hind III site, which encodes an additional KL. The second PCR product was amplified using a primer to the T3 promoter and primer 27860 (5′-GGAAGCTTGGCTATACAGACCCAAGAGC). This generated a PCR product containing the C terminal end of occludin starting with an engineered 5′ Hind III site followed by the last three amino acids of the first extracellular loop followed by the second transmembrane domain and the rest of the occludin protein. These PCR products were cloned into Bluescript SK+ and the inserts combined using the engineered Hind III site. The first extracellular loop is predicted to include 47 residues encompassing residues $D^{90}$ to $R^{135}$. The truncated first extracellular loop contained 11 amino acids, $D^{90}$RGKLGYTDPR$^{135}$ resulting from deletion of residues 93 to 129 and insertion of residues KL (from the Hind III site) that are not found in the authentic occludin sequence. To minimize potential PCR-generated sequence errors, a piece of this construct containing the truncated loop (Nsi I–Bgl II), was transferred into the authentic occludin cDNA in the pTRE vector (Clontech) for mammalian expression studies. The construct containing a truncated form of the second extracellular loop, Occ2(−) was made using the same approach; and the occludin primers used for PCR were primer 27861 (5′-CCAAGCTTAGTTGGGTTCACTCCCATTATATAGAC) and primer 27862 (5′-CCAAGCTTGATCCCCAGGAGGCCATTGCC); a Bgl II– NcoI fragment was used to replace the second extracellular loop in full length occludin. The second loop is predicted to include 48 residues from $G^{196}$ to $E^{243}$. The truncated second extracellular loop contained the amino acids $G^{196}$VNPTKLDPQE$^{243}$, resulting from deletion of residues 201 to 239 and insertion of residues KL not found in occludin. A construct containing truncations of both extracellular loops (pTRE1(−)/2(−) was generated by digestion of the pTRE1(−) and pTRE2(−) vectors with Nsi I and Bgl II and ligating the 150 bp fragment containing the truncated loop one from pTRE1(−) into pTRE2(−). All cloned PCR products were confirmed by sequencing in both directions.

## Cell Culture and Generation of Stable Lines

The glycophorin C constructs were expressed in MDCK cells using the mammalian expression vector pCB6 (Karl Matter, University of Geneva, Switzerland); stable lines were generated after DNA lipofection by selection in 0.8 mg/ml G418. For studies with truncated occludins Tet-OFF MDCK cells (Clontech) were transfected with pTRE alone (Clontech) or one of the pTRE-occludin vectors plus the pTK-Hyg selection plasmid (Clontech) using the lipofectamine protocol (GIBCO/BRL). Vector-alone or occludin-transfected cells were selected in 0.2 mg/ml hygromycin B and resistant clones were maintained in high-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 5 mM penicillin, 1 μg/ml puromycin, 0.1 mg/ml hygromycin B, 20 ng/ml doxycycline. Expression of transgenes was induced by removal of doxycycline. Cell lines were screened by immunofluorescence using the monoclonal VSV-G antibody (*see below*).

Rat-1 fibroblasts stably expressing pUHD15-1 neo plasmid which contains both the tetracycline transactivator gene and the neomycin resistance gene were obtained from the ATCC (Rat-1/R12 cells; CRL-2210). These cells were also doubly transfected with the expression vectors of the Tet-Off gene expression system (Clontech) using lipofection (GIBCO/BRL). Stable lines were selected in 0.15 mg/ml hygromycin B. Rat-1 cell lines were maintained in high glucose DMEM, 10% fetal bovine serum, 0.1 mg/ml G418 and 0.1 μg/ml hygromycin B. Two separate clones each containing the pTRE vector alone or expressing full length human occludin were analyzed, and at least 4–5 clones expressing the mutant occludins were used for immunofluorescence and immunoblot analysis.

## Immunoblot Analysis

To determine the expression profiles of the occludin constructs in the MDCK cell lines, transfected MDCK cell lines were plated at subconfluent density onto 6-well tissue culture plates. Protein expression was induced by doxycycline removal for 0, 24, 48, and 96 hr; all cells were collected at time 0. Doxycycline at this dose had no effect on cell growth (*data not shown*). Cells were washed twice with ice-cold phosphate-buffered saline (PBS), were scraped into 1 ml PBS and pelleted in a microcentrifuge. The PBS was then aspirated and the cells were lysed by adding 0.5 ml of SDS loading buffer (0.0625 M Tris, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 1% bromophenol blue, 5% 2-mercaptoethanol), immediately heated to 95°C in a heating block and stored at −80°C. Cells were thawed on ice, boiled for an additional 5 min and equal volumes of lysate immediately subjected to SDS-polyacrylamide gel electrophoresis (10%). Protein was then transferred to nitrocellulose and probed with a rabbit polyclonal VSV-G antibody (MBL International, Watertown, MA). Antigen-antibody complexes were detected using a rabbit horseradish peroxidase-linked secondary antibody (Amersham) according to the ECL Western blotting protocol (Amersham).

## Proteinase K Treatment

To probe the membrane topology of the mutant occludins, we determined their accessibility to extracellular Proteinase K, a broad spectrum protease. Transfected MDCK cells were plated onto 12-mm filter inserts (Costar, 2 filters for each cell line) and transgene expression induced in the absence of doxycycline for 4 days. On the fourth day, cells were washed twice in serum-free media and the apical and basolateral media of one set of filters was replaced with proteinase K media (0.02 mg/ml proteinase K, 25 mM HEPES in serum-free DMEM), while the media of the second set of filters was replaced with 25 mM HEPES in serum-free DMEM. All filters were incubated at 37°C for 30 min, placed in ice-cold stop buffer (0.5 mM phenylmethylsulfonyl fluoride in PBS) and kept on ice. The filters were cut out of the well inserts and immediately plunged into SDS loading buffer (described above), heated at 95°C for 5 min, then stored at −80°C until used for immunoblot analysis.

## Immunofluorescence

To analyze the localization of the glycophorin C and occludin constructs in MDCK cells, transfected MDCK cell lines were plated onto acid-washed 18-mm$^2$ glass coverslips and transgenes were induced for 48 hr with 5 mM sodium butyrate (for pCB6 glycophorin C vectors) or 4 days by removal of doxycycline (pTRE-containing cells). Cells were prepared for immunofluorescence as described previously (Fanning et al., 1998). The VSV-G-tagged occludin constructs were visualized with a mouse monoclonal anti-VSV-G antibody (P5D4; 1:100 dilution, a gift from Dr. Thomas Kreis [deceased], University of Geneva, Switzerland) and a donkey anti-mouse Cy2 conjugated secondary antibody (affinity-purified, species-specific; Jackson Immunoresearch). Endogenous ZO-1 was detected using a rat monoclonal antibody against ZO-1 (R40.76 cell supernatant, 1:50 dilution) and a donkey anti-rat Cy5 conjugated secondary antibody (Jackson). The coverslips were then

mounted onto glass slides (Corning) in Mowiol containing 1% n-propylgallate.

### Semithin Cryosectioning

To determine the localization of the occludin transgenes at a higher resolution, MDCK cell lines expressing the constructs were analyzed after semithincryo sectioning. Cells were plated onto acid-washed 18 × 40 mm glass coverslips and were maintained in the absence of doxycycline for 4 days to induce protein expression. The cells were then fixed in 3% paraformaldehyde, scraped from coverslips using a razor blade, and transferred to a microcentrifuge tube containing PBS with 0.05% sodium azide for storage at 4°C. The pellets were then transferred to microcentrifuge tubes containing 25% polyvinylpyrrolidine (in saturated sucrose) for cryoprotection and incubated on ice overnight. The pellets were then cut into ~1 mm long sections using a razor blade and the pieces individually mounted onto nail heads and immediately frozen and stored in liquid $N_2$ until further processing. For sectioning, the nails were mounted onto a Leica cryomicrotome, the pellets were sectioned at a thickness of 0.8–1.0 μm at −65°C and the sections mounted onto 3-aminopropyltriethoxysilane-coated 18 × 40 mm glass coverslips. These sections were stored in PBS containing 0.05% sodium azide at 4°C until further analysis. For immunofluorescence, the coverslips were quenched using 50 mM ammonium chloride, permeabilized by 0.2% Triton X-100, and then labeled with the primary and secondary antibodies listed above and with TRITC-phalloidin to visualize endogenous actin. The coverslips were then mounted onto glass slides in Mowiol containing 1% n-propylgallate.

### Light Microscopy

All microscopy was performed on a Nikon Microphot FX microscope using a 60X PlanApo lens and images captured using a Sensys cooled CCD camera (Photometrics). Images were then processed using Image Pro Plus 2.0 (Media Cybernetics) and Adobe Photoshop 5.0. Alternatively, (for glycophorin C constructs) the slides were viewed using a Bio-Rad MRC1024 confocal microscope on a Zeiss Axiovert using a 63× PlanApo lens.

### Freeze Fracture Electron Microscopy

MDCK cells, grown to confluence on 24 × 40 mm coverslips, were rinsed briefly in PBS followed by fixation with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4, for 20 min at room temperature. After removal of the fixed MDCK cell monolayers with a razor blade, cells were pelleted and rinsed three times for 10 min in cacodylate buffer. Freeze fracture replication was performed as described previously (Rahner et al., 1996). Briefly, cell pellets were cryoprotected with 25% glycerol in cacodylate buffer. Specimens were mounted on double replica gold specimen supports, rapidly frozen at −210°C in subcooled liquid $N_2$ (nitrogen slush) and transferred to a vacuum chamber (BFA 400D, Bal-Tec AG, Balzers, Lichtenstein). After fracturing at −100°C, specimens were shadowed with 2 nm platinum/carbon at an angle of 45° and replicated with 20 nm carbon at 90°. After thawing, replicas were cleaned with 50% chromic-sulfuric acid overnight, washed 6 times in double distilled water, and mounted on hexagonal 200 mesh copper grids. Replicas were examined and electron micrographs recorded on a Philips CM10 electron microscope.

## Results

### Occludin-Glycophorin C Chimeras Localize to the Lateral Membranes of MDCK Cells

To confirm that the cytosolic tail of occludin contains lateral targeting information but is not sufficient for incorporation into tight junctions, we created a chimera between glycophorin C and occludin. Glycophorin C was chosen because it is a single spanning transmembrane protein which, like occludin, has its C terminus within the cell. Unlike occludin, it lacks any cell-to-cell adhesion and does not aggregate within the membrane bilayer, beyond forming a dimer (Fisher, Engelman & Sturgis, 1999). When full-length glycophorin C tagged with a VSV-G epitope was expressed in MDCK cells it was localized on both the apical and basolateral plasma membranes in a nonpolarized fashion (Fig. 1*a*). ZO-1, in comparison, was highly restricted to the tight junction (Fig. 1*b*). In contrast, a chimera containing the extracellular and transmembrane sequences of glycophorin C fused to the cytosolic C-terminal 150 amino acids of occludin localized predominantly on the lateral membrane (Fig. 1*c*) below ZO-1 (Fig. 1*d*). This C-terminal domain of occludin has previously been shown to interact with the ZOs. These data confirm previous work and are consistent with the idea that in MDCK epithelial cells the C-terminal tail of occludin contains basolateral targeting information, but by itself is not sufficient to localize proteins at the tight junction (Matter & Balda, 1998). In addition, these results suggest that the transmembrane and/or extracellular domains of occludin may be required for localization at tight junctions, perhaps through their ability to aggregate (Balda & Matter, 1998; Chen et al., 1997) and bind occludin on other cells (Van Itallie & Anderson, 1997).

### Characterization of Inducible Occludin Constructs in MDCK Cells

To test the role of the extracellular domains in the localization of occludin, we used an inducible vector to express occludin with truncations in one or both of these domains. Initial experiments in which the entire first or second loops, including the transmembrane domains, were removed failed to produce any clonal cell lines expressing these proteins (*data not shown*). This led us to modify our approach and make mutant occludins which retained all four transmembrane segments but which lacked most of the extracellular domains, i.e., 36 of the 47 residues in the first loop (positions 92–129) and 41 of the 48 residues in the second loop (positions 200–240). Full length occludin is designated FL or Occ, while occludin containing the truncated first extracellular loop but a normal second loop is designated Occ1(−); that with a normal first extracellular loop but containing the truncated second extracellular loop is designated Occ2(−); and occludin with both extracellular loops truncated is named Occ1(−)/2(−). Because we were concerned that truncated versions of occludin might have dominant negative effects on tight junction assembly, we used a tetracycline suppressible transgene expression system (Bujard, 1999) to avoid potential problems asso-

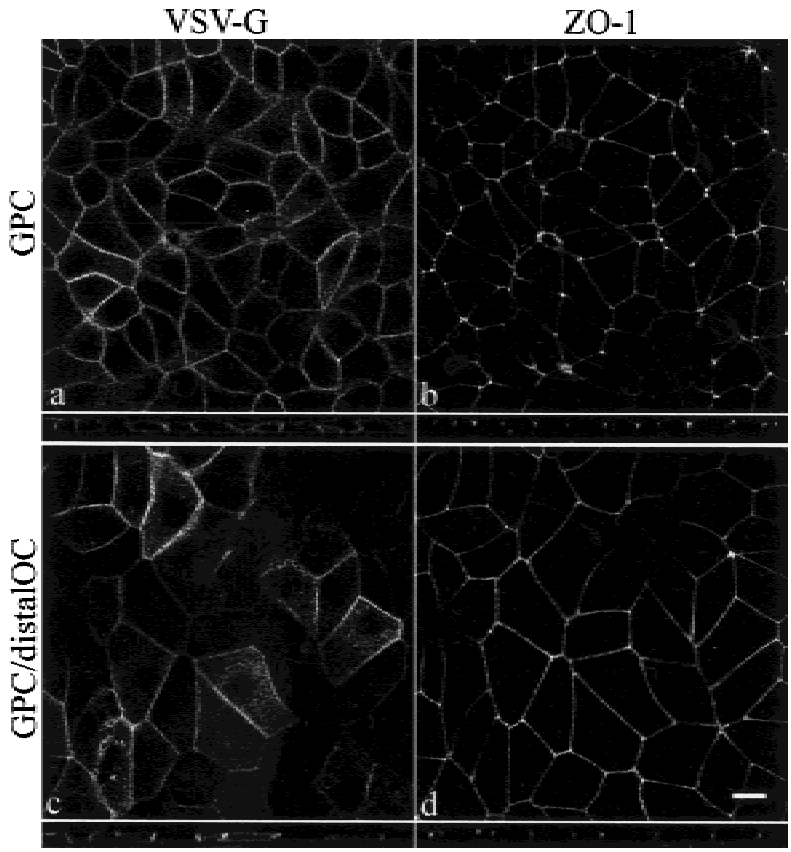


**Fig. 1.** Immunofluorescent confocal localization of glycophorin C (*a*) and glycophorin C/distal occludin chimera (*c*), colocalized with ZO-1 (*b, d*), in stably transfected MDCK cells. X-Y and Z-axis images are provided for each. Images are focused on ZO-1 at the tight junctions. Full length glycophorin C shows no polarity and is located on both the apical and basolateral plasma membranes. Glycophorin C/distal occludin chimeras are highly concentrated on the lateral membrane but not concentrated at the tight junction.

ciated with constitutive expression of the truncated proteins. In addition, this allowed us to avoid clonal differences in morphology in the freeze fracture analyses, since induced cells could be compared to their uninduced controls. MDCK cells that already contained the tetracycline transactivator gene were transfected with occludin expression vectors containing a tetracycline response element as described in Materials and Methods. Removal of tetracycline or doxycycline from the media resulted in induction of transgene expression.

To confirm that the constructs were expressed in an inducible manner and that induced proteins were of the expected molecular weights, transfected MDCK cells were induced for varying lengths of time and cell lysates were subjected to immunoblot analysis (Fig. 2). A diagrammatic representation of the occludin constructs is shown in Fig. 2 below the immunoblot corresponding to each protein. In all cases VSV-G tagged occludin expression was markedly induced within 24 hr after removal of doxycycline and induction continued up to a maximal level at 4 days (longer times not shown). For the present study we chose cell lines which showed no mutant protein expression under suppression by doxycycline (time 0 hr). The cell line transfected with pTRE vector alone (Fig. 2, vector) exhibited no detectable signal at any of the time points. MDCK cells transfected with the pTRE-occludin vector expressed a protein of ~65 kD which is expected for full length occludin. Cells transfected with pTRE vectors containing the mutant extracellular loops contained VSV-G tagged proteins that migrated at molecular weights consistent with the size of the deletions. The endogenous canine occludin can be distinguished on immunoblots because it has a smaller apparent molecular weight than untagged endogenous occludin. In all cases the transfected occludin did not alter the endogenous levels at 4 days. Based on the results above, 4 days of induction was used for all studies reported here.

### Proteinase K Digestion Reveals Proper Insertion of Occludin Constructs Into the Plasma Membrane

To determine whether the truncated forms of occludin were properly folded and inserted into the plasma membrane we tested their sensitivity to digestion with Proteinase K applied to intact MDCK cells. Cleaved products were analyzed on immunoblots using antibodies directed against the VSV-G tag positioned at the C-terminus, thus only fragments containing the C-terminus are visualized. Proteinase K treatment of cells expressing either the full length or Occ1(−) yielded protein fragments of 42 kDa (Fig. 3), consistent with the second loop

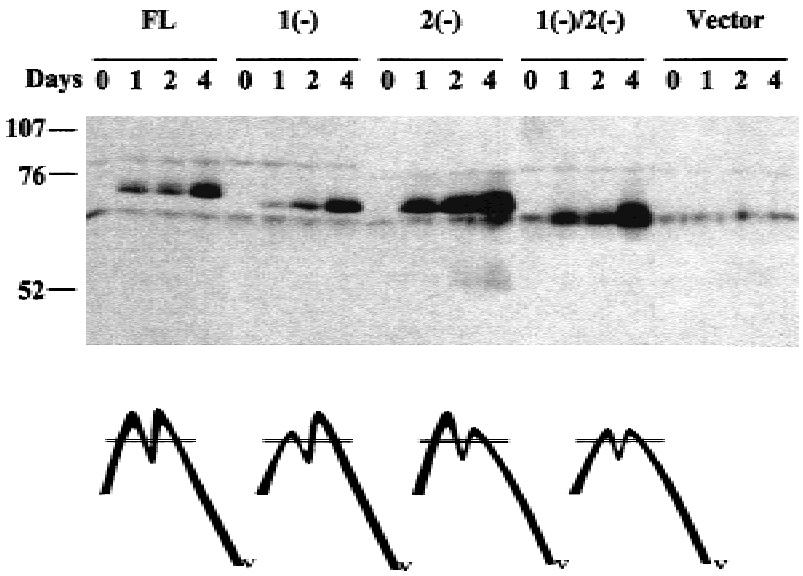


**Fig. 2.** Immunoblot analysis demonstrating inducible expression of VSV-G tagged occludin proteins in stably transfected MDCK cells. After removal of doxycycline, inducible expression is demonstrated over a 4-day period. FL, full-length VSV-tagged occludin; 1(−) lacks the first extracellular loop; 2(−) lacks the second loop and 1(−)/2(−) lacks both loops. Protein constructs are diagrammed below their immunoblot. Cells stably transfected with the empty pTRE vector show no specific inducible protein detectable by the VSV-G antibody. The background band migrating at the same size as the 1(−)/2(−) construct is a nonspecific signal visible to variable extent in all immunoblots probed with the VSV-G polyclonal antibody. Molecular weight standards are shown at the right in kDa.

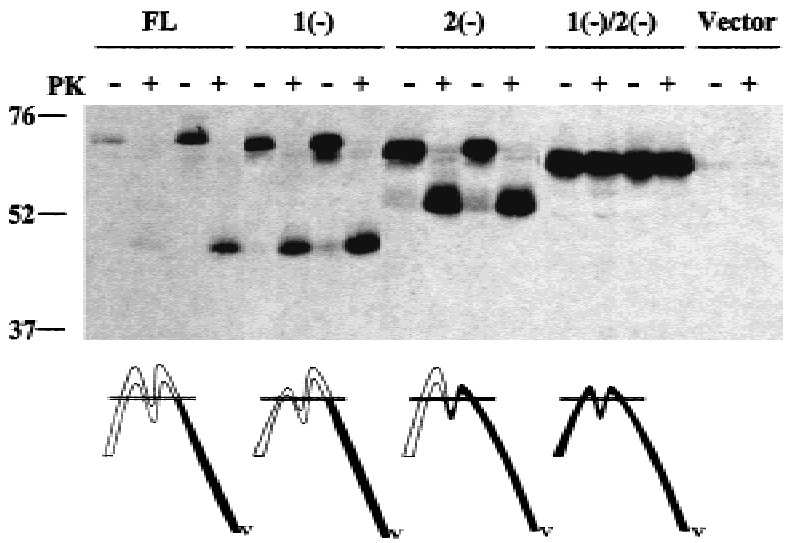


**Fig. 3.** Immunoblot analysis of occludin tagged at the C-terminus with a VSV-G epitope in intact MDCK cells before (−) and after (+) exposure to proteinase K (PK). Results from proteolysis of two separate clonal lines are shown for each construct of occludin. FL, full length; 1(−) lacks the first loop; 2(−) lacks the second loop and 1(−)/2(−) lacks both loops. Diagrams below each construct show the protein conformation and interpret which region of the protein was proteolyzed in solid black.

being accessible to the proteinase on the outside of the cell. Digestion of cells expressing occludin without the second loop, Occ2(−), produced a slightly larger fragment than the expected 48 kd (Fig. 3), but one that is consistent with the presence of proteinase K cleavage sites in the first loop, but not the truncated second loop. If the predicted topology is correct then this larger fragment is not expected after complete proteolysis of the full length occludin since the protein would also be cleaved in the second loop and result in the smaller 42 kDa fragment. The molecular weights of the digestion products are approximately consistent with the number of amino acids contained in each region and the predicted topology of occludin (Furuse et al., 1993). Truncation of both loops, Occ1(−)/2(−), resulted in a protein that was completely insensitive to digestion by proteinase K. A diagram interpreting the accessibility of the extracellular domains to proteinase K digestion is shown below the immunoblot for each construct. These results are consistent with the idea that truncation of either loop eliminates its sensitivity to proteolysis and indicate that all of the truncated proteins are present on the plasma membrane most likely in the tetra-spanning topology of native occludin. Thus, improper folding is unlikely to be the explanation if these proteins are functionally unable to localize to the tight junction.

### Occludin Constructs Colocalize with ZO-1 in Rat-1 Fibroblasts

We next investigated the localization of truncated occludin in Rat-1 fibroblasts to determine whether the trun-

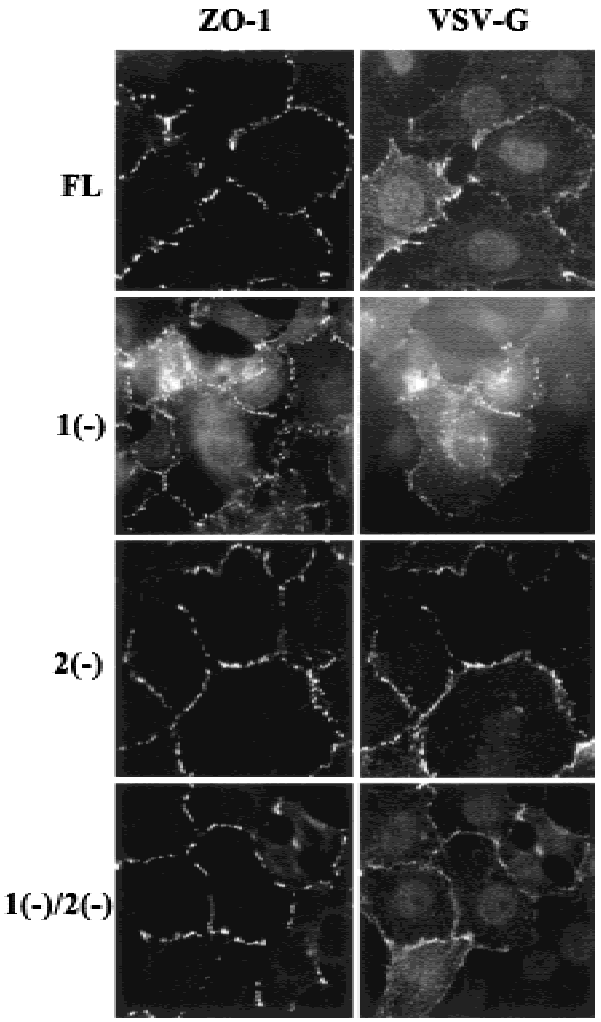


**Fig. 4.** Immunofluorescent localization of ZO-1 (left) and VSV-G tagged occludin (right) in stably transfected Rat-1 fibroblasts. ZO-1 and all forms of occludin colocalize at cell-to-cell contacts. FL, full-length occludin; 1(−) lacks the first loop; 2(−) lacks the second loop and 1(−)/2(−) lacks both loops.

cated proteins retain the functional ability of full length occludin to concentrate at ZO-1-enriched membrane contact sites. This should be another indication that the mutant proteins were correctly folded and could be used for localization studies. Rat-1 cells do not contain endogenous occludin (Van Itallie & Anderson, 1997) nor have claudins been identified in these cells (*data not shown*). As in most other fibroblast cell lines (Yonemura et al., 1995), the tight junction protein ZO-1 concentrates at cadherin-containing cell contacts, and transfected full length occludin also localizes to these contact sites. Rat-1 cells transfected with the pTRE vectors were maintained in the absence of doxycycline for 4 days to induce protein expression. As shown in Fig. 4, Occ, Occ1(−), Occ2(−), or Occ1(−)/2(−) all colocalize with ZO-1 at sites of cell–cell contact. Since occludin without the cytoplasmic tail does not colocalize with ZO-1 (*not shown*) these results indicate that a cytoplasmic interaction is required for localization in fibroblasts and that all of the constructs with extracellular deletions are still capable of this interaction. Occludin lacking both loops appeared less intensely focused at cell contacts and did not colocalize with ZO-1 in all cells, suggesting that the extracellular loops might stabilize occludin within the contact. Together with the proteinase K sensitivity results we take these data to indicate that the truncated forms of occludin are completely exposed on the plasma membrane, have the proper tetra-span topology and retain the ability to functionally interact with ZO-1-containing cell contacts.

### The Presence of the Second Extracellular Loop is Required for Integration of Occludin into MDCK Cells Tight Junctions

Although the full length and truncated occludin constructs localize with ZO-1 in fibroblasts, we next asked if they localized with ZO-1 at tight junctions of epithelial cells. MDCK cells transfected with the various pTRE vectors were maintained in the absence of doxycycline for 4 days to induce protein expression, fixed and subjected to immunofluorescent analysis by double-labeling with antibodies against the VSV-G tag and ZO-1. In all cell lines, the endogenous tight junction protein ZO-1 localized at the tight junction at the most apical aspect of the lateral plasma membrane (Fig. 5). In these images, the focal plane was set to give the sharpest appearance of ZO-1 and the apical junction complex. This same staining pattern was seen in cells transfected with the pTRE vector alone (*data not shown*) and when protein expression was not induced (*data not shown*).

In MDCK cells transfected with the pTRE-occludin vector, the distribution of exogenous full-length occludin colocalizes with endogenous ZO-1 (Fig. 5). This is consistent with several studies (Furuse et al., 1993, 1994; Balda et al., 1996; McCarthy et al., 1996). In addition, some lateral membrane staining of full length occludin was observed and increased as the level of expression increased (*not shown*). Lateral staining for occludin has also been observed in vivo in the intestinal epithelium (Sakakibara et al., 1997). In contrast, all three truncated forms of occludin showed a more diffuse localization including obvious staining on the lateral membrane. There is a suggestion that occludin lacking loop 1, Occ1(−), retained some colocalization with ZO-1 (Fig. 5), but this was difficult to assess using confocal technology. Consequently, in order to achieve higher resolution imaging and to obtain lateral images where junctional and lateral membrane staining could be resolved, we next visualized these cells using standard epifluorescence microscopy after semithin cryosectioning.

Figure 6 shows lateral images of cell monolayers

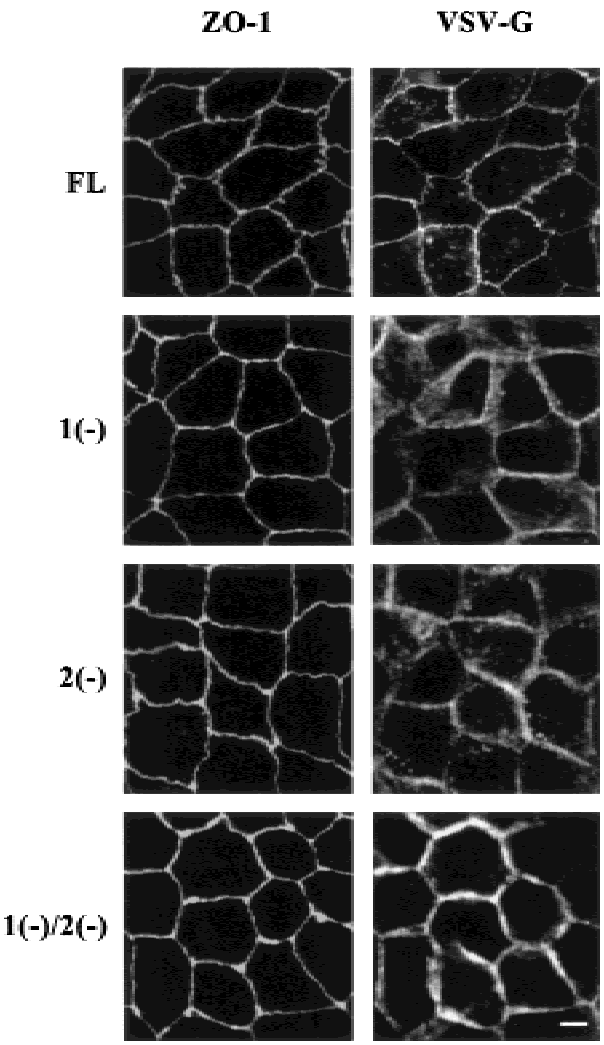


**Fig. 5.** Immunofluorescent confocal localization of ZO-1 (left) and VSV-G tagged occludin (right) in stably transfected MDCK cell monolayers. All images are focused on ZO-1 at the apical ends of the cells. Most of the full-length occludin (FL) colocalizes sharply with ZO-1. Some of the construct lacking the first loop, 1(−), appears to colocalize with ZO-1 and some does not. The construct lacking either the second loop, 2(−), or both loops, 1(−)/2(−), is more diffuse and neither colocalizes with ZO-1.

after semithin cryosectioning. Because the cell monolayers were scraped and pelleted prior to sectioning, multiple spatial orientations are possible. Perspectives are presented that reveal the longest lateral membrane depth and have the apical surfaces oriented upward. In all cells the distribution of ZO-1 was highly restricted to the tight junction indicating that expression of the various occludin constructs does not disrupt the localization of ZO-1. As previously reported, the VSV-G tagged full length occludin is concentrated with ZO-1 at the tight junction and shows some lateral and basal membrane staining (Fig. 6). The truncated occludins, however, showed clear differences in localization. Occludin lacking the first loop, Occ1(−), shows a localization that is very similar to the full length protein. It colocalizes with ZO-1, as determined by the yellow signal in merged images (Fig. 6*d*), and is also found along the basolateral plasma membrane. In contrast, occludin lacking the second loop, 2(−), or both loops, 1(−)/2(−), localizes to the basolateral plasma membrane and shows only a minor overlap with ZO-1 distribution at the most basal point of ZO-1 staining. These constructs are otherwise completely excluded from the tight junction. This distribution of the occludin mutants was observed in all cells in all clones for each construct; more examples are shown in Fig. 7. These results suggest that the second but not the first extracellular loop is sufficient to allow junctional localization.

### Overexpression of Full-Length but not Truncated Occludin Induces Morphologic Changes in Tight Junction Fibrils

To determine the effects of overexpressing full-length and truncated occludin on the ultrastructure of tight junction fibrils, we performed freeze fracture analysis of MDCK cells expressing certain constructs. The absence and presence of protein induction was confirmed by immunoblotting and immunolocalization on matched samples. MDCK cells transfected with the pTRE vector showed no difference in the freeze fracture pattern before (Fig. 8*a*) or after (*b*) induction by removal of doxycycline. In contrast, expression of full-length occludin (Fig. 8*d*) resulted in a striking side-to-side aggregation of fibrils. In some cases multiple fibrils associated in parallel (arrows). Similar results were previously observed when chicken occludin was overexpressed in MDCK cells (McCarthy et al., 1996). Expression of high levels of occludin lacking both loops caused no detectable changes in the fibril morphology (Fig. 8*e* and *f*), consistent with its absence from the junction as determined by immunofluorescence (Figs. 6 and 7). Finally, although there are high levels of full-length and doubly truncated occludin on the lateral membranes of these cells, no fibrils were ever detected on the lateral surface.

## Discussion

In this paper, we examine the role of the extracellular sequences of occludin as determinants for localization to the tight junction. Previous studies have addressed other aspects of cytoplasmic, intramembrane and extracellular interactions in this process. The results presented here, using chimeric and truncated versions of occludin, suggest both cytoplasmic scaffolding and extracellular adhesive interactions are important and specifically dem-

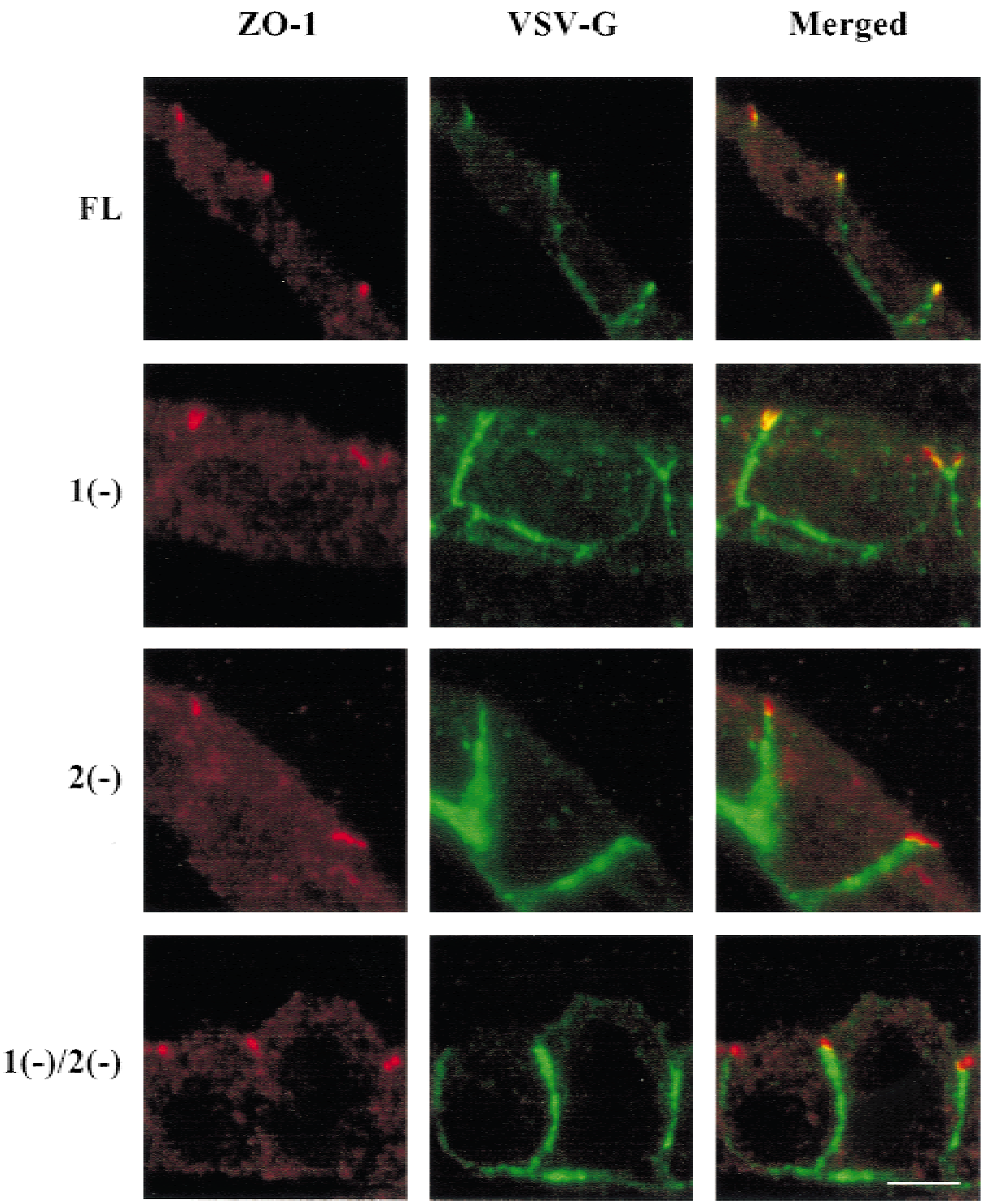


**Fig. 6.** Double immunolocalization of ZO-1 (red) and VSV-G tagged occludin (green) viewed laterally in 0.8 micron semithin cryosections of MDCK cells. Apical membranes and tight junctions are oriented toward the top. Colocalization of signals in merged images (right panels) results in yellow signal. Full-length (FL) occludin colocalizes with ZO-1 and is also present on the basolateral membrane. Occludin lacking the first loop, 1(−) also colocalizes with ZO-1 and is present on the basolateral membrane. Occludins lacking the second, 2(−), or both loops 1(−)/2(−) are present on the basolateral membranes but excluded from the tight junction.

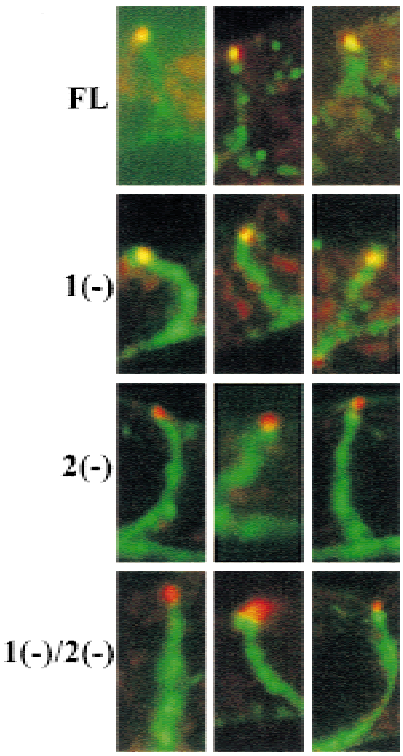


**Fig. 7.** Double immunolocalization of ZO-1 (red) and VSV-G tagged occludin (green) in various MDCK cell clones. Each image is a lateral membrane example from a confluent, filter-grown MDCK cell monolayer, with the ZO-1 signal at the top. Images of full length VSV-G tagged occludin and ZO-1 are examples from a single clone; 1(−), 2(−) and 1(−)/2(−) images are each examples from three separate MDCK cell clones expressing the respective mutant occludins.

onstrate the requirement for the second extracellular loop for stable occludin integration into tight junctions.

Our previous results (Mitic et al., 1999) demonstrated the importance of the carboxyl terminal ZO binding domain of occludin in tight junction localization. Chimeras composed of the transmembrane and extracellular domains of connexin-32 coupled to the carboxyl terminus of occludin were efficiently incorporated into tight junction fibrils. In the present study, a glycophorin C/occludin chimera containing the ZO binding domain was found in the lateral membrane, not at the tight junction, similar to the distribution of an Fc receptor/occludin chimera seen in MDCK cells (Matter & Balda, 1998). The ability of ZOs to direct localization to the tight junction is therefore context dependent, strengthening the idea that incorporation of the connexin/occludin chimeras into the tight junction might depend on the ability of the membrane and/or extracellular domains of connexins to interact laterally or transcellularly.

In this study, we determined the requirement of the extracellular loops of occludin for tight junction localization. Previous studies had demonstrated that occludin containing the transmembrane and extracellular domains but lacking the ZO binding domain interacted with both endogenous occludin (Balda et al., 1996; Chen et al., 1997; Matter & Balda, 1998), and claudins (C.M. Van Itallie & J.M. Anderson, *unpublished results*). The specific importance of the extracellular domains in occludin localization was indirectly demonstrated in several studies. Furuse et al. (1998) documented homophilic occludin interactions between cells, likely dependent on its extracellular domains. Other experiments have demonstrated that peptides homologous to the loops of occludin can influence the localization of occludin at the membrane (Wong & Gumbiner, 1997), impair occludin-dependent adhesion (Van Itallie & Anderson, 1997) or impair tight junction resealing (Lacaz-Viera et al., 1999). In addition, Wong & Gumbiner (1997) found that administration of a peptide that was homologous to the second loop of occludin not only disrupted transepithelial electrical resistance in MDCK cells, but also resulted in the selective disappearance of occludin detected on immunoblots. These data are consistent with the idea that interactions involving the extracellular domain of occludin may be critical for stable localization in the tight junction. The results presented in this paper demonstrate that the presence of the second extracellular loop is critical for tight junction localization. This may reflect the requirement of this loop for lateral or transcellular interactions of occludin with itself or other proteins.

One possible interpretation of the mislocalization of occludin 2(−) and 1(−)/2(−) is that these mutant proteins are not properly folded or inserted. The proteinase K accessibility studies were consistent with the correct insertion of occludin, occludin 1(−) and occludin 2(−) into the membrane. In addition, the ability of the ZO binding domain to target all the mutant occludin constructs to sites of cell contact in rat-1 cells demonstrates that these mutant proteins can be localized to the same site as full-length occludin in nonepithelial cells. It is still possible that introduction of the truncations deforms the conformation of the occludin transmembrane domains and interferes with their ability to interact with endogenous binding partners. Truncations of the extracellular loops, however, do not affect the ability of occludin to interact with claudin-2, since cotransfection of a construct with both extracellular domains truncated together with a plasmid encoding claudin-2 into L-cells results in coaggregation of the encoded proteins at cell contact points (*unpublished results*, C.M. Van Itallie and J.M. Anderson).

Our results suggest that removal of the first loop does not interfere with the ability of the second loop to induce localization of occludin to the tight junction. This appears to be in conflict with the results of Balda et al. (2000) where they made small deletions of 13 and 11 residues from the first and second loop, respectively, and neither localized to the junction. The reason for this dif-

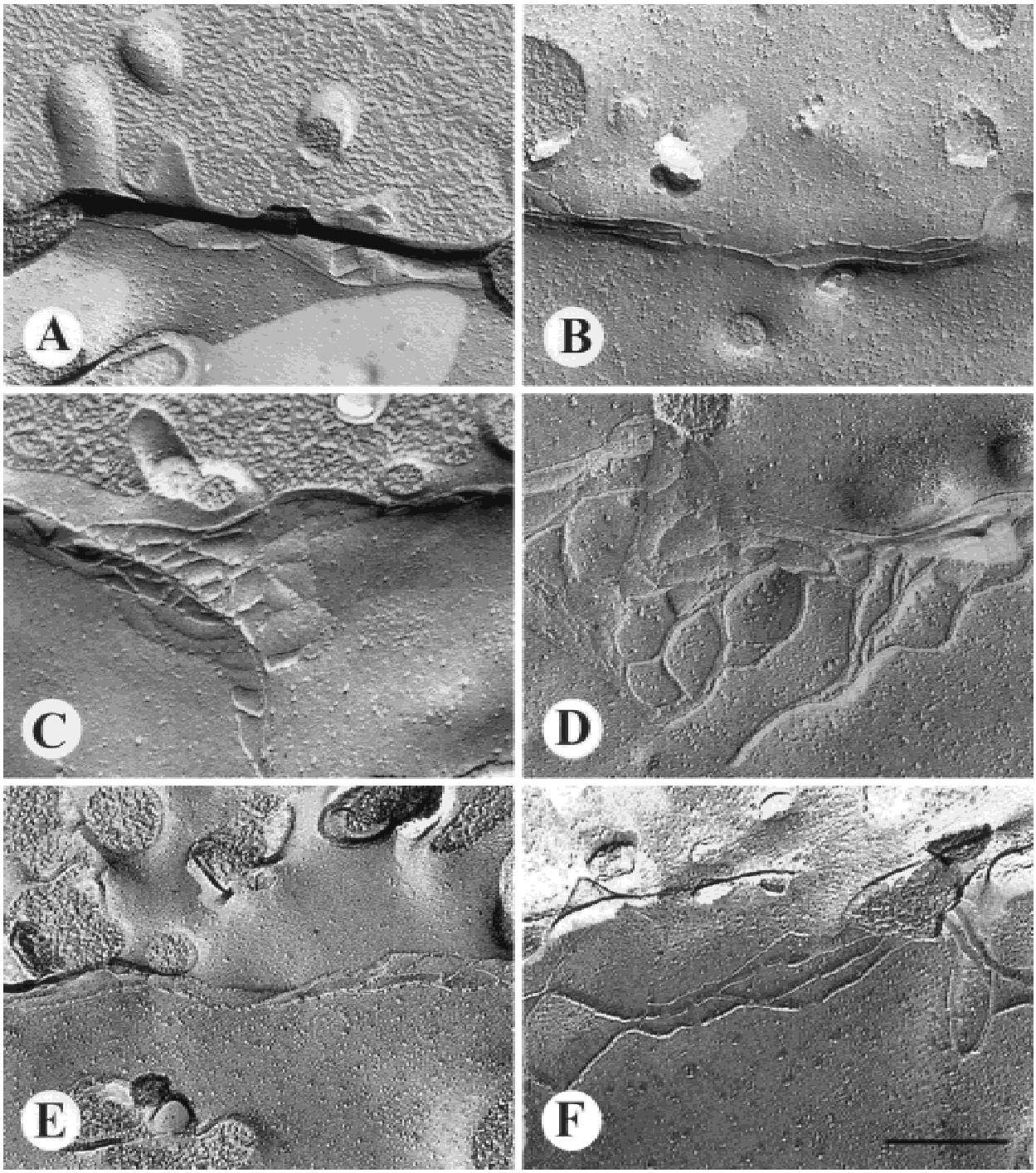


**Fig. 8.** Freeze fracture replica electron micrographs of the tight junctions from MDCK cells before (left) and after (right) induction of occludin transgene proteins. Cells transfected with empty vector (*a*, *b*) show no difference in fibril morphology with induction. Induction of high levels of full-length occludin (*d*) results in an increased number of side-to-side aggregation of fibrils compared with uninduced cells (*c*). The image in (*c*) demonstrates a tricellular junction where a larger number of fibrils are typically observed. Induction of the 1(−)/2(−) occludin does not affect fibril morphology (*e*, uninduced and *f*, induced). No fibrils were observed on the lateral cell surface following induction of high levels of any of the occludin constructs.

ference is not clear. Perhaps the small deletion in loop 1 caused it to misfold and interfere with function of loop 2. In our constructs most of the loop was removed. A second difference is that these authors used constitutively expressing cell lines and ours were inducible. If the interactions of the mutant occludins with endogenous tight junction proteins were less stable than with full length occludin, then it is possible that one would only see incorporation of mutant occludin into the junction after a large pulse of protein expression. We did not examine mutant occludin localization after longer periods of induction. A second study also appears to be in conflict. Bamforth et al. (1999) found that a truncated occludin containing only the last 14 amino acids of the second extracellular loop, the last transmembrane domain and the C-terminus of occludin was targeted to ZO-1 containing sites of cell contact. The reason for the discrepancy between their results and ours are unclear; however, their construct does contain more amino acid residues from the critical second extracellular domain than does our truncated loop construct.

The freeze fracture electron microscopic analysis presented in this paper shows that when transfected full-length or truncated occludin was located on the lateral cell surface, it did not form fibrils. In addition, the doubly deleted occludin does not affect normal tight junction fibril formation. This is in agreement with the results presented by Tsukita and coworkers (Furuse et al., 1998) in which in the absence of claudin, transfected occludin did not organize into tight junction-like fibrils. As demonstrated in the occludin-null ES cells (Saitou et al., 1998) and in OSP/claudin-11 deficient mice (Gow et al., 1999), claudins appear to make up the backbone of the freeze fracture fibril. Occludin, although adhesive (Furuse et al., 1996; Van Itallie & Anderson, 1997), apparently lacks the ability to form extended lateral membrane polymers. In this context it is surprising that overexpressing full-length occludin induces lateral aggregation of fibrils. Although the organization of the particle component of the tight junction fibril is unknown, one possibility is that excess occludin can copolymerize with other fibril constituents and deform the normal fibrillar organization. These results have been noted previously to a lesser extent (McCarthy et al., 1996); it is possible the higher level of transgene expression in the tetracycline-inducible cells may have made it more obvious.

Formation and maintenance of the tight junction is clearly a process dependent on interactions between a number of proteins. The simplest model for occludin localization is that an initiating interaction may be with ZOs. Fleming and coworkers (Sheth et al., 2000) have demonstrated that during early mouse embryogenesis occludin is delivered to tight junctions in association with ZO-1. In addition, in cells lacking tight junctions, the presence of the ZO binding domain of occludin is both necessary and sufficient for localization at cell contacts. However, ZO binding is not absolutely required in the face of preformed tight junctions, because occludin lacking this domain is efficiently localized to this apical junction complex. The protein interactions involved in this ZO-independent localization to the tight junction appear to be required for maintaining the protein at the junction. These interactions are apparently dependent on interactions mediated by the extracellular domains of occludin, particularly the second loop, since the results presented in this paper demonstrate the requirement for this domain. One possibility is that proteins that lack the necessary interaction domains are sterically excluded from the tight junction. Although recent results demonstrate the overwhelming importance of claudins in the formation and function of the tight junction, a critical challenge remains to understand the structural and functional role of occludin.

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