A Synthetic Peptide Corresponding to the Extracellular Domain of Occludin Perturbs the Tight Junction Permeability Barrier

Vivian Wong*‡ and Barry M. Gumbiner*

*Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York 10021; and‡Department of Physiology, University of California at San Francisco, San Francisco, California 94120

Abstract. Occludin, the putative tight junction integral membrane protein, is an attractive candidate for a protein that forms the actual sealing element of the tight junction. To study the role of occludin in the formation of the tight junction seal, synthetic peptides (OCC1 and OCC2) corresponding to the two putative extracellular domains of occludin were assayed for their ability to alter tight junctions in *Xenopus* kidney epithelial cell line A6. Transepithelial electrical resistance and paracellular tracer flux measurements indicated that the second extracellular domain peptide (OCC2) reversibly disrupted the transepithelial permeability barrier at concentrations of < 5 μM. Despite the increased paracellular permeability, there were no changes in gross epithelial cell morphology as determined by scanning EM. The OCC2 peptide decreased the amount of occludin present at the tight junction, as assessed by indirect immunofluorescence, as well as decreased total cellular content of occludin, as assessed by Western blot analysis. Pulse-labeling and metabolic chase analysis suggested that this decrease in occludin level could be attributed to an increase in turnover of cellular occludin rather than a decrease in occludin synthesis. The effect on occludin was specific because other tight junction components, ZO-1, ZO-2, cingulin, and the adherens junction protein E-cadherin, were unaltered by OCC2 treatment. Therefore, the peptide corresponding to the second extracellular domain of occludin perturbs the tight junction permeability barrier in a very specific manner. The correlation between a decrease in occludin levels and the perturbation of the tight junction permeability barrier provides evidence for a role of occludin in the formation of the tight junction seal.

THE tight junction, also known as *zonula occludens*, is the apical-most component of the junctional complex of epithelial and endothelial cells. It is a region where the plasma membrane of adjacent cells forms a series of contacts that appears to completely occlude the extracellular space as observed by transmission EM. These contact points of the tight junction correspond to a network of intramembrane fibrils, when studied by freeze-fracture EM, which completely circumscribes the apices of cells (9).

Two main functions have been attributed to the tight junction. First, the tight junction seals the intercellular space and is responsible for the separation of apical and basolateral fluid compartments of epithelia and endothelia. Macromolecules of radii ≥ 15 Å cannot flow through the tight junction (23). However, depending on the properties of each individual epithelium, small ions can pass through the tight junction to varying degrees, as indicated by differences in transepithelial electrical resistance in various epithelial and endothelial cells (5 Ω cm^2 to $> 5{,}000$ Ω cm^2) (13, 29). Therefore, the tight junction is crucial for the formation of blood–tissue barriers, such as the blood–brain barrier and the blood–retinal barrier, which are absolutely essential for the normal functioning of the organism (30, 34). Second, the tight junction functions as a diffusion barrier to plasma membrane lipids and proteins, which helps to define apical and basolateral membrane domains of these polarized epithelial and endothelial cells (18, 20). Therefore, the tight junction is crucial for the epithelium to generate chemical and electrical gradients across the cell monolayer that is necessary for vectorial transport processes such as absorption and secretion.

The tight junction is regulated in response to various physiological and tissue-specific needs. For example, the tight junction permeability to nutrients has been shown to be increased by intestinal luminal glucose after food intake, suggesting that the regulation of tight junction permeability has a role in absorption of nutrients in the intestine (4, 6). A number of hormones have been shown to affect the tight junction, including transforming growth factors (α and β) (8, 35) and glucocorticoids (37). Disruption of the tight junction has been found to occur in many

Address all correspondence to Barry M. Gumbiner, Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 564, New York 10021. Tel.: (212) 639-6146. Fax: (212) 717-3047.

diseases including hepatitis, Celiac Spruce, Crohn's disease, and gastritis, in which the tight junction intramembrane fibrils of the respective epithelia manifest discontinues and poor organizations (21, 24, 28, 33). On the other hand, increases in tight junctional depth in intestinal epithelium in Blind Loop syndrome correlate with a decrease in permeability to nutrients (31). In addition, the tight junction is regulated in various physiological processes, such as leukocyte transmigration across an endothelium (26) and intestinal cell division (5) and extrusion (7), to ensure minimal disruption of the tight junction barrier. Therefore, the tight junction appears to function as more than just a paracellular seal, and instead is regulated in physiologically important processes.

While the physiological significance of the tight junction is well recognized, the molecular component(s) involved in the formation of a functional tight junction barrier are not yet established. Several cytoplasmic peripheral membrane proteins (ZO-1, ZO2, cingulin, 7H6, and rab 13) (1, 2, 11, 12, 20, 32, 36, 38, 39) and one integral membrane protein (occludin) (15) have been found to localize at the tight junction. Occludin was shown to localize to junctional fibrils by immunogold labeling of freeze-fracture replicas of tight junctions (14). The cytoplasmic tail of occludin is necessary for its localization to cell–cell contacts, perhaps via binding to ZO-1 and ZO-2 (16). However, how these proteins function in the formation of the tight junction permeability barrier is unclear.

Occludin, being the only putative integral membrane protein so far identified, is a candidate for the formation of the functional intercellular seal of the tight junction. The primary amino acid sequence of chick occludin predicts four membrane-spanning regions and two 44–amino acid extracellular loops. Both extracellular domains of occludin consist solely of uncharged residues with the exception of one or two charged residues adjacent to the transmembrane regions (3). The nonpolar nature of the extracellular domains and the conservation of their sequences between five species (human, mouse, dog, chick, and rat–kangaroo) suggest that the extracellular domains have important functional roles, perhaps in the formation of the actual contact seal of the tight junction. To test this hypothesis, we made synthetic peptides corresponding to each of the putative extracellular domains of chick occludin and assayed for their ability to alter tight junction barrier function.

Materials and Methods

Cell Culture, Calcium Switch Assay, and Measurement of Transepithelial Resistance

The A6.2 subclone of the *Xenopus* kidney epithelial A6 cell line (27) was grown on Transwell filters (Costar Corp., Cambridge, MA) in 89% DME (1 g/liter glucose) supplemented with 0.74 g/liter bicarbonate, 5.95 g/liter Hepes (pH 7.4), and 5% FCS and maintained at 28°C and 1% CO_2. For the calcium switch assay (17, 19, 25), A6 cells were allowed to grow in normal growth medium to confluency and were subsequently changed to low calcium medium for 18 h. At the end of the low calcium (< 50 μM) incubation, A6 cells were replenished with normal calcium media, and the formation of tight junctions was monitored by the generation of transepithelial electrical resistance (TER).[1] TER was measured directly in normal growth media in Transwell wells. A short 4-μA current pulse was passed across the cell monolayer using a pair of calomel electrodes via KCl salt bridges, and the voltage was measured by a conventional voltmeter across the same cell monolayer using a pair of Ag/AgCl electrodes via KCl salt bridges. TER was calculated from the measured voltage and normalized by the area of the monolayer. The background TER of blank Transwell filters was subtracted from the TER of cell monolayers.

Peptide Synthesis and Treatment of Cells

Peptides OCC1 corresponding to amino acids 81–125 of chick occludin (44 amino acids [aa] = DYGYGLGGAYGTGLGGFYGSNYYGSGLSYSYGYGGYYGGVNQRT) and OCC2 corresponding to amino acids 184–227 of chick occludin (44 aa = GVNPQAQMSSGYYYSPLLAMCSQAYGSTYLNQYIYHYCTVDPQE) are the entire first and second putative extracellular domains of chick occludin, respectively. Two different peptide forms of the second extracellular domain were synthesized. The first peptide, OCC2, was modified at the two cysteine residues of the extracellular domain by covalent linkage to acetamidomethyl (*underlined*) to prevent formation of disulfide bond(s). An unmodified form of the second extracellular domain peptide without the protection groups at the two cysteine residues, OCC2(U), was also synthesized. A scrambled peptide OCC2(S) composed of a scrambled sequence of the same residues as OCC2, including the acetamidomethyl modification at cysteines, was also synthesized (44 aa = GACQVYDPYMSGNYPAQSLMYQNLQYLVSGIHTYPECSYATQSY). Peptides were prepared as 10 mM stock solutions in DMSO and were added to both sides of the Transwell bathing wells. All peptides were synthesized by the Microchemistry Core Facility at the Memorial Sloan-Kettering Cancer Center (New York).

Paracellular Tracers Flux Assay

Flux assays were performed on 6.5-mm Transwell (in 24-well cell culture dishes). Four different paracellular tracers, [^{3}H]mannitol (Amersham Corp., Arlington Heights, IL), [^{14}C]inulin (Amersham Corp.), neutral dextran (mol wt 3,000) conjugated with Texas red (Molecular Probes, Eugene, OR), and neutral dextran (mol wt 40,000) conjugated with Texas red (Molecular Probes), were used. At the beginning of the flux assay, both sides of the bathing wells of Transwell filters were replaced with fresh media without peptides (containing 5 mM unlabeled mannitol [mol wt 184] and 1 mM unlabeled inulin [mol wt 5,200]). Each tracer was added to a final concentration of 3.6 nmol for [^{3}H]mannitol, 0.36 nmol for [^{14}C]inulin, 25 μg/100 μl for dextran (mol wt 3,000), or 50 μg/100 μl for dextran (mol wt 40,000) to the apical bathing wells that contained 100 μl of media. The basal bathing well had no added tracers and contained 700 μl of the same flux assay media as in the apical compartment. All flux assays were performed at 25°C with gentle circular agitation. Cell monolayers were first allowed to equilibrate for 30 min after the addition of tracers. At each of the following 15-min time intervals, the entire Transwell filter cup was removed from the basal bathing well and transferred to a fresh basal bathing well containing 700 μl of the flux assay media. Three 15-min flux sampling intervals were taken, and the mean was used for the calculation of paracellular tracer flux that is taken as [amount of tracer in the basal bathing media]/[time]. For [^{3}H]mannitol and [^{14}C]inulin flux, the entire 700 μl of the basal media was added to 5 ml of scintillation fluid, and ^{3}H and ^{14}C were counted. The concentration of [^{3}H]mannitol and [^{14}C]inulin in the basal bathing media was calculated from a titration curve of known concentration of the same tracers. For dextran (3 kD and 40 kD), the concentration was calculated from the amount of fluorescence emission at 610 nm (excitation at 587 nm) using a titration curve of known concentration of the same tracers.

Immunofluorescence Staining and Western Blotting

A6 cells were grown on Transwell filters and TER was measured before and after peptide treatment. For immunofluorescence, cells were fixed with 100% methanol at −20°C for 30 min and dried with 100% acetone at −20°C for 5 min. Filters were blocked with immunofluorescence staining buffer (1% nonfat dry milk in 0.5% Triton X-100, 5 mM EDTA, 0.15 M NaCl, and 20 mM Hepes, pH 7.0) before incubation with primary antibodies. Rabbit anti–occludin antibodies were raised against a glutathione-*S*-transferase fusion protein of the cytoplasmic domain of chick occludin (255–510 aa). Rabbit anti–ZO-1 (No. 10153) and –ZO-2 (No. 9989) (22) antibodies were gifts from D. Goodenough (Harvard Medical School, Cambridge, MA). Rabbit anti–cingulin antibodies were gifts from S. Citi (University of Padua, Italy). Monoclonal mouse anti–*Xenopus* E-cadherin

1. *Abbreviations used in this paper*: aa, amino acid; TER, transepithelial electrical resistance.

antibodies (5D3) were raised against the extracellular domain of E-cadherin (10). FITC-conjugated secondary antibodies were obtained from Molecular Probes. For Western blot analysis, cells were rinsed twice in PBS and extracted directly in SDS-PAGE sample buffer (50 mM Tris-HPO_4, pH 6.8, 2.5 mM EDTA, 15% sucrose, 2% SDS, and 50 mM DTT) containing protease inhibitors (5 mM PMSF, 5 μg/ml pepstatin A, 1 μg/ml *N*-α-*p*-tosyl-L-lysine-chloromethyl ketone (TLCK), 10 μg/ml leupeptin, 20 μg/ml aprotinin, 50 μg/ml antipain, 2 mM benzamidine, 50 μg/ml soybean trypsin inhibitor, and 2.5 mM iodoactamide). Samples were boiled for 10 min, and cooled to room temperature before the addition of iodoacetic acid to a final concentration of 125 mM, and then SDS-PAGE was performed. Western blots for occludin, ZO-1, ZO-2, cingulin, and E-cadherin were done using the same primary antibodies as those for immunofluorescence stainings. Secondary antibodies conjugated with HRP (Bio Rad Laboratories, Hercules, CA) were developed by enhanced chemiluminescence (Amersham Corp.).

Metabolic Labeling, Immunoprecipitations, and Fluorography

A6 cells were grown on 75-mm Transwell filters. To examine the turnover of occludin, each monolayer was labeled with 0.8 mCi [^{35}S]methionine in methionine-free media (supplemented with 5% FBS) for 22 h, and protein turnover was monitored by replacing labeling media with fresh media in the presence or absence of 10 mM OCC2 for 12 h. Cells were extracted for immunoprecipitation either immediately at the end of the labeling period (t = 0) or at 12 h after the end of the labeling period (t = 12 h). For examination of occludin synthesis, cells were rinsed once with methionine-free media and incubated in methionine-free media for 30 min before metabolic labeling. Each Transwell cell monolayer was then briefly labeled for 2 h with 1.5 mCi [^{35}S]methionine in methionine-free media (supplemented with 5% dialyzed FBS) in the presence or absence of 5 μM OCC2 before extraction for immunoprecipitation. Pulse-labeling experiments were also performed on cells pretreated with 5 μM OCC2 for 20 h. For immunoprecipitation, cells from each 75-mm Transwell cell monolayer were extracted with 2 ml 1% SDS containing 5 mM EDTA and protease inhibitors (5 mM PMSF, 5 μg/ml pepstatin A, 1 μg/ml TLCK, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 50 μg/ml antipain, 2 mM benzamidine, 50 μg/ml soybean trypsin inhibitor, and 2.5 mM iodoactamide). Each sample was boiled for 10 min before the addition of Triton X-100, deoxycholate, NaCl, and Hepes to a final concentration of 0.2% SDS, 1% Triton X-100, 0.5% deoxycholate, 0.15 M NaCl, and 20 mM Hepes (pH 7.4). Immunoprecipitation was performed with protein A–Sepharose (Sigma Chemical Co., St. Louis, MO) in the presence of rabbit anti–chick occludin antibodies (see above) or preimmune serum. Immunoprecipitates were prepared for SDS-PAGE as described above. Polyacrylamide gels were fixed with 50% methanol and 10% acetic acid for 1 h and incubated in Amplify (Amersham Corp.) for 45 min before being dried under vacuum. Dried gels were exposed to Hyperfilm-MP (Amersham Corp.) at −80°C.

Scanning EM

A6 cells were plated on Thermanox coverslips (Nunc, Inc., Napaville, IL) coated with polylysine and were allowed to grow to confluency. Cells were either untreated or treated for 40 h with 10 μM OCC1 or 10 μM OCC2. Subsequently, the cells were rinsed twice in PBS and fixed with 2.5% glutaraldehyde/20 mM Pipes/pH 7.5 at room temperature overnight. Coverslips were rinsed in 20 mM Pipes/pH 7.5, followed by dehydration in a graded series of alcohol (50%, 75%, 95%, through absolute alcohol) and critical point dried in a DCP-1 critical point dryer (Denton Vacuum Inc., Cherry Hill, NJ). The samples were sputter coated with gold/palladium in a Hummer VI sputtering system (Technic Inc., Providence, RI). The samples were photographed using a JSM 35 scanning electron microscope (JEOL USA, Peabody, MA). All EM was performed by Nina Lampen in the Electron Microscopy Facility at the Memorial Sloan-Kettering Cancer Center.

Results

Expression and Localization of Occludin in A6 Cells Correlate with the Development of Transepithelial Resistance

The *Xenopus* kidney epithelial A6 cell line formed mono-

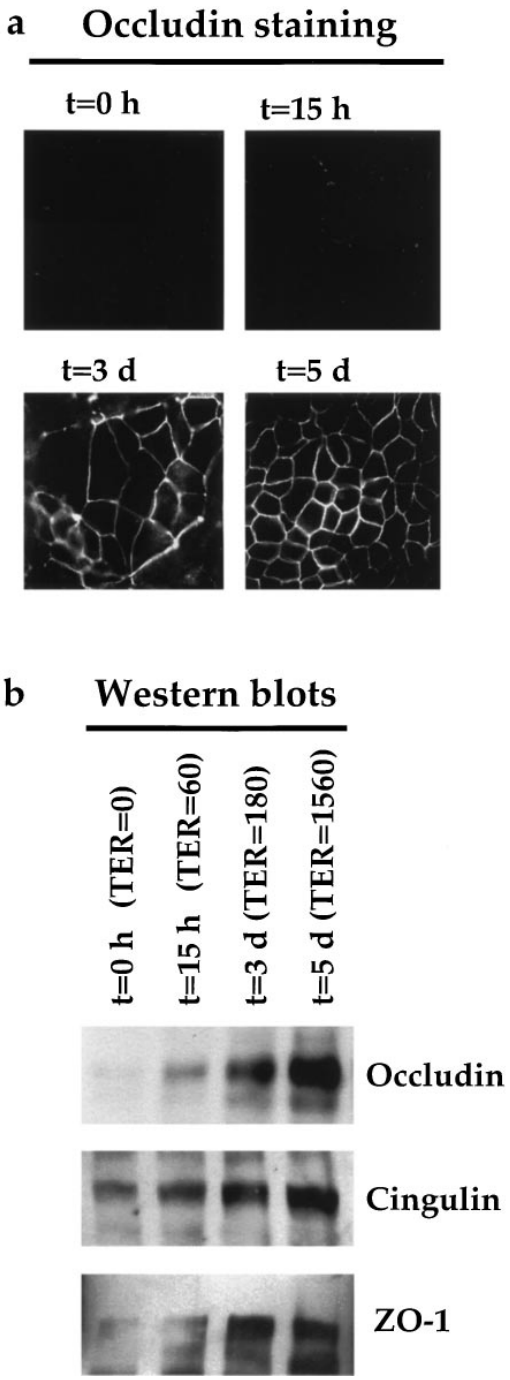

Figure 1. Expression and junctional localization of occludin correlated with the development of tight junctions in *Xenopus* A6 kidney epithelial cells. A6 cells were allowed to grow to confluency in Transwell filters in normal medium and were subsequently changed to low calcium medium for 18 h. Then the medium was replenished with normal calcium (t = 0), and the formation of tight junctions was monitored by measuring TER at t = 0 h (*TER* = Ωcm^2), t = 15 h (*TER* ~0 Ωcm^2), t = 3 d (*TER* ~100 Ωcm^2), and t = 5 d (*TER* > 1,000 Ωcm^2). (*a*) Indirect immunofluorescence of occludin in A6.2 cells at t = 0, 15 h, 3 d, and 5 d. (*b*) Western blots of A6 cell lysate for occludin, cingulin, and ZO-1 at t = 0, 15 h, 3 d, and 5 d.

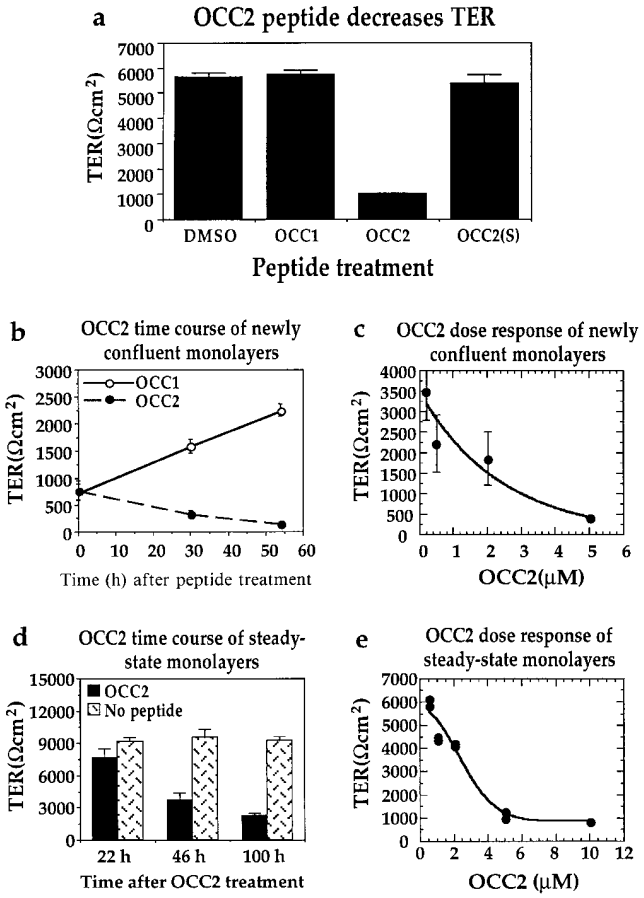

Figure 2. A synthetic peptide (*OCC2*) corresponding to the entire second extracellular domain of chick occludin decreased TER of A6 cell monolayers. (*a*) Effect of various synthetic peptides on TER. OCC1 (corresponding to the entire first extracellular domain of chick occludin), OCC2 (corresponding to the entire second extracellular domain of chick occludin), OCC2(S) (corresponding to the scrambled sequence of the entire second extracellular domain of chick occludin), and DMSO solvent control were used. Newly confluent A6 cell monolayers (starting *TER* ~1,000 Ωcm^2) that were still developing TER were used. Cell monolayers were treated with a final concentration of 5 μM OCC1, 5 μM OCC2, 5 μM OCC2(S), or DMSO (0.05%) for 66 h, and peptides were replenished every 24 h. At the end of the 66-h peptide incubation, TER for control monolayers reached ~5,000–6,000 Ωcm^2. $n = 6$ for each condition. (*b*) Time course of effect of OCC2 peptide on TER of A6 cells that were still developing TER. Cell monolayers that had attained TER ~750 Ωcm^2 were treated with a final concentration of 5 μM OCC1 ($n = 4$) or 5 μM OCC2 ($n = 5$) at $t = 0$. Peptides were replenished at 30 h. (*c*) Dose dependency of OCC2 peptide on TER in A6 cell monolayers that were still developing TER. A6.2 cells were allowed to grow to confluency in normal medium and were subsequently changed to low calcium medium for 18 h. The low calcium medium was replaced with normal calcium medium containing a final concentration of 0.2, 0.5, 2, and 5 μM OCC2. TER were measured after 4 d when control cell monolayers developed TER of ~3,000 Ωcm^2. $n = 3$ for all concentrates tested. (*d*) Time course of OCC2 peptide on TER of steady-state A6 cell monolayers that were confluent for ~2 wk (*TER* ~8,000 Ωcm^2). Cells were treated with a final concentration of 5 μM OCC2 at $t = 0$. Untreated monolayers were done in parallel as control. Peptides were replenished at 22 and 76 h. $n = 3$ for all conditions. (*e*) Dose dependency of OCC2 peptide on TER in steady-state A6 monolayers (*TER* ~6,000 Ωcm^2). Cell monolayers were treated with a final concentration of 0.5, 1, 2, 5, and 10 μM OCC2. TER was measured at 40 h after peptide addition. The TER of each individual monolayer is plotted. Each concentration of OCC2 was done on duplicate monolayers. All error bars represent standard error.

layers that had a very high TER of ~8,000 Ωcm^2 and were impermeable to macromolecules of mol wt ≥ 40 kD. After induction of synchronized intercellular junction formation by a calcium switch assay (see Materials and Methods for details), occludin localization at cell boundaries correlated with the formation of tight junctions as monitored by measurements of TER (Fig. 1 *a*). Western blot analysis of A6 total cell lysates using polyclonal antibodies raised against the cytoplasmic domain of chick occludin showed a single band of mol wt ~60 kD corresponding to *Xenopus* occludin. A minor band of lower molecular weight is detected only occasionally, and therefore is presumed to be a degradation product of occludin. The expression levels of occludin during tight junction formation correlated roughly with the increase in TER (Fig. 1 *b*). The increase in occludin expression plateaued as TER reached maximal steady-state levels (not shown). By Western blot analysis, ZO-1 and cingulin levels also increased during tight junction development (Fig. 1 *b*), but their expression levels changed less dramatically and tended to plateau earlier than the TER or occludin levels. The time course of occludin localization and expression was consistent with the hypothesis that occludin participates in the formation of the tight junction.

TER Is Reduced by a Synthetic Peptide (OCC2) Corresponding to the Second Extracellular Domain of Occludin

The predicted topology of occludin from the chick occludin amino acid sequence consists of two short extracellular domains, each one having 44 residues. Synthetic peptides corresponding to either the entire first or second extracellular domain were made and assayed for their ability to affect tight junctions as assessed by measurements of TER. Treatment of A6 cell monolayers with the second extracellular domain peptide (OCC2) caused a substantial reduction of TER from ~6,000 Ωcm^2 to ~900 Ωcm^2 (Fig. 2 *a*). In contrast, the first extracellular domain peptide (OCC1) did not alter TER as compared with the DMSO only control (Fig. 2 *a*). Additionally, a control peptide containing a scrambled amino acid sequence from the second extracellular domain, OCC2(S), had no effect on TER (Fig. 2 *a*). Therefore, the peptide corresponding to the second extracellular domain of chick occludin (OCC2) specifically reduced TER in *Xenopus* kidney epithelial A6 cell monolayers.

OCC2 was synthesized with a protection group, acetamidomethyl, covalently linked to the sulfhydryls of the two cysteine residues of the peptide. When the peptide was synthesized without the protection groups, OCC2(U) (U stands for unmodified), no change in TER was observed at concentrations that worked maximally for the protected peptide OCC2 (data not shown). The addition of acetamidomethyl protection groups to the two cysteine residues increased the hydrophilicity of OCC2 (as judged by a mobility shift using HPLC; data not shown) and pre-

vented intermolecular disulfide bond formation (as judged by formation of a ladder of higher molecular weight forms using SDS-PAGE; data not shown). Either effect may contribute to the enhanced effectiveness of the protected peptide.

The magnitude of change in TER by treatment with OCC2 depended on the growth state of the cells and the dosage used. The time course and dose response of the OCC2-induced decrease in TER were assayed on both newly formed monolayers that were developing TER (TER ~1,000 Ωcm^2) and steady-state monolayers that had completely formed maximal TER (TER ~8,000–10,000 Ωcm^2). Newly formed monolayers were studied either by plating A6 cells at confluent density or by inducing junction formation with the low calcium switch assay (see Materials and Methods); both procedures yielded the same results. For newly formed monolayers that were still developing TER (see figure legends for details), OCC2 treatment resulted in a ~10-fold lower TER of ~250 Ωcm^2 as compared with ~2,500 Ωcm^2 in 2 d (Figure 2 *b*). Treatment with the first extracellular domain peptide, OCC1, had no effect (Fig. 2 *b*). The ability of OCC2 to decrease TER in newly formed monolayers was dose dependent with a maximal inhibition at the final concentration of 5 μM OCC2, resulting in a ~10-fold lower TER of ~400 Ωcm^2 as compared with ~3,500 Ωcm^2 (Fig. 2 *c*). For steady-state monolayers that had been confluent for >10 d and attained a maximal TER (~10,000 Ωcm^2), OCC2 decreased TER from ~10,000 Ωcm^2 to ~2,000 Ωcm^2, whereas untreated monolayers retained a stable maximal TER (Fig. 2 *d*). However, the effect of OCC2 on steady-state monolayers took much longer than it did for newly formed monolayers. It took 5 d to attain an approximately fivefold decrease in TER in steady-state monolayers as compared with 2 d for newly formed monolayers. The effect of OCC2 on steady-state monolayers was also dose dependent with a maximal inhibition at 5 μM when TER decreased from ~6,000 Ωcm^2 to ~800 Ωcm^2 (Fig. 2 *e*). This agreed well with the dose required for newly formed monolayers, suggesting that OCC2 peptide acted in similar manner in both growth states. (It is important to point out that the OCC2 peptide is somewhat water insoluble, and therefore its actual effective concentration in solution is unclear.) In conclusion, the second extracellular domain peptide of chick occludin (OCC2) decreased the TER of *Xenopus* A6 cell monolayers in a time- and dose-dependent manner, and the magnitude and time course of the effect depended on the growth state of the cells. Additional experiments carried out using the calcium switch method, in which peptides were added before TER development, showed that OCC2 also inhibited the generation of TER (data not shown).

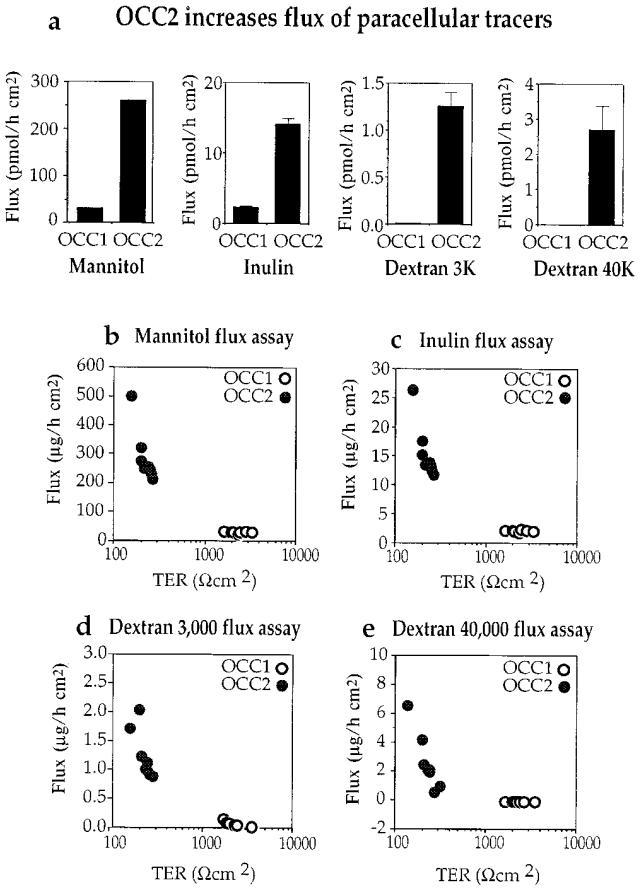

Figure 3. OCC2 increased the paracellular flux of membrane-impermeant tracer molecules. (*a*) Effects of OCC2 on the flux of [^{3}H]mannitol, [^{14}C]inulin, Texas red–conjugated neutral dextran (mol wt 3,000), and Texas red–conjugated neutral dextran (mol wt 40,000). OCC1 was used as control peptide. A6 cell monolayers were allowed to grow until TER reached ~1,200 Ωcm^2. Cell monolayers were then treated with a final concentration of 5 μl OCC1 or OCC2 for 36 h. TER was measured and tracers flux assays were performed as described in Materials and Methods. For all four tracers, $n = 8$ and error bars represent SEM. (*b–e*) The relationship between tracer flux and TER changes induced by OCC2 treatment. Absolute flux values for individual A6 cell monolayers were plotted against TER of the same monolayer. (*b*) [^{3}H]mannitol, (*c*) [^{14}C]inulin, (*d*) neutral dextran (mol wt 3,000) conjugated with Texas red, and (*e*) neutral dextran (mol wt 40,000) conjugated with Texas red.

OCC2 Increases Paracellular Permeability

The decrease in TER caused by the OCC2 peptide could be attributed to either an increase in paracellular tight junction permeability or transcellular plasma membrane permeability to ions. To distinguish between the two possibilities, we assessed the flux of membrane-impermeant paracellular tracer molecules across A6 cell monolayers. Four different paracellular flux tracers were used: mannitol (mol wt 184), inulin (mol wt 5,200), dextran 3K (mol wt 3,000), and dextran 40K (mol wt 40,000). Newly formed A6 monolayers (starting TER of ~1,000 Ωcm^2) were treated with 5 μM OCC1 or OCC2 for 36 h. At the end of the 36 h of peptide treatment (when control TER developed to ~2,500 Ωcm^2), paracellular tracer flux assays were performed. As before, treatment of monolayers with OCC2 resulted in a ~10-fold reduction in TER from ~2,500 Ωcm^2 to ~250 Ωcm^2. In the same monolayers, OCC2 caused a ~10-fold increase in the flux of paracellular tracers (Fig. 3 *a*). The flux of mannitol (hydrodynamic radius ~4 Å), inulin (hydrodynamic radius ~10–14 Å), and dextran 3K all increased ~10-fold after OCC2

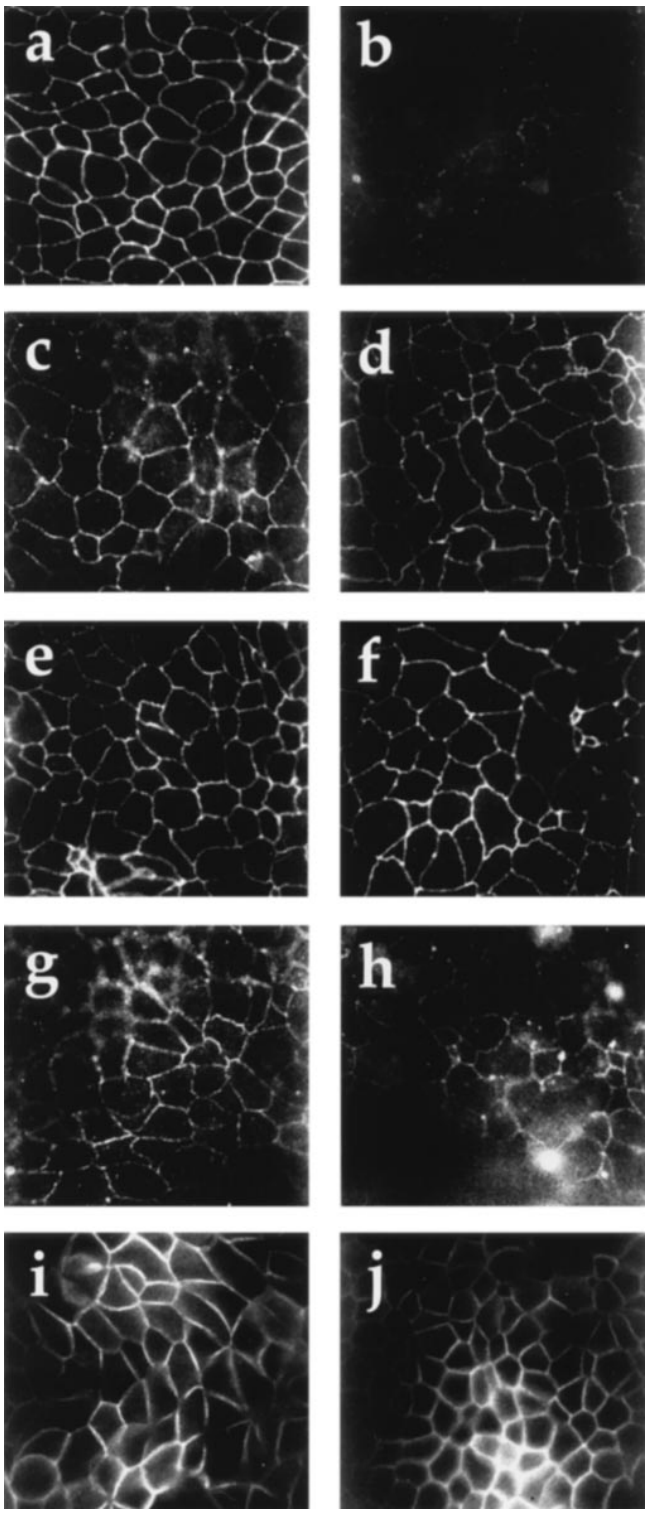

Figure 4. OCC2 reduced junctional stainings of occludin but not ZO-1, cingulin, ZO-2, and E-cadherin. A6 cell monolayers from the paracellular tracer flux assays described in Fig. 3 were processed for indirect immunofluorescence microscopy at the end of the flux assays. OCC1-treated monolayers had TER of ~2,500 Ωcm^2, and OCC2-treated monolayers had TER of ~250 Ωcm^2. OCC1-treated (*a*, *c*, *e*, *g,* and *i*) and OCC2-treated (*b*, *d*, *f*, *h*, and *j*) monolayers were immunostained in parallel for occludin (*a* and *b*), ZO-1 (*c* and *d*), cingulin (*e* and *f*), ZO-2 (*g* and *h*), and E-cadherin (*i* and *j*).

treatment. Therefore, the decrease in TER caused by OCC2 peptide was associated with an increase in paracellular permeability of the tight junction.

It was possible that OCC2 only altered the rates of movement of these relatively small paracellular tracers through the tight junction. To examine whether the functional tight junction barrier to macromolecules was disrupted by OCC2 peptide, the paracellular flux of dextran 40K, to which A6 cell monolayers are usually completely impermeable, was measured. Indeed, treatment of A6 cell monolayers with OCC2 opened the paracellular barrier to dextran 40K (Fig. 3 *a*). OCC1 treatment had no detectable effect on the flux of dextran 40K. Therefore, in addition to reducing TER, the tight junction permeability barrier to macromolecules of 40 kD was also compromised by OCC2 treatment.

For individual Transwell monolayers, there was a close correlation between tracer fluxes and the magnitude of the drop in TER. For this analysis, data from the experiments shown in Fig. 3 *a* was replotted for individual Transwell monolayers in Fig. 3, *b–e*. This correlation held for all four tracers: mannitol (Fig. 3 *b*), inulin (Fig. 3 *c*), dextran 3,000 (Fig. 3 *d*), and dextran 40,000 (Fig. 3 *e*). This correlation suggested that the decrease in TER caused by OCC2 was predominantly, if not exclusively, due to the increase in paracellular permeability. Therefore, we conclude that the peptide corresponding to the second extracellular domain of occludin perturbed the tight junction permeability barrier function of the A6 cell monolayers.

OCC2 Selectively Decreases the Level of Occludin in A6 Cells

To examine the potential effects of OCC2 on the tight junction at the molecular level, the localization and total cellular content of various known tight junction proteins were determined. A6 cell monolayers that were used in paracellular tracer flux assays (see above) were immediately processed for indirect immunofluorescence microscopy. OCC2-treated monolayers (TER ~250 Ωcm^2) had substantially less occludin present at cell boundaries as compared with OCC1-treated monolayers (TER ~2,500 Ωcm^2) (Fig. 4, *a* and *b*). On the other hand, ZO-1, ZO-2, cingulin, and E-cadherin distributions were not changed detectable by OCC2 treatment (Fig. 4, *c–j*). Therefore, it appeared that OCC2 selectively depleted occludin from the tight junction of A6 cells.

To confirm the depletion of occludin biochemically, we performed Western blot analysis of various tight junction proteins in A6 cell lysates that were treated for 24 h with OCC1 (10 μM), OCC2 (10 μM), or DMSO solvent control (0.1%). OCC2 selectively reduced total cellular occludin levels but did not alter the levels of ZO-1, ZO-2, cingulin, or E-cadherin (Fig. 5 *a*). Moreover, OCC1 had no effect on the level of occludin. Total cellular occludin levels were also unaltered by the scrambled peptide OCC2(S) and the unmodified peptide OCC2(U) (Fig. 5 *b*). These results suggested that the perturbation of the tight junction permeability barrier correlated with the selective reduction of total cellular occludin levels but not the levels of other junctional proteins, ZO-1, ZO-2, cingulin, and E-cadherin.

To determine whether the decreased occludin levels by OCC2 treatment were due to an inhibition of occludin

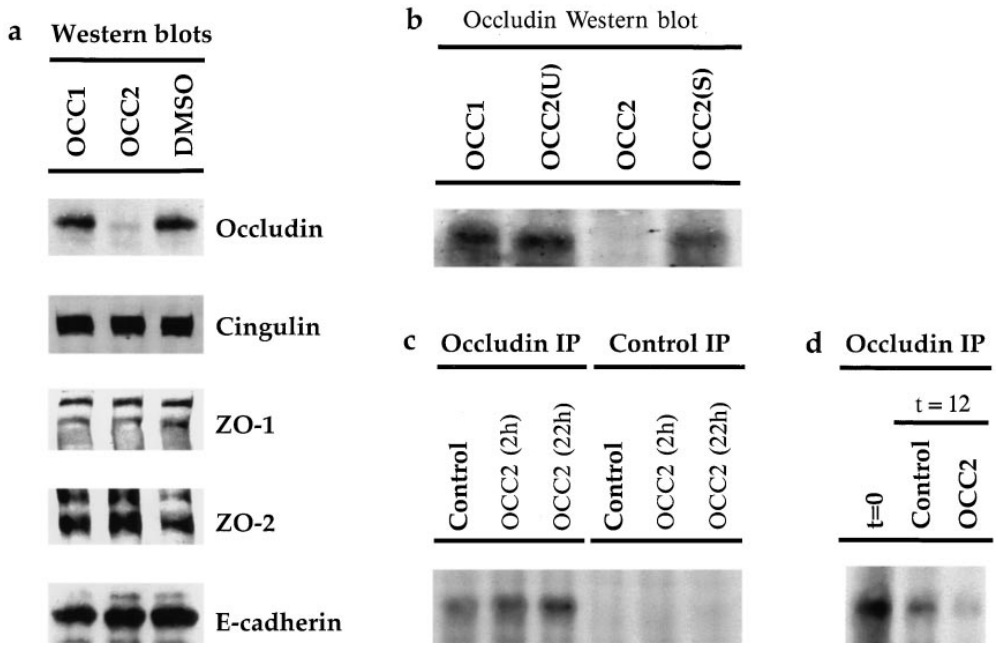

Figure 5. OCC2 specifically decreased total cellular occludin levels. (*a*) Western blots of occludin, cingulin, ZO-1, ZO-2, and E-cadherin of total cell lysates from monolayers that were treated with OCC1, OCC2, or DMSO solvent control. A6 cells were allowed to grow until TER reached ~1,000 Ωcm^2, and monolayers were treated with 10 μM of OCC1, 10 μM OCC2, or 0.1% DMSO for 24 h. (*b*) Only the peptide that decreased TER also caused a decrease in occludin levels. Western blot of occludin in A6 total cell lysates of monolayers that were treated with OCC1, OCC2(U) (unmodified), OCC2, or OCC2(S) (scrambled). A6 cells were allowed to grow to confluency in normal medium and were subsequently changed to low calcium medium for 18 h. A6 cells were then replenished with normal calcium media containing peptides at a final concentration of 5 μM. OCC2(U), unmodified OCC2, and OCC2(S), scrambled sequence of OCC2. Peptides were replenished every 24 h, and cells were extracted for analysis at 4 d after initial peptide treatment. (*c*) Occludin synthesis was not reduced by OCC2 treatment. A6 cells that were either untreated or treated for either 2 or 22 h with a final concentration of 5 μM OCC2 were subsequently labeled for 2.5 h with [^{35}S]methionine followed by immunoprecipitation (*IP*) of occludin. (*d*) Turnover of occludin was enhanced by OCC2 treatment. A6 cells were metabolically labeled 20 h with [^{35}S]methionine. At the end of the labeling period (t = 0), fresh media (without [^{35}S]methionine) containing 10 μM OCC2 was added for 12 h followed by immunoprecipitation (*IP*) of occludin. Untreated A6 cells were used in parallel as a control.

protein synthesis or an enhancement of occludin turnover, the effect of OCC2 on occludin synthesis and turnover was tested. The synthesis of occludin was examined by briefly metabolically labeling cellular proteins with [^{35}S]methionine (2.5-h pulse) followed by immunoprecipitation of occludin. Incorporation of [^{35}S]methionine into occludin was not altered by either 2 or 22 h of OCC2 treatment relative to untreated cells. Therefore, it is unlikely that OCC2 decreased total cellular occludin levels by inhibiting occludin synthesis (Fig. 5 *c*).

The effect of OCC2 on occludin turnover was determined by analyzing the disappearance of [^{35}S]methionine-labeled occludin from steady-state labeled cells (22 h). OCC2 peptide was added to the chase medium and, within 12 h of chase, the amount of labeled occludin was noticeably lower than that of untreated cells, suggesting that OCC2 caused occludin to turn over faster (Fig. 5 *d*). Therefore, the depletion of cellular occludin by OCC2 was attributed to an increase in occludin turnover.

TER Recovery after OCC2 Removal

If OCC2 decreased TER and occludin levels by specifically promoting occludin turnover rather than nonspecific toxicity, A6 cells would be expected to remain healthy and to be capable of reforming the tight junction permeability barrier after the removal of OCC2. Therefore, we tested the ability of A6 cell monolayers to recover TER after the removal of OCC2 peptide. Newly formed monolayers were treated with 5 μM OCC2 for 24 h, and TER dropped from ~2,000 Ωcm^2 to ~180 Ωcm^2. OCC2-containing medium was then removed from the cells and replaced with fresh OCC2-free medium. After OCC2 removal, the TER slowly increased and recovered to the initial pre-OCC2 treatment value in 48 h (Fig. 6 *a*). In fact, for the OCC2 peptide to maintain a continuous effect on TER, it is necessary to replenish OCC2 every 48 h because cells that were treated with only one dose of OCC2 recovered TER readily. Immunofluorescence analysis of occludin showed that the recovery of TER correlated with the reappearance of occludin at the tight junction (Fig. 6 *b*). The reversibility of the effect of OCC2 on both TER and occludin localization suggested that OCC2 only transiently altered the ability of A6 cells to form tight junctions. Furthermore, the correlation of TER recovery with occludin reappearance at the tight junction again provided evidence for a role of occludin in the formation of the tight junction permeability barrier.

Cell Morphology after OCC2 Treatment

The OCC2 peptide could act by disrupting the tight junction extensively, causing adjacent cells to separate from each other. Alternatively, OCC2 might selectively perturb the tight junction sealing element, leaving the overall tight junction morphology relatively intact. Scanning EM was used to examine the effect of OCC2 on A6 cell contacts. Examination of gross cell morphology by scanning EM did not reveal any detectable difference between cell monolayers that were treated for 30 h with DMSO (0.1%),

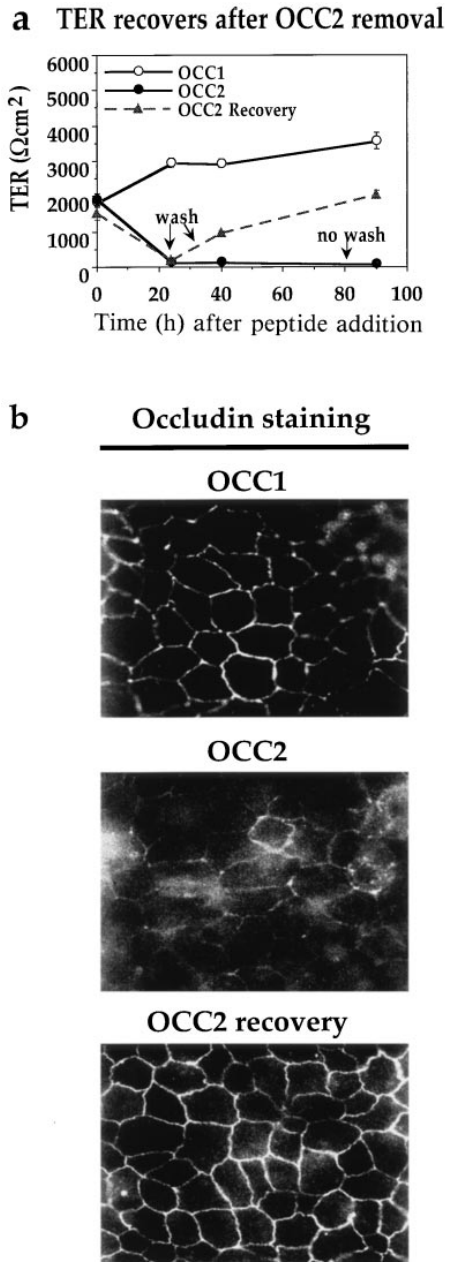

Figure 6. The effects of OCC2 on TER and occludin accumulation were reversible. (*a*) Reversibility of TER after OCC2 removal. A6 cell monolayers that had TER of ~1,700 Ωcm^2 were treated at $t = 0$ with a final concentration of 5 μM OCC1 or OCC2. At $t = 24$ h, peptides were either replenished (*OCC1* and *OCC2*) or removed (*OCC2 Recovery*) from the cells. OCC1 ($n = 6$), OCC2 ($n = 6$), and OCC2 recovery ($n = 3$). (*b*) Recovery of junctional stainings of occludin after OCC2 removal. A6 cell monolayers that had TER ~1,000 Ωcm^2 were treated at $t = 0$ with a final concentration of 5 μM OCC1 or OCC2. At $t = 24$ h, peptides were either replenished (*OCC1* and *OCC2*) or removed (*OCC2 recovery*) from the cells. At $t = 60$ h, cells were processed for indirect immunofluorescence microscopy of occludin. OCC1 (TER ~2,200 Ωcm^2), OCC2 (TER ~250 Ωcm^2), and OCC2 recovery (TER ~2,300 Ωcm^2).

OCC1 (10 μM), or OCC2 (10 μM) (Fig. 7). All monolayers appeared intact and where characterized by a high density of microvilli at cell boundaries. In addition, in preliminary experiments, no changes in the tight junction, as observed by transmission EM of thin-sections, were detected (unpublished observations). However, a much more extensive study would be necessary to determine the effects of OCC2 at the ultrastructural level.

Discussion

A synthetic peptide (OCC2) corresponding to the entire second extracellular domain of chick occludin was able to consistently and significantly decrease TER when added to *Xenopus* kidney epithelial A6 cell monolayers. The decrease in TER was attributed to a disruption of the tight junction permeability barrier because it was associated with an increase in paracellular flux of membrane-impermeant tracers. The effect of OCC2 on the tight junction permeability barrier correlated with a selective depletion of total cellular levels and junctional localization of occludin. On the other hand, the total cellular levels and localization of cytoplasmic components of the tight (ZO-1, ZO-2, and cingulin) and adherens (E-cadherens) junctions were not affected. Furthermore, cell morphology, as observed by scanning EM, was not altered. These results suggested that the second extracellular domain peptide of occludin (OCC2) acted specifically to perturb the permeability barrier function of the tight junction. The correlation of the physiological effects of OCC2 with the selective reduction of occludin provides evidence for a role for occludin in the formation of a functional tight junction seal.

The effect of OCC2 did not appear to be caused by general cell toxicity or perturbation of the plasma membrane. First of all, the perturbation of epithelial permeability by OCC2 occurred with only a maximal effective concentration of 5 μM. Secondly, the slow time course of the effect of OCC2, developing over 24 h of incubation, suggested that the peptide did not act by perturbing plasma membrane integrity, an effect that would be expected to be immediate. Thirdly, incubation of OCC2 for up to 5 d did not cause changes in overall epithelial cell morphology, suggesting that OCC2 was not detrimental to the cells. In fact, if the observed increase in the flux of 40-kD dextran had been caused by leaking through the plasma membrane, the cells would not be expected to survive. Moreover, cells that were treated with OCC2 excluded the vital dye, trypan blue, indicating that they remained intact and alive (data not shown). Fourthly, the effect of OCC2 was reversible after removal of the peptide, indicating that OCC2 did not impair the cells permanently but only temporarily perturbed the tight junction permeability barrier. In conclusion, OCC2 did not appear to be toxic to the cells or disruptive to the integrity of the plasma membrane in a nonspecific manner.

The effect of OCC2 appeared to be due to specific perturbation of the tight junction permeability barrier. First of all, the effect was specific to the amino acid sequence of OCC2 because only OCC2, but not OCC2(S), the scrambled sequence of the same amino acid composition as OCC2, was able to decrease the TER of cell monolayers. Secondly, the decrease in TER caused by OCC2 was asso-

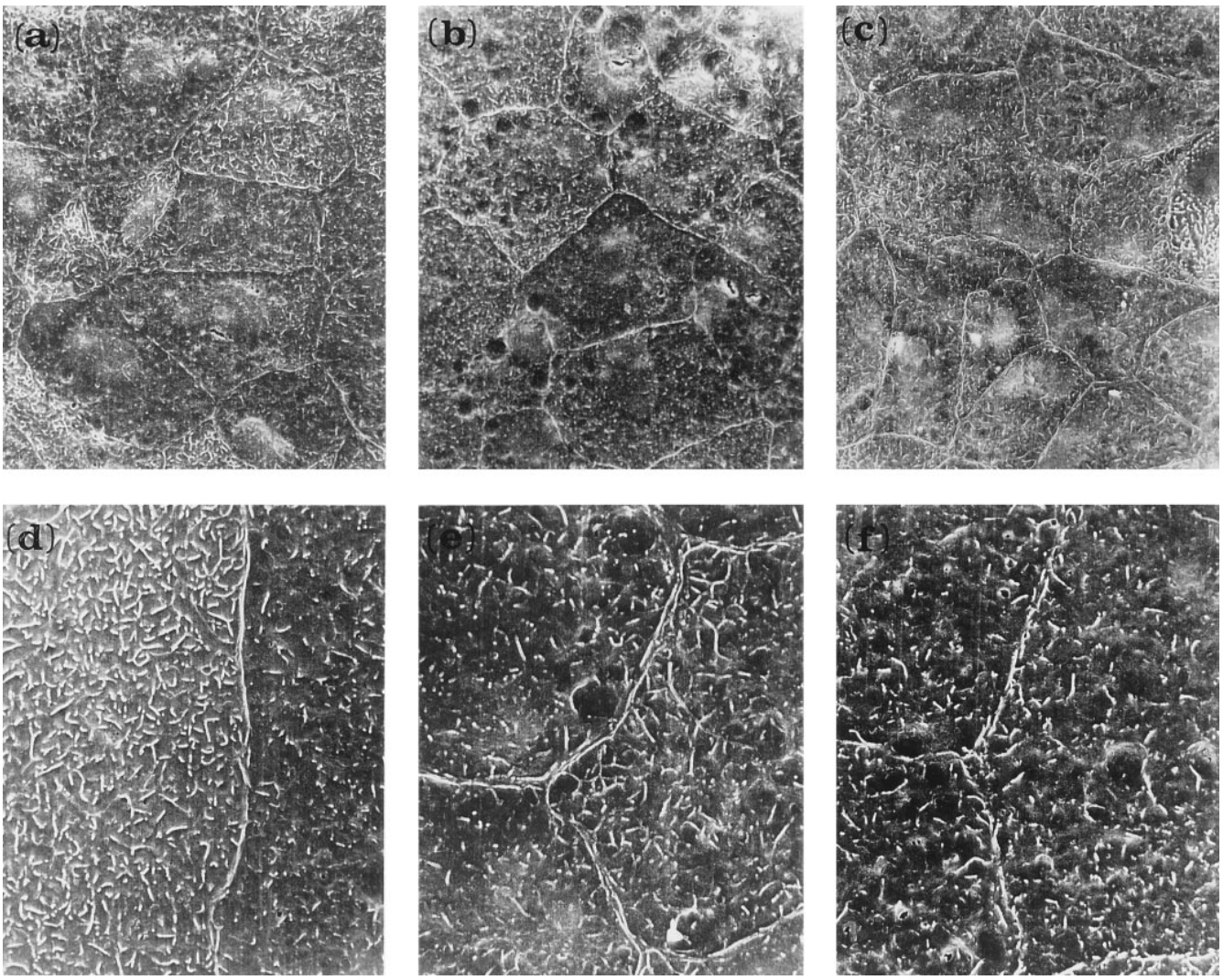

Figure 7. OCC2 did not cause morphological changes in A6 cell monolayers as observed by scanning EM. Confluent A6 cells grown on polylysine-coated coverslips were treated 24 h with a final concentration of 10 μM OCC1 (*a* and *d*), 10 μM OCC2 (*b* and *e*), and 0.1% DMSO (*c* and *f*). Cells were then processed for scanning EM. A6 cells were ~7–10 μM in diam.

ciated with an increase in the flux of membrane-impermeant tracers, demonstrating that OCC2 affected the paracellular tight junction barrier. Thirdly, OCC2 did not cause observable changes in epithelial cell morphology, suggesting that the perturbation of the tight junction barrier was not an indirect effect resulting from some gross morphological alteration of the cells. Furthermore, OCC2 did not disrupt the entire epithelial junctional complex, because neither the cellular levels nor the localization of various known tight (ZO-1, ZO-2, and cingulin) and adherens (E-cadherin) junction proteins were altered. Therefore, the physiological effects of OCC2 appeared to be due to specific perturbation of the permeability seal of the tight junction.

Although OCC2 caused cell monolayers to become permeable to macromolecular tracers, including dextran 40K, the TERs of treated monolayers remained at significant levels (~250 Ωcm^2). This suggests that the effect of OCC2 on cells within the monolayer was not homogeneous. It is expected that regions of the monolayer responsible for high tracer fluxes would also be areas of low electrical resistance. Consistent with this explanation are the nonhomogeneous patterns of residual occludin staining after OCC2 treatment (Fig. 4, *a* and *b*). Therefore, it is likely that a fraction of the cells in the monolayers were partially resistant to perturbation of the permeability barrier by OCC2 treatment.

OCC2 had specific reproducible effects on occludin, the protein from which it is derived. OCC2 selectively depleted the cellular level and junctional localization of occludin without influencing the levels of other junctional proteins examined. Control peptides that did not perturb the tight junction permeability barrier, including a scrambled version of OCC2, OCC2(S), did not alter occludin levels or localization. In addition, the amount of reduction in occludin levels at various time points after OCC2 treatment closely correlated with the magnitude of drop of TER (data not shown). Moreover, after the removal of OCC2, occludin expression and localization recovered completely to normal levels. In fact, the time course of both the deple-

tion and recovery of occludin correlated with the time course of the physiological effects of OCC2. Thus, the selective depletion of occludin from the tight junction appeared to be a biochemical effect consistently associated with the physiological action of the peptide. Therefore, we propose that the depletion of occludin by OCC2 is the mechanism by which OCC2 perturbed the tight junction permeability barrier.

OCC2 behaves as if it is a competitive inhibitor of occludin function that competes with endogenous occludin for its receptor(s) or binding protein(s). Two possible mechanisms of action of OCC2 could account for its perturbation of the tight junction barrier. First, OCC2 could bind an occludin receptor by intercalating into the tight junction and directly interfering with the normal function of a functional seal. Secondly, OCC2, by binding to occludin receptor(s), could cause the release of occludin from its normal stabilized interactions in the tight junction, which subsequently leads to gradual disassembly of tight junction sealing elements. Our results are consistent with the second mechanism because the effect of OCC2 correlated with the depletion of occludin, suggesting that disassembly of sealing elements is the mechanism of OCC2 action.

Our results strongly implicate occludin in the formation of the tight junction permeability barrier. Both the specificity of the OCC2 peptide sequence and the correlation of occludin levels and TER support this contention. This conclusion is consistent with the findings that occludin is present at the tight junction contact points and the intramembrane fibrils (14, 15), the region defined as the occluding barrier where paracellular tracers do not permeate through. The simplest model to explain how occludin might participate in sealing the tight junction is that occludin polymerizes in the plane of the plasma membrane and completely circumscribes the apex of cells. However, we do not known whether occludin is the only component of the fibrils or some other unidentified protein(s) also participate(s) in the formation of these fibrils. We also do not know how occludin forms the cell–cell contact at the tight junction. Does it bind to another occludin from the adjacent cell in a homophilic interaction or to an unidentified protein in a heterophilic manner? Interestingly, preliminary data indicate that OCC2 peptide–coupled beads can form a large aggregate, which might suggest that OCC2 binds to itself, perhaps reflecting either a homophilic binding event or polymerization of occludin (Wong, V., unpublished data). Regardless of the mechanism, our findings provide strong evidence for the functional role of occludin in the tight junction permeability barrier.

The fact that occludin can be depleted from the tight junction without affecting other known tight junction proteins suggests that its incorporation into the tight junction can be regulated separately. This can provide a potential mechanism to regulate the permeability barrier function of the tight junction without affecting the rest of the tight junction structure. Instead of assembling and disassembling the whole tight junction complex, the tight junction permeability barrier could be regulated by recruiting and removing occludin to and from the tight junction. This could also help to explain the dynamic nature of the tight junction, which must rapidly open and close during physiological processes. Further experiments will be required to determine whether the incorporation of occludin into the tight junction is regulated physiologically.

The OCC2 peptide offers the possibility to selectively and transiently eliminate the tight junction permeability barrier without disrupting the general architecture of cells. OCC2 treatment is the most specific means identified so far to selectively perturb the paracellular barrier of the tight junction. The transient perturbation of the barrier function of the tight junction by OCC2 could be potentially useful in medical therapeutics such as the facilitation of drug delivery across the blood–brain barrier. The OCC2 sequence should be considered as just a starting point for potential pharmaceutical development. It may be possible to identify other occludin sequences or small molecules that can optimally perturb occludin and the physiological properties of the tight junction.

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