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FUNCTIONAL ROLE OF OCCLUDIN IN THE TIGHT JUNCTION PERMEABILITY BARRIER

by

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DISSERTATION

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To my parents, my husband, and my daughter

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Functional Role of Occludin in the Tight Junction Permeability Barrier

Vivian Wong

ABSTRACT

The tight junction of epithelial and endothelial cells defines the paracellular permeability barrier to ions and macromolecules, thus allowing epithelia and endothelia to act as biological barriers. Although several cytoplasmic/peripheral membrane proteins (ZO-1, ZO-2, cingulin, 7H6 antigen, and rab 13) and one integral membrane protein (occludin) of the tight junction have been identified, none of their functions have been elucidated. Occludin, being the only identified integral membrane protein of the tight junction, is a candidate for forming the actual contact seal of the paracellular pathway. In this study, the function of occludin in the formation of the tight junction permeability barrier has been examined.

The predicted structure of occludin contains four membrane spanning regions and two extracellular loops. The extracellular loops of occludin, which are conserved between five species (chick, rat-kangaroo, mouse, human, and dog), consist mainly of non-polar residues and, therefore, are candidates for protein-protein interactions. Synthetic peptides corresponding to the extracellular loops of chick occludin were assayed for their ability to perturb tight junctions in *Xenopus* kidney epithelial A6 cells. After treating cells with the peptides, the function and molecular structure of the tight junction as well as epithelial cell morphology were examined.

The peptide corresponding to the second extracellular loop of occludin was capable of reversibly disrupting the tight junction permeability barrier in a dose and time dependent manner. The disruption of the tight junction was

associated with a selective depletion of total cellular and junctional occludin. However, total cellular content and localization of junctional proteins ZO-1, ZO-2, and cingulin were not altered. Furthermore, epithelial cell morphology was also unaltered by this peptide. The selective depletion of occludin that is associated with the disruption of the tight junction permeability barrier implies that there is a functional role of occludin in the formation of the paracellular seal.

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LIST OF ABBREVIATIONS

A6	A *Xenopus* kidney epithelial cell line
CHO	A Chinese hamster ovary cell line
DMSO	Dimethly sulfoxide
EM	Electron microscopy
HMW	High molecular weight
kDa	Kilo Daltons
LMW	Low molecular weight
MDCK	The Madin-Darby canine kidney cell line
MDCK I	The strain I subclone of the Madin-Darby canine kidney cell line
MDCK II	The strain II subclone of the Madin-Darby canine kidney cell line
MW	Molecular weight
OCC1	A synthetic peptide corresponding to the putative first extracellular domain of chick occludin
OCC2	A synthetic peptide corresponding to the putative second extracellular domain of chick occludin that is protected at cysteine residues
OCC2(S)	A synthetic peptide corresponding to the scrambled sequence of OCC2
OCC2(U)	A synthetic peptide corresponding to the putative second extracellular domain of occludin that is not protected at cysteine residues
TER	Transepithelial electrical resistance
TX-100	Triton X-100

INTRODUCTION

The *Zonula occludens*, also known as the tight junction, is an intercellular junction of epithelial and endothelial cells. The tight junction has two main functions. Firstly, it acts as a gate which regulates the passage of ions and macromolecules through the paracellular pathway. This allows the separation of apical and basolateral compartments, resulting in the formation of biological barriers such as the blood-brain-barrier and the blood-retinal-barrier, that are essential for the maintenance of the internal milieu in different tissues of the organism (Sagaties, Raviola, Schaeffer & Miller, 1987; Wolburg, Neuhaus, Kniesel, Kraub & Schmid, 1994). Macromolecules of radii $\geq$ 15 Å cannot flow through the tight junction (Madara & Dharmsathaphorn, 1985). However, depending on the properties of individual epithelium, small ions can pass through the tight junction to varying degrees, as indicated by the differences in transepithelial electrical resistance (TER) in various epithelial and endothelial cells (5 Ω cm^2 to >5000 Ω cm^2) (Claude & Goodenough, 1973; Powell, 1981). Secondly, the tight junction acts as a barrier to plasma membrane lipids and proteins, thus preventing the mixing of apical and basolateral membrane components. By doing so, the tight junction helps to maintain the polarity of the plasma membrane, which is crucial for vectorial processes such as absorption and secretion that are carried out by epithelia.

The physical structure of the tight junction has been studied at the level of electron microscopy (EM). The tight junction, which usually locates at the apical-most of the cells, spans about 0.2 to 0.5 µm in depth along the lateral plasma membrane. In transmission EM of thin sections, the tight junction appears as a series of contacts of adjacent cells (Farquhar & Palade, 1963). These contact sites represent touching of the outer leaflets of the adjacent plasma membranes and demarcate the regions where paracellular tracer

molecules are excluded (Gumbiner, 1987; Stevenson, Anderson & Bullivant, 1988). In freeze fracture transmission EM, the tight junction appears as a network of branching and anastomosing intramembrane fibrils with particles seen in the P-face (inwardly facing outerleaflet) and complimentary grooves in the E-face (outwardly facing innerleaflet) (Claude & Goodenough, 1973). These tight junctional fibrils correspond to the actual contact points as seen in transmission EM of thin sections and completely circumscribe the apices of the cells (Gumbiner, 1987; Stevenson et al., 1988).

The tight junction appears to be regulated in various physiological processes. For example, when luminal concentrations of glucose and amino acids are elevated after food intake, the paracellular permeability of the intestinal epithelium to glucose and amino acids increases, suggesting that the tight junction permeability might be regulated to participate in nutrient absorption (Atisook, Carlson & Madara, 1990; Ballard, Hunter & Taylor, 1995)(Atisook et al., 1990; Ballard et al., 1995). The tight junction is also regulated in dynamic physiological processes such as intestinal cell division (Baker & Garrod, 1993) and intestinal cell extrusion (Baron & Miller, 1990) during which the intramembrane fibrils of the tight junction move in the plane of the plasma membranes as the cells slide along each other. This dynamic regulation of the tight junction fibrils might contribute to the continuous maintenance of the permeability barrier during cell division and extrusion. In conclusion, the tight junction functions more than simply a stagnant seal, but rather, is regulated dynamically.

Numerous substances have been shown to affect the tight junction; some of which might function as endogenous physiological regulators of the tight junction. Transforming growth factors (α and β) have been shown to disrupt the tight junction permeability barrier and to cause delocalization of ZO-1

from cell-cell contacts (Buse et al., 1995; Woo, Cha, Singer & Firestone, 1996). On the other hand, glucocorticoids have been shown to induce tight junction formation and the generation of high transpithelial electrical resistance (Zettl et al., 1992). Disruption of the tight junction has been found in various diseases such as hepatitis, Celiac Spruce, Crohn's disease, and gastritis in which the tight junction fibrils of the respective epithelia manifest discontinuities and poor organization (Hollander, 1988; Madara & Trier, 1980; Posalaky, Posalaky, McGinley & Meyer, 1989; Swift, Mukherjee & Rowland, 1983). Interestingly, an increase in tight junctional depth as seen in Blind Loop syndrome of the intestine is associated with a decrease of the permeability of the intestine to nutrients (Schulzke et al., 1990). However, it is not known whether these changes are the consequences or the causes of the diseases. To understand the functional role of tight junctions at the level of a tissue or an organism, it is important to examine the tight junction at the molecular level.

Five cytoplasmic/peripheral membrane proteins (ZO-1, ZO-2, cingulin, 7H6 antigen, and rab 13) and one integral membrane protein (occludin) of the tight junction have been identified (Anderson, Stevenson, Jesaitis, Goodenough & Mooseker, 1988; Anderson et al., 1989; Citi, Sabanay, Jakes, Geiger & Kendrick-Jones, 1988; Citi, Sabanay, Kendrick-Jones & Geiger, 1989; Furuse et al., 1993; Gumbiner, Lowenkopf & Apatira, 1991; Stevenson, Siliciano, Mooseker & Goodenough, 1986; Zahraoui et al., 1994; Zhong et al., 1994; Zhong et al., 1993). ZO-1 is a 225 kDa phosphoprotein that localizes at the immediate contact sites of the tight junction by transmission EM of immunogold labeled thin sections (Stevenson & Goodenough, 1984; Stevenson et al., 1986). Phosphorylation of ZO-1 is different in two strains of Madin-Darby canine cells (MDCK I and II) that have different TER, suggesting

a role of ZO-1 in the regulation of the tight junction (Stevenson, Anderson, Braun & Mooseker, 1989). Two isoforms of ZO-1 are present in varying ratios in cells that have different tight junction dynamics which further supports the notion that ZO-1 participates in the regulation of tight junctions (Balda & Anderson, 1993; Kurihara, Anderson & Farquhar, 1992; Willott, Balda, Heintzelman, Jameson & Anderson, 1992). Cingulin is a 140 kDa dimeric protein that is localized further from the tight junction contact sites than ZO-1 when assessed by transmission EM of immunogold labeled thin sections (Citi et al., 1988; Citi et al., 1989). Cingulin is extractable under moderately stringent conditions and is probably a cytoplasmic component rather than a peripheral membrane protein of the tight junction (Citi et al., 1988; Citi et al., 1989). ZO-2 is a 160 kDa phosphoprotein that also localizes to the tight junction contact sites by transmission EM of immunogold labeled thin sections. ZO-2 co-immunoprecipitates with ZO-1 from MDCK cells under moderately stringent conditions and appears to exist as a protein complex with ZO-1 because both ZO-1 and ZO-2 turnover with similar kinetics (Gumbiner et al., 1991). 7H6 antigen is a 155 kDa protein that is also localized to the tight junction contact sites by transmission EM of immunogold labeled thin sections (Zhong et al., 1994; Zhong et al., 1993). Rab 13, a small GTP binding protein, appears to be specifically localized to the tight junction when assessed by indirect immunofluorescence microscopy (Zahraoui et al., 1994). The integral membrane protein of the tight junction, occludin, is a ~65 kDa protein that localizes to the tight junction contact sites by transmission EM of immunogold labeled thin sections (Furuse et al., 1993) as well as to the intramembrane fibrils by transmission EM of immunogold labeled freeze fracture replicas (Fujimoto, 1995). Although many of the tight junction

molecular components were identified, none of their roles in the function of the tight junction have been elucidated.

Occludin, being the only integral membrane protein of the tight junction, is the best candidate for protein that participate in the formation of the actual contact seal of the paracellular pathway. In this study, the role of occludin in the function of the tight junction has been examined. The first chapter addresses the participation of occludin in the formation of the tight junction permeability barrier. The second chapter addresses the regulation of occludin and the potential role of occludin in dictating the permeability properties of the tight junction.

CHAPTER I

A SYNTHETIC PEPTIDE CORRESPONDING TO THE SECOND EXTRACELLULAR DOMAIN OF OCCLUDIN PERTURBS THE TIGHT JUNCTION PERMEABILITY BARRIER

I.1 ABSTRACT

Occludin, the only known tight junction integral membrane protein, is an attractive candidate for the 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, two 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 demonstrate that the second extracellular domain peptide (OCC2) reversibly disrupted the transepithelial permeability barrier at concentrations of less than 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 both the amount of occludin present at the tight junction, as assessed by indirect immunofluoresence, and the total cellular content of occludin, as assessed by antigen blot analysis. Pulse-labelling and metabolic chase analysis suggested that these decreases in occludin levels could be attributed to an increase in the turnover of cellular occludin rather than a decrease in occludin synthesis. The effects on occludin were 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 provide evidence for a role of occludin in the formation of the tight junction seal.

I.2 INTRODUCTION

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 electron microscopy (EM). These contact points of the tight junction correspond to a network of intramembrane fibrils when studied by freeze-fracture EM which completely circumscribe the apices of (Gumbiner, 1987; Stevenson et al., 1988).

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 (Madara & Dharmsathaphorn, 1985). 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 >5000 Ω cm^2) (Claude, 1978; Powell, 1981). 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 (Sagaties et al., 1987; Wolburg et al., 1994). 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 (Gumbiner & Simons, 1987; Powell, 1981). 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 permeability of tight junction to nutrients has been shown to be increased by intestinal luminal glucose after food intake suggesting that the regulation of tight junction permeability have a role in absorption of nutrients in the intestine (Atisook et al., 1990; Ballard et al., 1995). A number of hormones have been shown to affect the tight junction including transforming growth factors (α and β) (Buse et al., 1995; Woo et al., 1996)) and glucocorticoids (Zettl et al., 1992). Disruption of the tight junction has been found to occur in many diseases including hepititis, Celiac Spruce, Crohn's disease, and gastritis in which the tight junction intramembrane fibrils of the respective epithelia manifest discontinuities and poor organization (Hollander, 1988; Madara & Trier, 1980; Posalaky et al., 1989; Swift et al., 1983). On the other hand, an increase in tight junctional depth in the intestinal epithelium in Blind Loop syndrome correlates with a decrease in permeability to nutrients (Schulzke et al., 1990). In addition, the tight junction is regulated in various physiological processes, such as leukocyte transmigration across an endothelium (Milks, Conyers & Cramer, 1986) and intestinal cell division (Baker & Garrod, 1993) and extrusion (Baron & Miller, 1990) to ensure minimal disruption of the tight junction barrier. Therefore, the tight junction appears to function as more than just a paracellular seal but in addition 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 (ZO-1, ZO2, cingulin, 7H6, and rab 13) and one integral

membrane protein (occludin) have been found to localize at tight junctions (Anderson et al., 1988; Anderson et al., 1989; Citi et al., 1988; Citi et al., 1989; Furuse et al., 1993; Gumbiner et al., 1991; Stevenson et al., 1986; Zahraoui et al., 1994; Zhong et al., 1994; Zhong et al., 1993). Occludin was shown to localize to junctional fibrils by immunogold labeling of freeze-fracture replicas of tight junctions (Fujimoto, 1995). The cytoplasmic tail of occludin is necessary for its localization to cell-cell contacts, perhaps via binding to ZO-1 and ZO-2 (Furuse et al., 1994). However, how these proteins function in the formation of the tight junction permeability barrier is unclear.

Occludin, being the only 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 acids extracellular loops. Both extracellular domains of occludin consist solely of uncharged residues with the exceptions of one or two charged residues adjacent to the transmembrane regions. The non-polar nature of the extracellular domains and the conservation of their sequences between five species (human, mouse, dog, chick, and rat-kangaroo) (Ando-Akatsuka et al., 1996) suggest that the extracellular domains have important functional roles, perhaps the formation of the actual contact seal of the tight junction. To test this hypothesis, synthetic peptides corresponding to each of the putative extracellular domains of chick occludin were assayed for their ability to alter tight junction barrier function.

I.3 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 (Perkins & Handler, 1981) was grown on Costar Transwell filters (Fisher, Santa Clara, CA) in 89% DMEM (1 g/L glucose) supplemented with 0.74 g/L bicarbonate, 5.95 g/L HEPES (pH 7.4), and 5% FCS and maintained at 28°C and 1% CO_2. For the calcium switch assay (González-Mariscal, de Ramirez & Cereijido, 1985; Gumbiner & Simons, 1986; Meldolesi, Castiglioni, Parma, Nassivera & de Camilli, 1978), A6 cells were allowed to grow in normal growth medium to confluency and subsequently changed to low calcium medium for 18 hours. At the end of the low calcium (<50 μM) incubation, A6 cells were replenished with normal calcium media and the formation of tight junctions were monitored by the generation of transepithelial electrical resistance (TER). 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.

Peptides Synthesis and Treatment of Cells

Peptides OCC1 corresponding to amino acids 81-124 of chick occludin (44 a.a. = DYGYGLGGAYGTGLGGFYGSNYYGSGLSYSYGYGGYYGGVNQRT) and OCC2 corresponding to amino acids 184-227 of chick occludin (44 a.a. = 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 second 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) (44 a.a. = GACQVYDPYMSGNYPAQSLMYQNL QYLVSGIHTYPECSYATQSY) composed of a scrambled sequence of the same residues as OCC2 including the acetamidomethyl modification at cysteines was also synthesized . 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.

Paracellular Tracers Flux Assay

Flux assays were performed on 6.5 mm Costar Transwell filter chambers (Fisher, Santa Clara, CA). Four different paracellular tracers: ^{3}H-mannitol (Amersham Life Sciences, Inc., Arlington Heights, IL), ^{14}C-inulin (Amersham LIfe Sciences, Inc., Arlington Heights, IL), neutral Dextran-MW 3,000 conjugated with Texas Red (Molecular Probes, Inc., Eugene, OR), and neutral Dextran-MW 40,000 conjugated with Texas Red (Molecular Probes,

Inc., Eugene, OR), 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 (MW 184) and 1 mM unlabeled inulin (MW 5200)). 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-MW 3,000 or 50 μg/100 μl for Dextran-MW 40,000 to the apical bathing wells which contained 100 μl 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^{o}C with gentle circular agitation. Cell monolayers were first allowed to equilibrate for 30 min after the addition of tracers. After each following 15 min time interval, the entire Transwell filter cup was removed from the basal bathing well and transfer 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 which 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 kDa and 40 kDa, 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 Antigen Blotting

A6 cells were grown on Transwell filters and the 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% non-fat 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 GST fusion protein of the cytoplasmic domain of chick occludin (255-510 a.a.). Rabbit anti-ZO-1 (#10153) and ZO-2 (#9989) (Jesaitis & Goodenough, 1994) antibodies were gifts from D. Goodenough. Rabbit anti-cingulin antibodies were gifts from S. Citi. Monoclonal mouse anti-*Xenopus* E-cadherin antibodies (5D3) were raised against the extracellular domain of E-cadherin (Choi & Gumbiner, 1989). FITC-conjugated secondary antibodies were obtained from Molecular Probes. For antigen blot analyses, 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 dithiothreitol) containing 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). Samples were boiled for 10 min, and cooled to room temperature before the addition of iodoacetic acid to a final concentration of 125 mM. After SDS-PAGE, antigen blots for occludin, ZO-1, ZO-2, cingulin, and E-cadherin were performed using the same primary antibodies as for immunofluorescence stainings. Secondary antibodies conjugated with HRP (Biorad, Richmond, CA) were developed by enhanced chemiluminescence (Amersham Life Sciences, Inc., Arlington Heights, IL).

Metabolic labelling, Immunoprecipitations, and Fluorography

A6 cells were grown on 75 mm Costar Transwell filters (Fisher, Santa Clara, CA). To examine the turnover of occludin, each monolayer was labelled with 0.8 mCi ^{35}S-methionine in methionine-free media (supplemented with 5% fetal bovine serum) for 22 hours, and protein turnover was monitored by replacing labelling media with fresh media in the presence or absence of 10 mM OCC2 for 12 hours. Cells were extracted for immunoprecipitation either immediately at the end of the labelling period (t = 0) or at 12 hours after the end of the labelling period (t = 12 hr). For examination of occludin, cells were rinsed once with methionine-free media and incubated in methionine-free media for 30 min before metabolic labelling. Each Transwell cell monolayer was then briefly labelled for 2 hours with 1.5 mCi ^{35}S-methionine in methionine-free media (supplemented with 5% dialyzed fetal bovine serum) in the presence or absence of 5 μM OCC2 before extraction for immunoprecipitation. Pulse-labelling experiments were also performed on cells pretreated with 5 μM OCC2 for 20 hours. For immunoprecipitations, 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, 5μg/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). Immunoprecipitations were performed with Protein-A Sepharose (Sigma, St. Louis, Missouri) in the presence of rabbit anti-chick occludin anti-bodies (see above) or pre-immune serum. Immunoprecipitates were prepared for

SDS-PAGE as described above. Polyacrylamide gels were fixed with 50% Methanol and 10% acetic acid for one hour and incubated in Amplify (Amersham Life Sciences, Inc., Arlington Heights, IL) for 45 min before dried under vacuum. Dried gels were exposed to Hyperfilm-MP (Amersham Life Sciences, Inc., Arlington Heights, IL) at -80^{o}C.

Scanning Electron Microscopy

A6 cells were plated on Thermanox coverslips coated with polylysine and allowed to grow to confluency. Cells were either untreated or treated for 40 hours with 10 μM OCC1 or 10 μM OCC2. Subsequently, the cells were rinsed twice in 0.135 M NaCl/10 mM HEPES/pH 7.5 and fixed with 2.5% glutaldehyde/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 Denton DCP-1 Critical Point Dryer. The samples were sputter coated with gold/palladium in a Technics Hummer VI sputtering system. The samples were photographed using a Joel JSM 35 Scanning Electron Microscope. All electron microscopy was performed by Nina Lampen in the Electron Microscopy Facility at the Memorial Sloan-Kettering Cancer Center.

I.4 RESULTS

Expression and Localization of Occludin in A6 cells Correlates with the Development of Transepithelial Resistance

The *Xenopus* kidney epithelial A6 cell line formed monolayers that had very high transepithelial electrical resistance (TER) of ~8,000 Ωcm^2 and were impermeable to macromolecules of MW ≥ 40 kD. After induction of synchronized intercellular junction formation by a calcium switch assay (see methods for details), occludin localization at cell boundaries correlated with the formation of tight junctions as monitored by measurements of TER (Fig. 1.1a). Western blot analysis of A6 total cell lysates using polyclonal antibodies raised against the cytoplasmic domain of chick occludin showed a single band of apparent gel mobility of ~ 65 kDa corresponding to *Xenopus* occludin. Expression levels of occludin during tight junction formation correlated roughly with the increase in TER (Fig. 1.1b). The time course of occludin localization and expression was consistent with the hypothesis that occludin participates in the formation of the tight junction.

Transepithelial Electrical Resistance 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 having 44 residues. Synthetic peptides corresponding to either the entire first or second extracellular domains 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 ~6000 Ωcm^2 to ~900 Ωcm^2 (Fig. 1.2a). In contrast, the first extracellular domain peptide (OCC1) did not alter the TER as compared to the DMSO only control (Fig. 1.2a). Additionally, a control peptide containing a scrambled amino acid sequence from the second extracellular domain, OCC2(S), had no effect on the TER (Fig. 1.2a). Therefore, the peptide corresponding to the second extracellular domain of chick occludin (OCC2) specifically reduced the 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 were 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 high performance liquid chromotography, data not shown) and prevented inter-molecular 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 the TER by the 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 the TER were assayed on both newly formed monolayers that were developing TER (TER ~1000 Ωcm^2) and steady-state monolayers that had completely formed maximal TER (TER ~8,000 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 methods); both procedures yielded the same results. For newly formed monolayers that were still developing TER, OCC2 treatment caused a ~10 fold decrease in TER from ~2500 Ωcm^2 to ~250 Ωcm^2 in 2 days (Fig. 1.2b). Treatment with the first extracellular domain peptide, OCC1, had no effect (Fig. 2b). 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 causing a ~10 fold decrease in TER from ~3500 Ωcm^2 to ~400 Ωcm^2 (Fig. 1.2c). For steady-state monolayers that had been confluent for over 10 days and attained a maximal TER (~10,000 Ωcm^2), OCC2 decreased TER from ~10,000 Ωcm^2 to ~2000 Ωcm^2 whereas untreated monolayers retained a stable maximal TER (Fig. 1.2d). However, the effect of OCC2 on steady-state monolayers took much longer than it did for newly formed monolayers. It took 5 days to attain a ~5 fold decrease in TER in steady-state monolayers as compared to 2 days 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 ~6000 Ωcm^2 to ~800 Ωcm^2 (Fig. 1.2e). 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. Nevertheless, 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.

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 (MW 184), inulin (MW 5200), Dextran 3K (MW 3000), and Dextran 40K (MW 40,000). Newly formed A6 monolayers with a TER of ~1000 Ωcm^2 were treated with 5 μM OCC1 or OCC2 for 36 hours. At the end of the 36 hours of peptide treatment, the TERs were measured and paracellular tracer flux assays were performed. As before, treatment of monolayers with OCC2 caused a ~10 fold reduction in TER from ~2500 Ωcm^2 to ~250 Ωcm^2. In the same monolayers, OCC2 caused a ~10 fold increase in the flux of paracellular tracers (Fig. 1.3a). The flux of mannitol (hydrodynamic radius ~4 Å), inulin (hydrodynamic radius ~11-14 Å), and Dextran 3K all increased ~10 fold after OCC2 treatment. Therefore the decrease in TER caused by OCC2 peptide was associated with an increase in paracellular permeabilty of the tight junction.

It seemed 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. 1.3a). OCC1 treatment had no detectable effect on the flux of Dextran 40K. Therefore, in addition to reducing the TER, the tight junction

permeability barrier to macromolecules was also compromised by OCC2 treatment.

Analysis of individual Transwell monolayers showed a close correlation between the magnitude of the drop in TER and the amount of increase in flux of all four tracers: mannitol (Fig. 1.3b), inulin (Fig. 1.3c), Dextran 3000 (Fig. 1.3d), and Dextran 40,000 (Fig. 1.3e). This correlation suggested that the decrease in TER caused by OCC2 was due to predominantly, if not exclusively, the increase in paracellular permeability. Therefore, 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 to OCC1 treated monolayers (TER ~2500 Ωcm^2) (Fig. 1.4 a & b). On the other hand, ZO-1, ZO-2, cingulin, and E-cadherin distributions were not changed detectably by OCC2 treatment (Fig. 1.4 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 hours with OCC1 (10 μM), OCC2 (10 μM), or DMSO solvent control (0.1%). OCC2 selectively reduced total cellular occludin levels

but did not alter detectably the levels of ZO-1, ZO-2, cingulin, or E-cadherin (Fig. 5a). Moreover, OCC1 had no detectable 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. 1.5b). These results suggest that the perturbation of the tight junction permeability barrier correlates with the selective reduction of total cellular occludin levels but not the levels of other junctional proteins, ZO-1, ZO-2, cingulin, or E-cadherin.

To determine whether the decrease in occludin levels induced by OCC2 treatment was due to an inhibition of occludin protein synthesis or an enhacement of occludin turnover, the effect of OCC2 on occludin synthesis and turnover was tested. The synthesis of occludin was examined by briefly metabolically labelling cellular proteins with ^{35}S-methionine (2.5 hours pulse) followed by immunoprecipitation of occludin. Incorporation of ^{35}S-methionine into occludin was not altered significantly by either 2 or 22 hours of OCC2 treatment relative to untreated cells. Therefore, it is unlikely that OCC2 decreased total cellular occludin levels by inhibiting occludin synthesis (Fig. 1.5c).

The effect of OCC2 on occludin turnover was determined by analyzing the disappearance of ^{35}S-methionine labelled occludin from steady-state labelled cells (22 hours). OCC2 peptide was added to the chase medium and within 12 hours of chase, the amount of labelled occludin was noticeably lower than the amount in untreated cells, suggesting that OCC2 caused occludin to turnover faster (Fig. 1.5d). Therefore, the depletion of cellular occludin by OCC2 appears to be 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 non-specific toxicity, A6 cells would be expected to remain healthy and 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 hours and TER dropped from ~2000 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 hours (Fig. 1.6a). In fact, for the OCC2 peptide to maintain a continuous effect on TER, it is necessary to replenish OCC2 every 48 hours 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. 1.6 b-d). The reversibility of the effect of OCC2 on both TER and occludin localization suggested that OCC2 did not permanently, but only transiently, alter 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 electron microscopy (EM)

was employed 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 hours with DMSO (0.1%), OCC1 (10 μM), or OCC2 (10 μM) (Fig. 1.7). All monolayers appeared intact and were 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 observation). However, a much more extensive study would be necessary to determine the effects of OCC2 at the ultrastructural level.

I.5 DISCUSSION

A synthetic peptide (OCC2) corresponding to the entire second extracellular domain of chick occludin was able to consistently and significantly decrease the 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-cadherin) 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 with a maximal effective concentration of 5 μM is within a range consistent with physiological specificity. Secondly, the slow time course of the effect of OCC2, developing over 24 hours 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 days 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 kDa Dextran had been caused by leaking through the plasma membrane, the cells would not be expected to survive. Forthly, 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 non-specific 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 associated 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.

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 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 depletion and recovery of occludin correlated with the time course of the physiological effects of OCC2. In summary, 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, the depletion of occludin by OCC2 appeared to be 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. Firstly, OCC2 could bind an occludin receptor by intercalating into the tight junction and directly interfering with the normal formation of a functional seal. Secondly, OCC2, by binding to an occludin receptors(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. The results in this study 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.

The results in this study strongly implicate a role of 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 (Fujimoto, 1995; Furuse et al., 1993), the region defined as the occluding barrier where paracellular tracers do not permeate through. One simple 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, it is not known whether occludin is the only component of the fibrils or other unidentified protein(s) also participate in the formation of these fibrils. It is also not known 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 large aggregates, which might suggest that OCC2 binds to itself, perhaps reflecting either a homophilic binding event or a polymerization of occludin (V. Wong, unpublished data). Regardless of the mechanism, this work provides 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 depleting and enriching occludin at 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

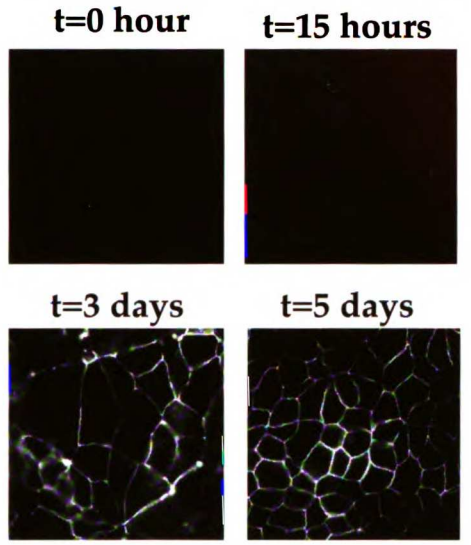
whether the incorporation of occludin into the tight junction is regulated physiologically.

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.

I.6 LEGENDS & FIGURES

Fig. 1.1. Expression and junctional localization of occludin correlates 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 subsequently changed to low calcium medium for 18 hours. Then, the medium was replenished with normal calcium (t=0) and the formation of tight junctions was monitored by measuring transepithelial electrical resistance (TER) at t=0 hours (TER = 0 Ωcm^2), t=15 hours (TER ~0 Ωcm^2), t=3 days (TER ~100 Ωcm^2), and t=5 days (TER >1000 Ωcm^2). (a) Indirect immunofluorescence of occludin in A6.2 cells at t=0, 15 hrs, 3 days, and 5 days. (b) Antigen blots of A6 cell lysate for occludin, cingulin, and ZO-1 at t=0, 15 hrs, 3 days, and 5 days.

(a) Occludin staining



(b) Western blots

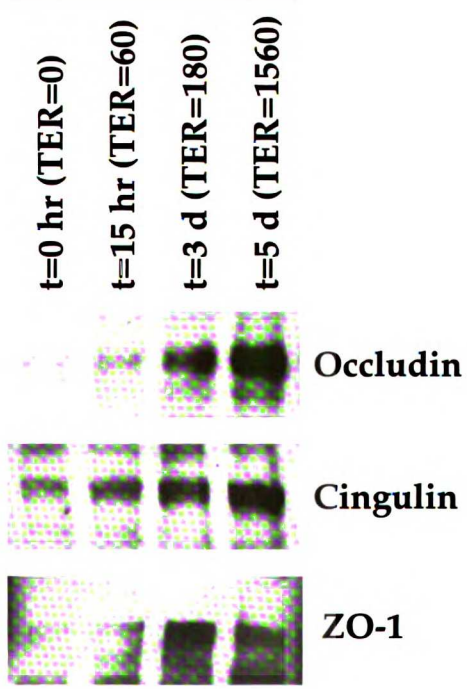


Fig. 1.2. A synthetic peptide (OCC2) corresponding to the entire second extracellular domain of chick occludin decreased TER of A6 cell monolayers. (a) Effect of various synthetic peptides on TER. OCC1 (corresponding to the entire first extracellular domain of chick occludin), OCC2 (corresponding to the entire second extracellular domain of chick occludin), and 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 (TER ~1000 Ωcm^2) that were still developing TER were used. Cell monolayers were treated with a final concentration of 5 μM OCC1/DMSO, 5 μM OCC2/DMSO, 5 μM OCC2(S)/DMSO, or DMSO alone (0.05%) for 66 hours and peptides were replenished every 24 hours. (n=6) for each condition. (b) Time course of OCC2 peptide on the TER of A6 cells that were still developing TER. Cell monolayers that had attained a TER of ~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 hrs. (c) Dose dependence of OCC2 peptide on the TER of A6 cell monolayers that were still developing TER. A6.2 cells were allowed to grow to confluency in normal medium and subsequently changed to low calcium medium for 18 hours. The low calcium medium was replaced with normal calcium medium containing a final concentration of 0.2, 0.5, 2, and 5 μM OCC2. The TER were measured after 4 days when control cell monolayers developed TER of ~3000 Ωcm^2. (n=3) for all concentrations tested. (d) Time course of OCC2 peptide action on the TER of steady-state A6 cell monolayers that were confluent for ~2 weeks (TER ~8000 Ωcm^2). Cells were treated with a final concentration of 5 μM OCC2 at t=0. Untreated monolayers were assayed in parallel as control. Peptides were replenished at 22 and 76 hrs. (n=3) for all conditions. (e) Dose dependence of OCC2 peptide on the TER of steady-state A6 monolayers (TER ~6000 Ωcm^2).

Cell monolayers were treated with a final concentration of 0.5, 1, 2, 5, or 10 μM OCC2. The TER was measured at 40 hours after peptide addition. The TER of each individual monolayer is plotted. Each concentration of OCC2 was assayed on duplicate monolayers. All error bars represent standard errors.

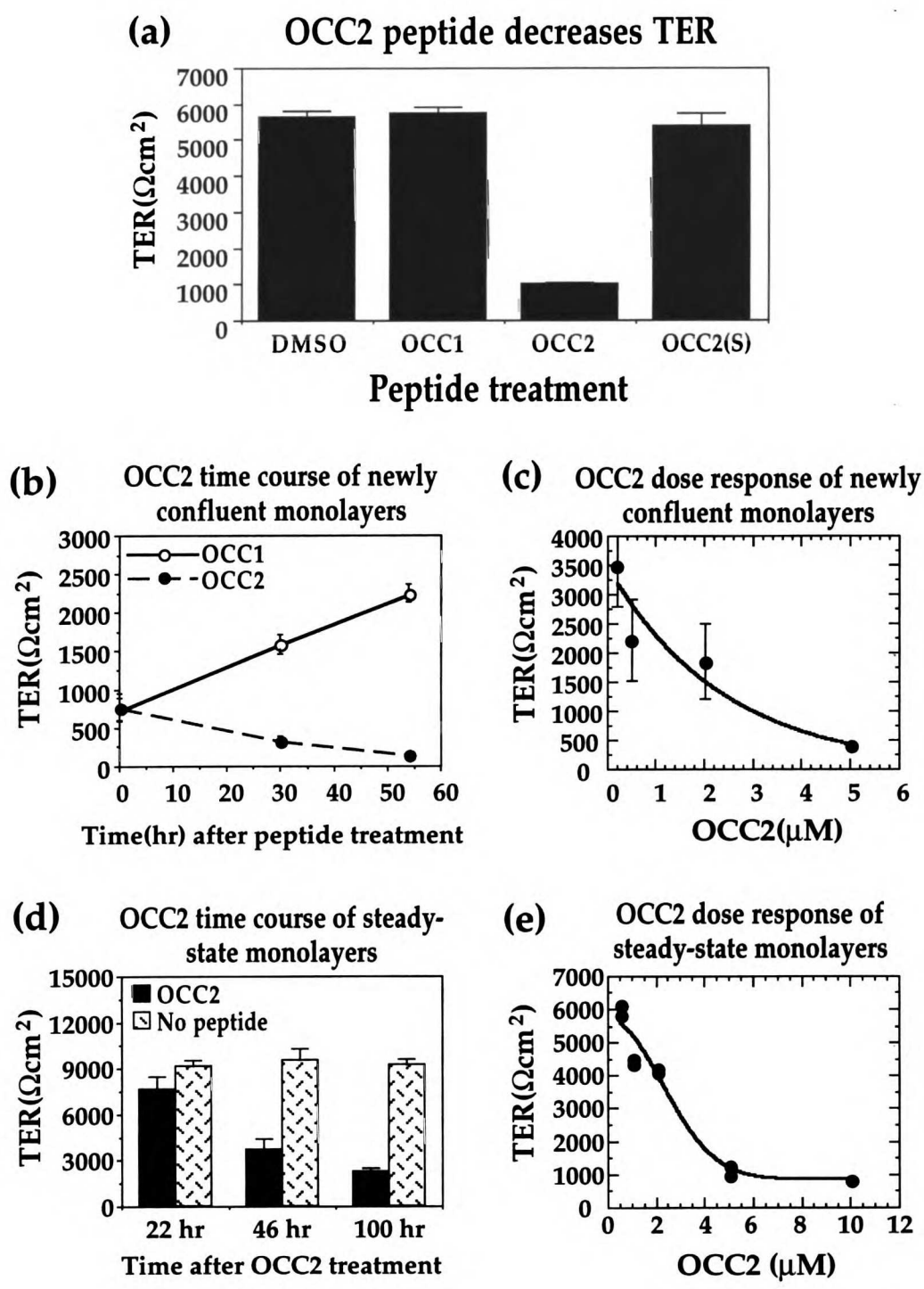

(a)
OCC2 peptide decreases TER
TER(Ωcm²)
7000
6000
5000
4000
3000
2000
1000
0
DMSO
OCC1
OCC2
OCC2(S)
Peptide treatment
(b)
OCC2 time course of newly confluent monolayers
TER(Ωcm²)
OCC1
OCC2
3000
2500
2000
1500
1000
500
0
0 10 20 30 40 50 60
Time(hr) after peptide treatment
(c)
OCC2 dose response of newly confluent monolayers
TER(Ωcm²)
4000
3500
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6
OCC2(μM)
(d)
OCC2 time course of steady-state monolayers
TER(Ωcm²)
OCC2
No peptide
15000
12000
9000
6000
3000
0
22 hr
46 hr
100 hr
Time after OCC2 treatment
(e)
OCC2 dose response of steady-state monolayers
TER(Ωcm²)
7000
6000
5000
4000
3000
2000
1000
0
0 2 4 6 8 10 12
OCC2 (μM)

Fig. 1.3. OCC2 increases 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-MW 3,000, and Texas red-conjugated neutral Dextran-MW 40,000. OCC1 was used as control peptide. A6 cell monolayers were allowed to grow until the TER reached ~1200 Ωcm^2. Cell monolayers were then treated with a final concentration of 5 μM OCC1 or OCC2 for 36 hours. The TER was measured and tracers flux assays were performed as described in Methods. The ratio of the absolute flux values were plotted as relative flux units. For all four tracers n=8 and error bars represent the standard error of the mean. (b-e) The relationship between tracer flux and TER changes induced by OCC2 treatment. Absolute flux values for individual A6 cell monolayer were plotted against TER of the same monolayer. (b) ^{3}H-mannitol, (c) ^{14}C-inulin, (d) neutral Dextran-MW 3,000 conjugated with Texas Red, and (e) neutral Dextran-MW 40,000 conjugated with Texas Red.

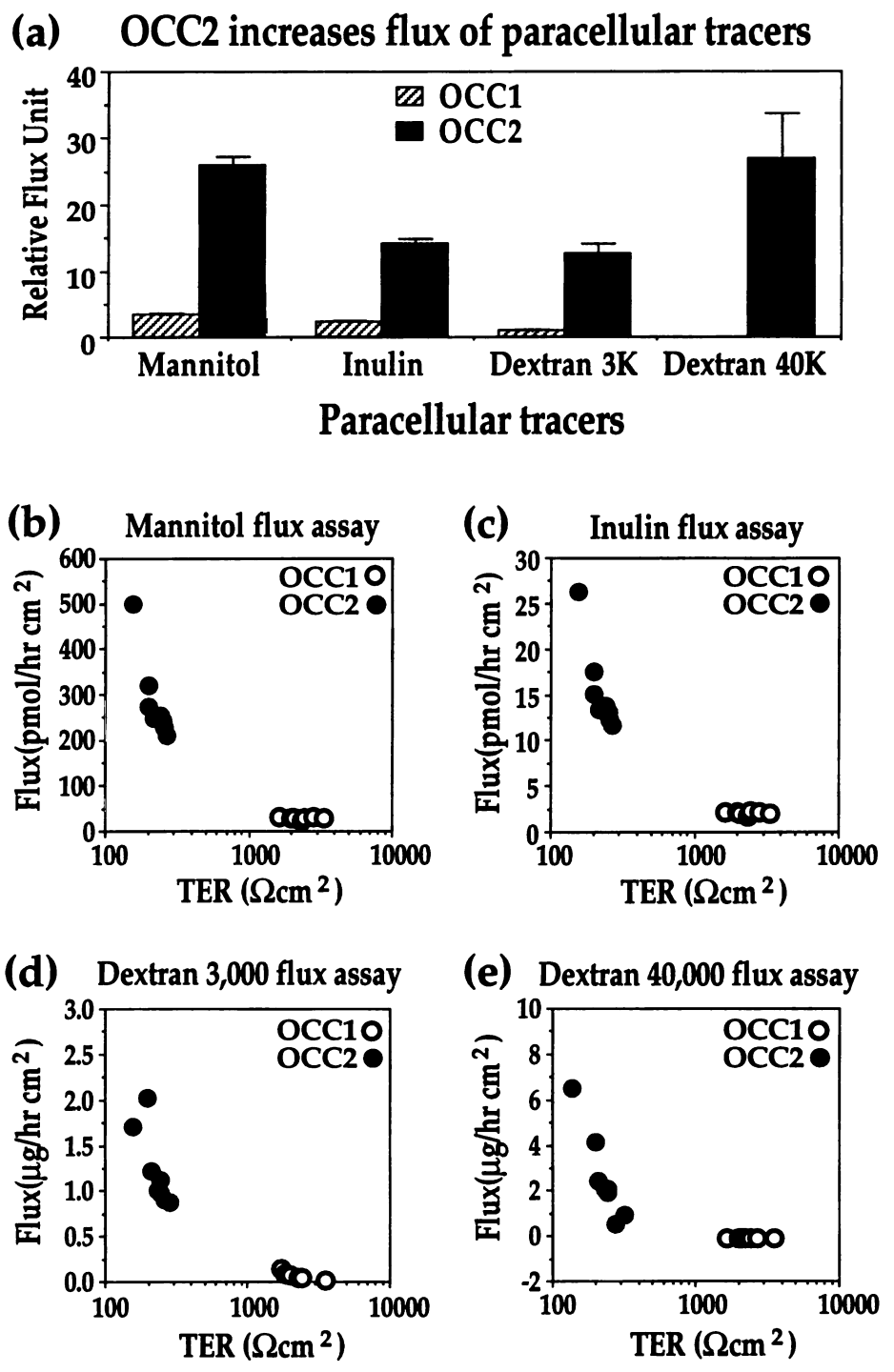

(a) OCC2 increases flux of paracellular tracers
Relative Flux Unit
40
30
20
10
0
OCC1
OCC2
Mannitol
Inulin
Dextran 3K
Dextran 40K
Paracellular tracers
(b) Mannitol flux assay
Flux(pmol/hr cm 2)
600
500
400
300
200
100
0
OCC1
OCC2
100
1000
10000
TER (Ωcm 2)
(c) Inulin flux assay
Flux(pmol/hr cm 2)
30
25
20
15
10
5
0
OCC1
OCC2
100
1000
10000
TER (Ωcm 2)
(d) Dextran 3,000 flux assay
Flux(μg/hr cm 2)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OCC1
OCC2
100
1000
10000
TER (Ωcm 2)
(e) Dextran 40,000 flux assay
Flux(μg/hr cm 2)
10
8
6
4
2
0
-2
OCC1
OCC2
100
1000
10000
TER (Ωcm 2)

Fig. 1.4. OCC2 reduces junctional staining of occludin but not ZO-1, cingulin, ZO-2, and E-cadherin. A6.2 cell monolayers from the paracellular tracers 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 ~2500 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 & b), ZO-1 (c & d), cingulin (e & f), ZO-2 (g & h), and E-cadherin (i & j). A6 cells were approximately 7-10 μM in diameter when grown on Transwell filters.

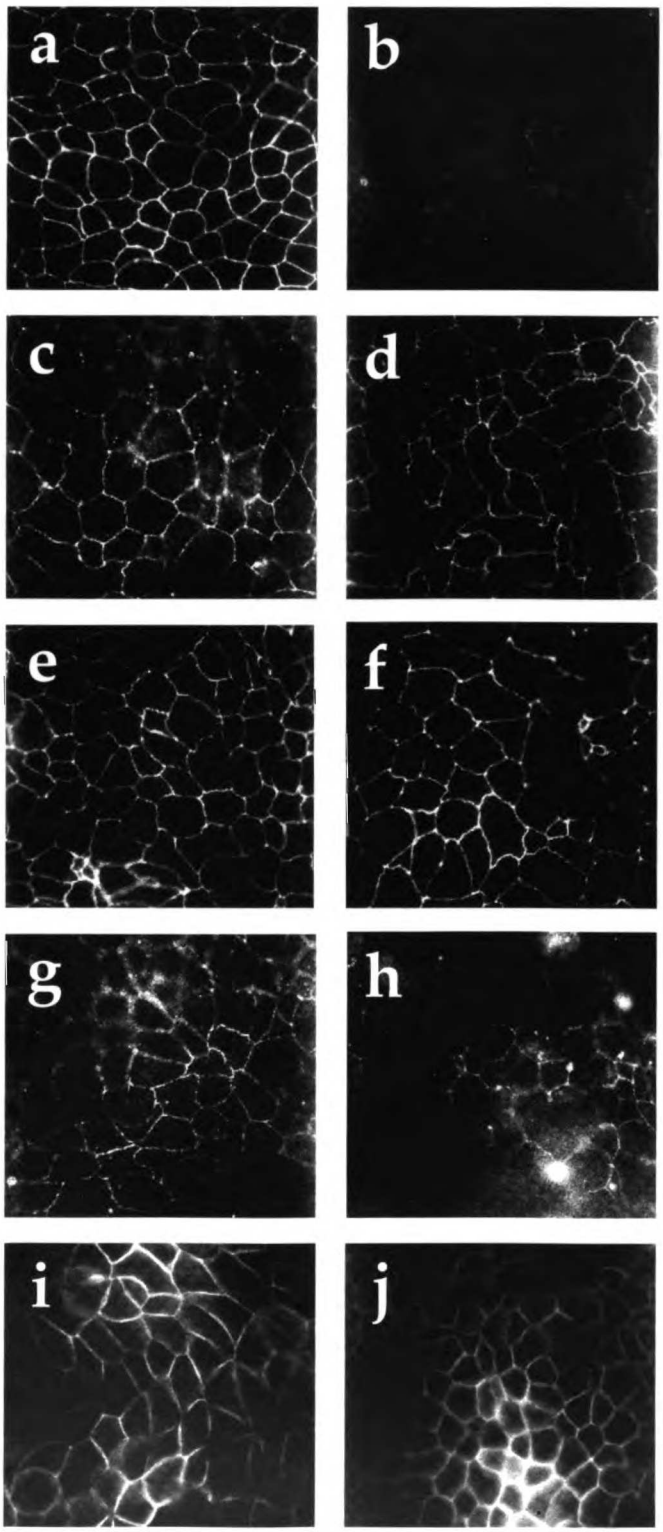

a
b
c
d
e
f
g
h
i
j

Fig. 1.5 OCC2 specifically decreases total cellular occludin levels. (a) Antigen blots of occludin, cingulin, ZO-1, ZO-2, and E-cadherin of total cell lysate from monolayers that were treated with OCC1, OCC2, or a DMSO solvent control. A6.2 cells were allowed to grow until the TER reached ~1000 Ωcm^2 and monolayers were treated with 10 μM of OCC1, 10 μM OCC2, or 0.1% DMSO for 24 hours. (b) Only the peptide that decreased the TER also caused a decrease in occludin levels. Antigen blots of occludin in A6.2 total cell lysates of monolayers that were treated with OCC1, unmodified OCC2(U), OCC2 or scrambled OCC2(S). A6.2 cells were allowed to grow to confluency in normal medium and subsequently changed to low calcium medium for 18 hours. 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 hours and cells were extracted for analysis at 4 days after initial peptide treatment. (c) Occludin synthesis was not reduced by OCC2 treatment. A6.2 cells that were either untreated or treated for either 2 hours or 22 hours with a final concentration of 5 μM OCC2 were subsequently labelled for 2.5 hours with ^{35}S-methionine followed by immunoprecipitation (IP) of occludin. (d) Turnover of occludin was enhanced by OCC2 treatment. A6.2 cells were metabolically labelled 20 hours with ^{35}S-methionine. At the end of the labelling period (t=0), fresh media (without ^{35}S-methionine) containing 10 μM OCC2 was added for 12 hours followed by immunoprecipitation (IP) of occludin. Untreated A6.2 cells were used in parallel as a control.

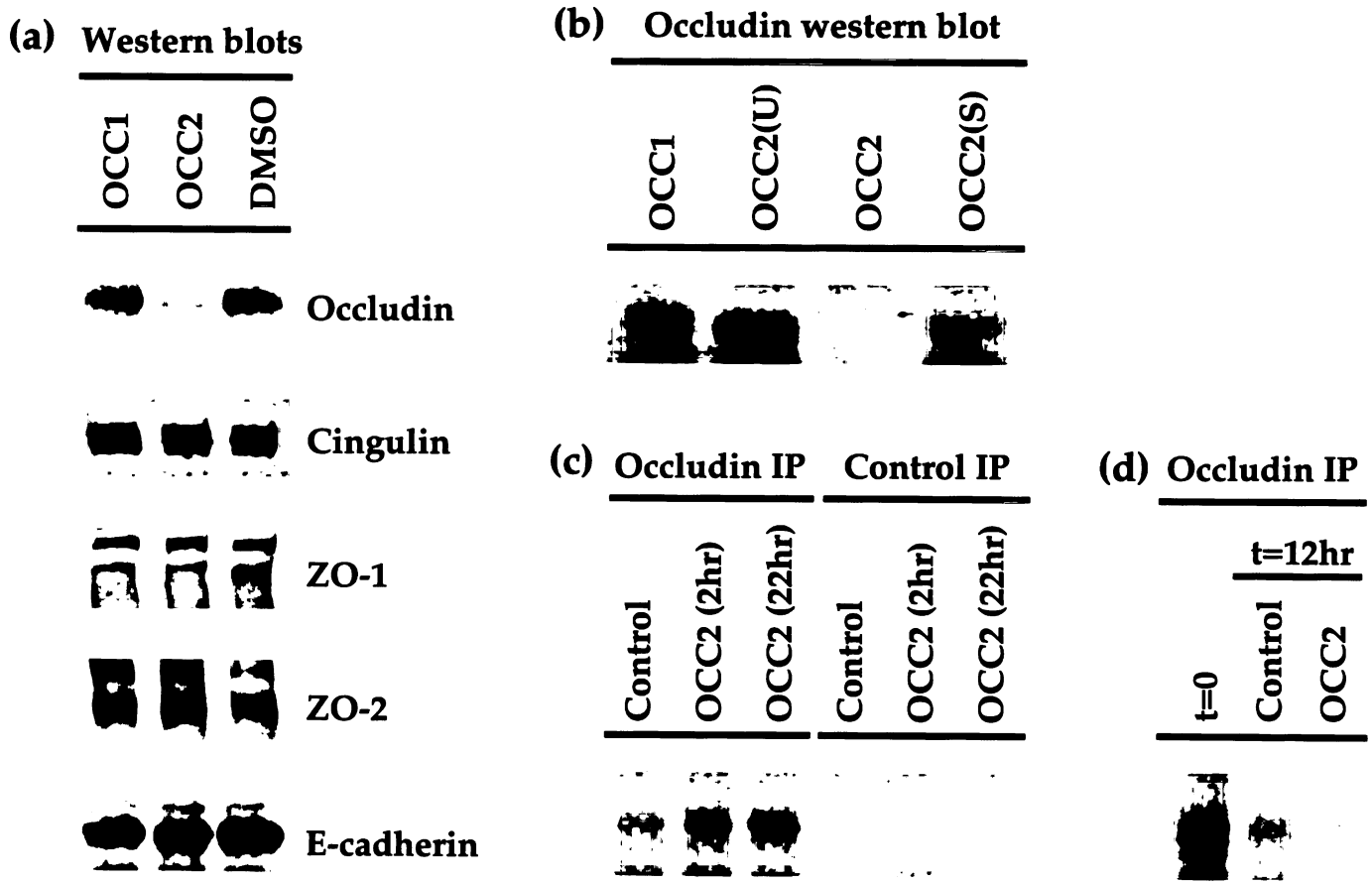
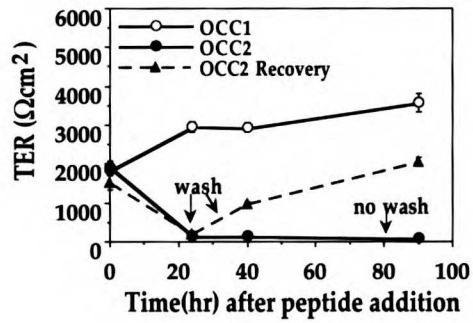

(a) Western blots
OCC1
OCC2
DMSO
Occludin
Cingulin
ZO-1
ZO-2
E-cadherin
(b) Occludin western blot
OCC1
OCC2(U)
OCC2
OCC2(S)
(c) Occludin IP
Control IP
Control
OCC2 (2hr)
OCC2 (22hr)
Control
OCC2 (2hr)
OCC2 (22hr)
(d) Occludin IP
t=12hr
t=0
Control
OCC2

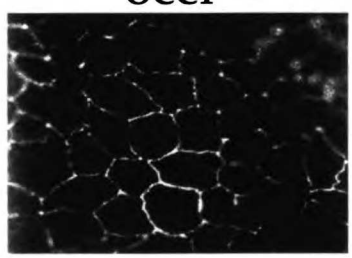
Fig. 1.6. The effects of OCC2 on TER and occludin accumulation are reversible. (a) Reversibility of TER after OCC2 removal. A6.2 cell monolayers that had TER of ~1700 Ωcm^2 were treated at t=0 with a final concentration of 5 μM OCC1 or OCC2. At t=24 hours, peptides were either replenished (OCC1 & OCC2) 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. A6.2 cell monolayers that had TER ~1900 Ωcm^2 were treated at t=0 with a final concentration of 5 μM OCC1 or OCC2. At t=24 hours, peptides were either replenished (OCC1 & OCC2) or removed (OCC2 recovery) from the cells. At t=60 hours, cells were processed for indirect immunofluorescence microscopy of occludin. OCC1 (TER ~2200 Ωcm^2), OCC2 (TER ~250 Ωcm^2), and OCC2 recovery (TER ~2300 Ωcm^2).

(a) TER recovers after OCC2 removal

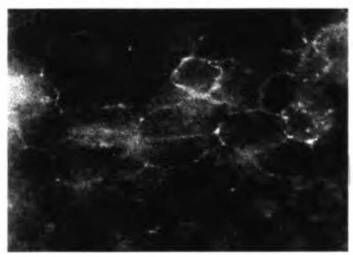


(b) **Occludin staining**

OCC1



OCC2



OCC2 recovery

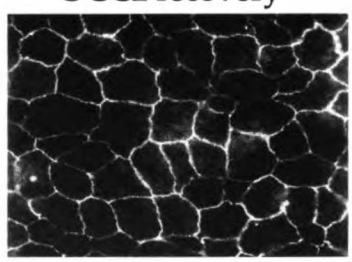
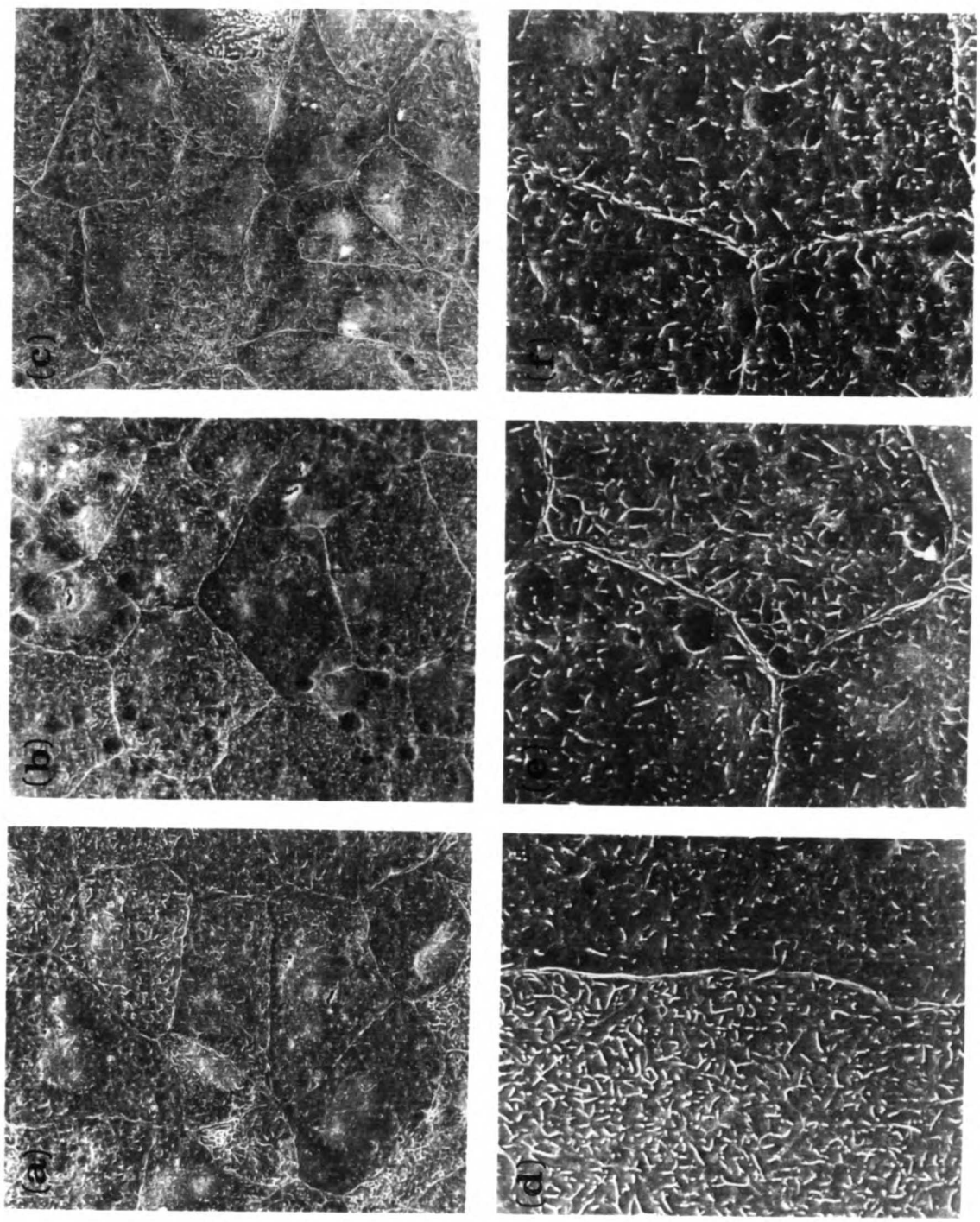


Fig. 1.7. OCC2 does not cause morphological changes in A6.2 cell monolayers as observed by scanning EM. Confluent A6.2 cells grown on polylysine-coated coverslips were treated 24 hours with a final concentration of (a & d) 10 μM OCC1, (b & e) 10 μM OCC2, and (c & f) 0.1% DMSO. Cells were then processed for scanning electron microscopy.


(c)
(f)
(b)
(e)
(a)
(d)

CHAPTER II

OCCLUDIN PHOSPHORYLATION IS A POTENTIAL MECHANISM FOR THE REGULATION OF OCCLUDIN FUNCTION AND TIGHT JUNCTION PERMEABILITY PROPERTIES

II.1 ABSTRACT

Occludin, an integral membrane protein of the tight junction, has been implicated in chapter I to play a role in the formation of the tight junction permeability barrier. In this second chapter, the basic biochemistry and regulation of occludin was investigated. Multiple forms of occludin (65-75 kD) was found in Madin-Darby canine kidney (MDCK) cells. The different forms of occludin appeared to be a result of differential phosphorylation. These multiple forms of occludin were different in the two strains of MDCK cells (I and II) that have different transepithelial electrical resistance (TER). A cluster of high molecular weight (HMW) forms of occludin (~72-75 kD in MDCK I cells and ~70-73 kD in MDCK II cells), which could be eliminated by phosphatase treatment, appeared to be the hyperphosphorylated forms of the low molecular weight (LMW) forms (~65-68 kD in both MDCK I and II cells). In both MDCK I and II cells, the LMW forms of occludin were constitutively expressed in the absence of cell-cell contacts, when the cells were maintained in low calcium (<20 μM) growth conditions. However, when the growth medium was switched to normal calcium to induce tight junction formation, the HMW forms of occludin were expressed. Triton X-100 (TX-100) fractionation of MDCK cells showed that the LMW forms of occludin were mostly TX-100 soluble whereas the HMW forms were TX-100 insoluble. Comparison of occludin staining in intact and TX-100 extracted MDCK cells showed that the majority of junction localized occludin ws present in the TX-100 insoluble pool. To confirm that the junction localized forms of occludin were the functional forms, a synthetic peptide that has been previously shown to deplete junctional occludin (see chapter I) was used. Treatment of MDCK cells with the peptide decreased TER and greatly reduced occludin

staining at tight junctions, as assessed by indirect immunofluorescence microcopy. Furthermore, the HMW forms of occludin were substantially decreased by the peptide. However, the LMW forms of occludin were only slightly reduced. This is consistent with the observations that after TX-100 extraction, the majority of the HMW and only a minority of the LMW forms of occludin contributed to the junction localized pool. In conclusion, the junction localized and functional forms of occludin appeared to be represented by the HMW forms. These results suggest that phosphorylation of occludin might be a mechanism by which occludin localization/function is regulated. Since the HMW forms of occludin appear to be the junctional localized and functional forms, the presence of different HMW forms in MDCK I and II cells that have different TER suggest a possible role of differential phosphorylation of occludin in regulating the TER.

II.2 INTRODUCTION

The tight junction of different epithelia have different permeability properties to ions and macromolecules (<15Å in diameter) (Madara & Dharmsathaphorn, 1985). For example, the epithelia of the blood-brain-barrier and blood-retinal-barrier have high transepithelial electrical resistance (TER) and are not permeable to macromolecules whereas the proximal tubule epithelium has low TER and is permeable to macromolecules (Arthur, Shivers & Bowman, 1987; Li & Poznansky, 1990; Milton & Knutson, 1990; Walker, MacKenzie, Wiggs, Montaner & Hogg, 1988; Wolburg et al., 1994). The different permeability properties of the tight junction in different epithelia appear to be important for the specific physiological functions of the epithelia.

The permeability of the tight junction has been proposed to correlate with its ultrastructure. In transmission electron microscopy (EM) of freeze fracture replicas, the tight junction is represented by rolls of anastomosing and branching networks of intramembrane fibrils that circumscribe continuously and completely the apices of the cells. These fibrils represent contact points of adjacent plasma membrane as seen in transmission EM of thin-sections and demarcate the region where paracellular tracers were excluded. When the tight junction fibrils of various epithelia were compared, the numbers and complexities of the organization of fibrils correlated with TER of the respective epithelium. Therefore, it was proposed that each fibril corresponds to a barrier and an increasing amount and complexity of fibrils would create epithelia of increasing TER, thus decreasing permeability (Claude, 1978; Claude & Goodenough, 1973).

However, the TER of an epithelium could also be changed without altering the number and organization of tight junction fibrils. The permeability of the tight junction of intestinal epithelium has been shown to increase by luminal glucose and amino acids in the absence of changes in the tight junction ultrastructure intestine (Atisook et al., 1990; Ballard et al., 1995). In addition, two strains of the Madin-Darby canine cell line (MDCK I and II) that have very different TER showed similar tight junction fibril structures (Stevenson, Anderson, Goodenough & Mooseker, 1988). Therefore, it was proposed that the permeability of the tight junction can be regulated within the fibrils.

The tight junction fibrils have been showed to be regulated in various physiological processes such as leukocyte transmigration across an endothelium (Nash, Stafford & Madara, 1987; Nash, Stafford & Madara, 1988). During these dynamic processes, the tight junction permeability barrier is temporarily disrupted but subsequently resealed. The resealing process is relatively quick, usually within an hour, suggesting that resealing is accomplished by assembly of preexisting elements rather that resynthesis of new proteins. In addition, the tight junction fibrils are found to be present after the the permeability barrier was disrupted, supporting the notion that the tight junction sealing element is only temporarily perturbed or uncoupled. To begin to understand the dynamics of the tight junction, it is important to understand the regulation of the sealing element of the tight junction permeability barrier.

The tight junction integral membrane protein, occludin, has been shown to localize to the tight junction fibrils by transmission EM of immunogold labelled freeze-fracture replicas (Fujimoto, 1995). It has also been implicated to participate in the formation of the actual tight junction permeability barrier

(see chapter I). Therefore, occludin is an attractive candidate for the regulation of the permeability properties of the tight junction that has been proposed to reside in the fibrils. To examine the regulation of occludin and its potential role in regulating tight junction permeability, the basic biochemistry of occludin in two strains of MDCK cells (I and II) that have different TER has been studied.

II.3 METHODS

Cell Culture, Calcium Switch Assay and Measurements of Transepithelial Electrical Resistance

Madin-Darby kidney epithelial cell line MDCK (strains I and II) were gifts from Kai Simons. Cells were grown on Costar Transwell filters (Fisher, Santa Clara, CA) in MEM medium supplemented with 5% fetal bovine serum and maintained at 37°C and 5% CO_2. For the calcium switch assay, cells were initially grown in normal calcium growth medium to confluency and subsequently changed to low calcium medium (calcium-free MEM medium supplemented with 10% dialyzed fetal bovine serum) for 48 hours. At the end of the low calcium switch, cell monolayers were replenished with normal calcium medium and the formation of tight junctions was monitored by the generation of transepithelial electrical resistance (TER). The TER was measured directly in normal growth media in Transwell wells. A short 4 uA 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. The 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 monolayers.

Triton X-100 Extraction, Immunofluorescence Microscopy and Antigen Blotting

MDCK cells were grown on Costar Transwell filters (Fisher, Santa Clara, CA) and the TERs were measured before preparation of cells for indirect immunofluorescence microscopy and SDS-PAGE. For Triton X-100 (TX-100) extractions, cells were incubated in TX-100 extraction buffer (0.5% TX-100, 5mM EDTA, and 0.15 M NaCl) for 3 hours at 4°C. The TX-100 insoluble fraction of cells that was left attached onto the Transwell filters were rinsed two times in 0.15 M NaCl and subsequently processed for immunofluorescence microscopy and SDS-PAGE in parallel with non-extracted cells. For immunofluorescence, cells were fixed on filters 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% non-fat dry milk in 0.5% Triton X-100, 5 mM EDTA, 0.15 M NaCl, 20 mM HEPES , and pH 7.0) before incubation with primary antibodies. Rabbit anti-occludin antibodies were raised against a GST fusion protein of the cytoplasmic domain of chick occludin (255-510 a.a.). FITC-conjugated secondary antibodies were obtained from Molecular Probes, Inc. (Eugene, OR). For SDS-PAGE, cells were 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 dithiothreitol) containing 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). SDS-PAGE samples were boiled for 10 min, and cooled to room temperature before addition of iodoacetic acid to a final concentration of 125 mM. All SDS-PAGE are performed with 4-15% gradient gels. Western blots for occludin were done using the same primary antibodies as for immunofluorescence microscopy. Rabbit anti-ZO-1 (#10153) and anti-ZO-2 (#9989) antibodies were gifts from D. Goodenough. Rabbit anti-cingulin antibodies were gifts from S.

Citi. Secondary antibodies conjugated with HRP (Biorad) were developed by enhanced chemiluminescence (Amersham Life Sciences, Inc., Arlington Heights, IL). For blot competition, 20 μg/ml of the antigen (GST-occludin cytoplasmic tail) or 20 μg/ml GST were added to the blots along with anti-occludin antibodies.

Metabolic-labelling, Immunoprecipitations, Phosphatase Treatment and Fluorography

MDCK cells were grown on 75 mm Transwell filters (Fisher, Santa Clara, CA). To study the phosphorylation of occludin, each monolayer was labelled with 100 μCi ^{32}P-orthophosphate in phosphate-free media (supplemented with 5% fetal bovine serum) for 22 hours. Each cell monolayer was extracted for immunoprecipitation immediately at the end of the labelling period 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 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). Immunoprecipitations were performed with Protein-A Sepharose (Sigma, Santa Clara, CA) in the presence of anti-occludin (see above) or pre-immune serum. Immunoprecipitates were washed four times in immunoprecipitation buffer before extraction for SDS-PAGE. Phosphatase treatments of occludin were done by incubating occludin immunoprecipitates (washed four times in immunoprecipitation buffer) with 50 μg of potato acid phosphatase (CalBiochem, San Diego, CA) in 1 ml 10

mM HEPES, pH 6.0, for 2 hours at 25 ^{o}C. Phosphatase treated immunoprecipitates were directly extracted in sample buffer for SDS-PAGE. For metabolic labelling of occludin, each monolayer was incubated with 1 mCi ^{32}S-methionine in methionine-free media (supplemented with 5% fetal bovine serum) for 22 hours. Immunoprecipitation and sample preparation for SDS-PAGE were performed as above. Polyacrylamide gels were fixed with 50 % MEOH and 10 % HOAc for one hour and incubated in Amplify (Amersham Life Sciences, Inc., Arlington Heights, IL) for 45 min before dried under vacuum. Dried gels were exposed to Hyperfilm-MP (Amersham Life Sciences, Inc., Arlington Heights, IL) at -80^{o}C.

Peptides Synthesis and Peptide Treatment of Cells

Peptides OCC1 (44 a.a. = DYGYGLGGAYGTGLGGFYGSNYYGSGLSYSYGY GGYYGGVNQRT) and OCC2 (44 a.a. = GVNPQAQMSSGYYYSPLLAMCSQAY GSTYLNQYIYHYCTVDPQE) correspond to the entire first and second putative extracellular domains of chick occludin, respectively. OCC2 was modified by covalent linkage to acetamidomethyl groups at the two cysteine residues (underlined) to prevent formation of disulfide bond(s). Peptides were prepared as 10 mM stock solutions in DMSO and were added to both sides of the Transwell bathing wells at a final concentration of 10 μM for 24 hours. All peptides were synthesized by the Microchemistry Core Facility at the Memorial Sloan-Kettering Cancer Center.

II.4 RESULTS

Multiple Forms of Occludin in MDCK Cells

To examine the possible role of occludin in dictating TER, the basic biochemistry of occludin including expression levels and localization was studied in two strains of MDCK cells that have different TER (~10,000 Ωcm^2 for MDCK I cells and ~50 Ωcm^2 for MDCK II cells). Indirect immunofluorescence microscopy for occludin showed that occludin was present at the tight junction in both MDCK I and II cells and the levels between the two strains were not significantly different (see Fig. 2.3b & 2.4a). The result suggested that the amount of occludin localization at the tight junction did not account for the differences in the TER between MDCK I and II cells.

Western blotting for occludin of MDCK cell lysates showed multiple bands at MW ~65-75 kD (Fig. 2.1a). In MDCK I cells , two prominent clusters of bands at ~65-68 kD (LMW) and ~72-75 kD (HMW) were present. In MDCK II cells, two prominent clusters of bands at ~65-68 kD (LMW) and ~70-73 kD (HMW) were present. To confirm that the multiple bands were antigenically related to chick occludin, antigen blot analysis were performed in the presence or absence of excess amount of the antigen (a GST fusion protein containing the cytoplasmic residues 255-510 of chick occludin) or GST only as control (Fig. 2.b). Binding to both the LMW and HMW bands was competed by the antigen suggesting that each was specific to the antibodies and immunologically related to chick occludin cytoplasmic domain. However, the overall amounts of occludin in the two strains of MDCK cells were not significantly different suggesting that it was not the level of expression of

occludin that was responsible for the differences in TER in the two strains of MDCK cells.

These results suggested that neither the localization nor the expression levels of occludin could account for the great difference in TER of MDCK I and II cells. However, the presence of the various HMW forms in the two strains of cells might have a potential role in regulating TER.

High MW Forms of Occludin Correlates with Tight Junction Formation

To examine the role of the multiple forms of occludin, antigen blot analyses for occludin were performed from lysates of cells that had formed or were devoid of cell-cell contacts. When a confluent monolayer of MDCK cells were incubated in low calcium growth medium for 48 hours (see methods), intercellular junctions were essentially absent and the cells mainly expressed the LMW (~ 65-68 kD) forms of occludin (Fig. 2.1c). Both strains of MDCK cells barely expressed their respective HMW forms of occludin in the absence of cell contacts. After tight junctions formation was induced by switching the low calcium medium to normal calcium medium for 48 hours (TER ~3300 Ωcm^2 for MDCK I cells and ~30 Ωcm^2 for MDCK II cells), both the LMW and HMW forms of occludin were expressed. The patterns of bands that were induced after the calcium switch were essentially identical to those of control MDCK cells that were continuously maintained in normal calcium in a parallel experiment, demonstrating that the HMW bands induced by the calcium switch were the normally expressed forms of occludin. The differential expression of LMW and HMW forms of occludin in the presence or absence of functional tight junctions suggested that the multiple forms have differential roles. The HMW forms of occludin that correlated with the

induction of TER suggested their participation in the formation of the actual tight junction permeability seal.

High MW Forms are the Hyperphosphorylated Forms of Occludin

The multiple forms of occludin in MDCK cells could be due to either the presence of isoforms or various post-translational modifications such as phosphorylation. To examine whether phosphorylation contributed to the existence of these multiple forms of occludin, metabolic labelling experiments with ^{32}P-orthophosphate were performed. Immunoprecipitations of occludin showed that both the LMW and HMW forms of occludin were phosphorylated in MDCK I and II cells (Fig. 2.2). These multiple phosphorylated bands were not co-immunoprecipitated products of occludin because the cells were extracted directly in 1% SDS before immunoprecipitation to eliminate protein-protein interactions and ensure immunoprecipitation of only occludin protein. Furthermore, the HMW forms of occludin appeared to be hyperphosphorylated because the ratio of phosphorylation of the HMW forms to the LMW forms were much greater than that of ^{35}S-methionine labelled occludin (Fig. 2.2). In addition, phosphatase treatment that removed most of the ^{32}P activities from the HMW forms also eliminted the ^{35}S-labelled HMW forms. Antigen blot analyses for occludin of phosphatase treated and untreated ^{32}P-occludin immunoprecipitates confirmed that the HMW forms were efficiently eliminated by phosphatase treatment (Fig. 2.2). Therefore, it appeared that the HMW forms were the hyperphosphorylated forms of occludin. The elimination of the HMW forms by phosphatase treatment suggested that the HMW forms were the hyperphosphorylated forms of the LMW forms.

High MW Forms of Occludin are Triton X-100 Insoluble

To determine whether the biophysical properties of the various forms of occludin in MDCK cells were different, Triton X-100 (TX-100) solubility of occludin were analyzed. Incubation of confluent monolayers of MDCK (I and II) cells with 0.5% TX-100 (see methods) showed that the HMW forms of occludin were mostly fractionated into the TX-100 insoluble fraction whereas the LMW forms of occludin were mostly TX-100 soluble (Fig. 2.3a). The different TX-100 solubility of the LMW and HMW forms of occludin suggested that there were two cellular pools of occludin. The TX-100 insoluble pool of occludin consisted of the HMW forms and only a small fraction of the LMW forms whereas the TX-100 soluble pool consisted of the majority of the LMW forms.

To confirm that the TX-100 insoluble HMW forms of occludin were the hyperphosporylated forms, ^{32}P-orthophosphate labelled occludin was compared in intact or TX-100 extracted MDCK (I and II) cells. Immunoprecipitates of occludin showed that the hyperphosphorylated forms of occludin were all retained in the TX-100 insoluble fraction (Fig. 2.3a). The correlation between hyperphosphorylation and TX-100 insolubility suggested that phosphorylation might be a mechanism of regulating the incorporation of occludin into the TX-100 pool.

Triton X-100 Insoluble Occludin Represents the Tight Junction Localized Forms

To determine the subcellular localization of the multiple forms of occludin, indirect immunofluorescence microscopy for occludin were performed in intact and TX-100 extracted MDCK (I and II) cells. Occludin was localized to the tight junction in both intact and TX-100 extracted cells (Fig. 2.3b). However, the TX-100 soluble pool of occludin was not detected by immunofluorescence, perhaps lost during fixation procedures. In addition, the intensity of occludin stainings in both the intact and TX-100 extracted cells was similar, suggesting that the TX-100 insoluble pool of occludin represented the junctional localized pool. These results suggested that the HMW forms and only a small fraction of the LMW forms of occludin were the junction localized species of occludin. Hence, the majority of the LMW forms of occludin appeared to be functionally irrelevant for the formation of the tight junction seal.

High MW Forms of Occludin Participate in the Formation of the Tight Junction Permeability Barrier

To confirm that the junction localized HMW forms of occludin indeed participated in the formation of the functional tight junction permeability barrier, a synthetic peptide OCC2 (corresponding to the second extracellular domain of chick occludin) was used to deplete junction localized occludin. A control peptide, OCC1, and solvent control, DMSO, were used in parallel experiments. Treatment of MDCK cells with OCC2 peptide (TER decreased from ~10,000 to ~200 Ωcm^2 for MDCK I cells but did not changed in MDCK II cells, TER ~50 Ωcm^2) greatly reduced the HMW forms of occludin (Fig. 2.4a). However, the LMW forms were only slightly decreased. As a control for OCC2 selectivity to influence only occludin, two peripheral membrane

proteins of the tight junction, ZO-1 and cingulin, were also blotted and were shown to be unaltered. Indirect immunofluorescence microscopy for occludin confirmed that junctional occludin in both MDCK I and II cells were greatly reduced by OCC2 (Fig. 2.4b). These results confirmed that the depletion of occludin at the tight junction correlated with the perturbation of the tight junction permeability barrier. The depletion of the majority of the HMW forms and only a minority of the LMW forms of occludin was consistent with the TX-100 extraction results and supported the hypothesis that the HMW forms and only a small fraction of the LMW forms of occludin localized to the tight junction and participated in the formation of the permeability barrier.

II.5 DISCUSSION

The basic biochemistry of occludin was studied in two strains of Madin-Darby canine cell line (MDCK I and II) that had different transepithelial electrical resistance (TER). Antigen blot analyses showed that there were multiple forms of occludin in MDCK cells. In the absence of cell-cell contacts, a cluster of low molecular weight (LMW) bands (~ 65-68 kD) were present in both MDCK I and II cells. Upon induction of junction formation, in addition to the LMW bands, a cluster of high molecular weight (HMW) bands (~72-75 kD for MDCK I cells and ~70-73 kD for MDCK II cells) were also expressed. The HMW bands, which could be eliminated by phosphatase treatment, appeared to be the hyperphosphorylated species of occludin. These hyperphosphorylated HMW bands were Triton X-100 (TX-100) insoluble whereas most of the LMW bands were TX-100 soluble. The TX-100 insoluble pool of occludin appeared to represent the tight junction localized forms of occludin. In addition, OCC2 peptide that depleted junctional occludin also reduced the HMW forms of occludin but only slightly decreased the LMW forms. In conclusion, the selective localization of the hyperphosphorylated forms of occludin at the tight junction suggested a role of phosphorylation in occludin targeting/assembly/function. Since the HMW forms of occludin were different in the two strains of MDCK cells that had different TER, it is possible that differential phosphorylation of occludin contributes to the regulation of the TER.

The correlation between the expression of the hyperphosphorylated forms of occludin and the formation of the tight junction permeability barrier suggested a role of phosphorylation in the regulation of occludin function. Phosphorylation of occludin could stabilize occludin-protein interactions and

result in assembly of occludin at the tight junction. Fig. 2.5 shows a hypothetical model for the regulation of occludin by phosphorylation. Two main pathways could be used; one represents recruiting occludin from the plasma membrane while the other pathway represents recruiting occludin from intracellular vesicles. Three possible mechanisms could be involved in recruiting occludin from the plasma membrane. Phosphorylation of occludin could stabilize the assembly of occludin in the formation of a continuous barrier in the plane of the plasma membrane, an event that resembles the polymerization of occludin (A). Alternatively, phosphorylation of occludin could stabilize the binding of occludin to its extracellular binding protein to form the functional seal of the tight junction (B). Furthermore, phosphorylation of occludin could stabilize interaction of occludin cytoplasmic domain to cytoplasmic tight junction proteins such as ZO-1 and ZO-2 to allow incorporation of occludin into the tight junction (C). The second pathway that involves recruiting occludin from intracellular vesicles represents a mechanism similar to docking of occludin containing vesicles to the tight junction, perhaps via interaction with the cytoplasmic proteins of the tight junction (D). Each or a combination of the above possible mechanisms could allow regulation of occludin in the formation of the tight junction. The possibility that occludin is regulated via phosphorylation provides future directions in understanding the assembly of occludin at the tight junction.

The regulation of occludin localization by phosphorylation could allow dynamic control of the tight junction permeability barrier. It was suggested in chapter I that the tight junction permeability barrier could be regulated by localization and delocalization of occludin. However, this could be accomplished either by degradation/resynthesis of occludin or by

assembly/disassembly of occludin, perhaps via phosphorylation. The potential rapid regulation of the tight junction barrier provided by phosphorylation/dephosphorylation of occludin is consistent with the observation that tight junctions could be open and reseal within a short time (~1 hour) during dynamic physiological processes such as leukocyte transmigration across an endothelium. Therefore, it is possible that the tight junction permeability barrier is regulated by assembly/disassembly of occludin at the tight junction via phosphorylation/dephosphorylation of occludin.

The different HMW forms of occludin that were expressed in MDCK I and II cells provided insights into the regulation of TER, a parameter for the permeability properties of the tight junction. The paradox is that despite the very different TER, both MDCK I and II cells had a similar tight junction fibril number and organization. Since occludin has been implicated in the formation of the tight junction seal, it is a candidate for the regulation of the tight junction sealing properties. Interestingly, the results of this study were consistent with the paradox that occludin localization at the tight junction was similar in MDCK I and II cells. However, there were different HMW forms of occludin present in MDCK I and II cells. These HMW forms represented the junctional localized and functional forms of occludin, and therefore, were the structural components for the formation of the physical tight junction seal. The presence of the different HMW forms in MDCK I and II cells could result in the formation of tight junction seals that had different permeability properties which might help to explain the difference in TER of MDCK I and II cells.

The different hyperphosphorylated forms of occludin in MDCK I and II cells suggested that the TER might be regulated via differential occludin

phosphorylation. Fig. 2.6 shows a hypothetical model for the regulation of TER via differential phosphorylation of occludin. The differences in hyperphosphorylation of occludin in MDCK I and II cells could reflect the presence of different occludin kinases and phosphatases in the two strains of MDCK cells. Alternatively, the differences in TER of MDCK I and II cells could be due to a gain or a loss of phosphorylation sites that influenced the TER. In conclusion, our results suggested that differential phosphorylation of occludin might be a potential mechanism to regulate tight junction permeability properties.

II.6 LEGENDS AND FIGURES

Fig. 2.1. Multiple forms of occludin are present in MDCK I and II cells. (a) Antigen blots of total cell lysates. Two clusters of bands, low MW (~60-65 kD in both MDCK I and II cells) and high MW (~72-75 kD in MDCK I cells and ~70-73 kD in MDCK II cells), were present. (b) The multiple bands were immunologically related to chick occludin. Western blot of total cell lysates of MDCK I and II cells were performed in the presence or absence of 50 μg/ml of the antigen (GST/Occ, a GST fusion protein containing the cytoplasmic tail of chick occludin) or GST alone. (c) Expression of the high MW bands correlated with the formation of tight junctions, whereas expression of the low MW bands were constitutive in both the presence and absence of cell-cell contacts. Confluent MDCK monolayers that were incubated in low calcium medium for 48 hours (t=0) were induced to form intercellular junctions by switching to normal calcium medium for 2 days (t=2d). Positive (+) control were monolayers that were grown in parallel but had not been subjected to the calcium switch manipulation.

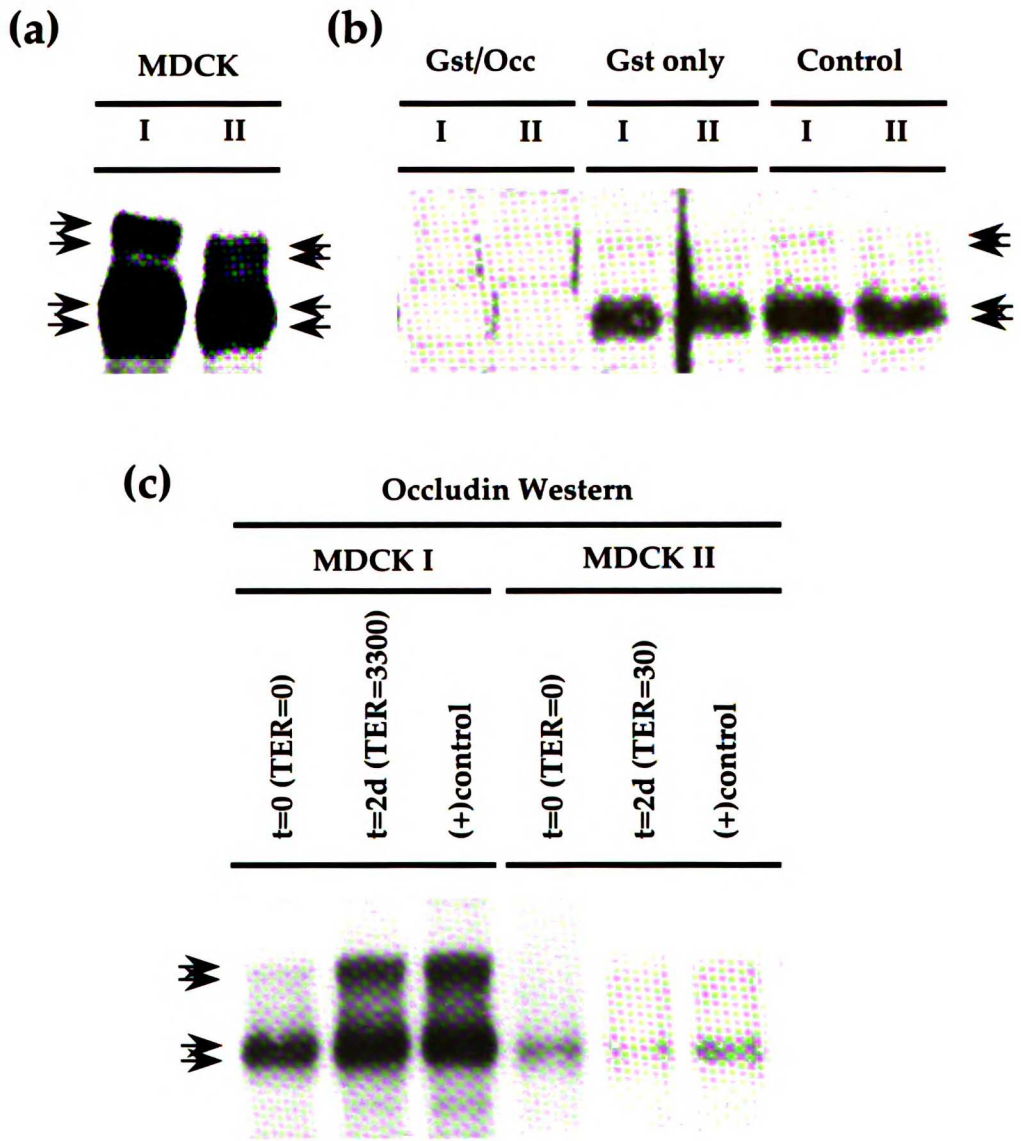

(a)
MDCK
I
II
(b)
Gst/Occ
Gst only
Control
I
II
I
II
I
II
(c)
Occludin Western
MDCK I
MDCK II
t=0 (TER=0)
t=2d (TER=3300)
(+)control
t=0 (TER=0)
t=2d (TER=30)
(+)control

Fig. 2.2. The high MW forms of occludin are hyperphosphorylated species of occludin. Autoradiograph (^{32}P), fluorograph (^{35}S), and antigen blots of occludin immunoprecipitates that were treated (+) or untreated (-) with phosphatase. Phosphatase treatment eliminated the high MW forms of both ^{32}P-labelled and ^{35}S-labelled occludin. In addition, antigen blot analyses of the ^{32}P-labelled immunoprecipitates showed that dephosphorylation was sufficient to eliminate the high MW forms of occludin. The low MW bands of occludin were masked by the co-migrated IgG band.

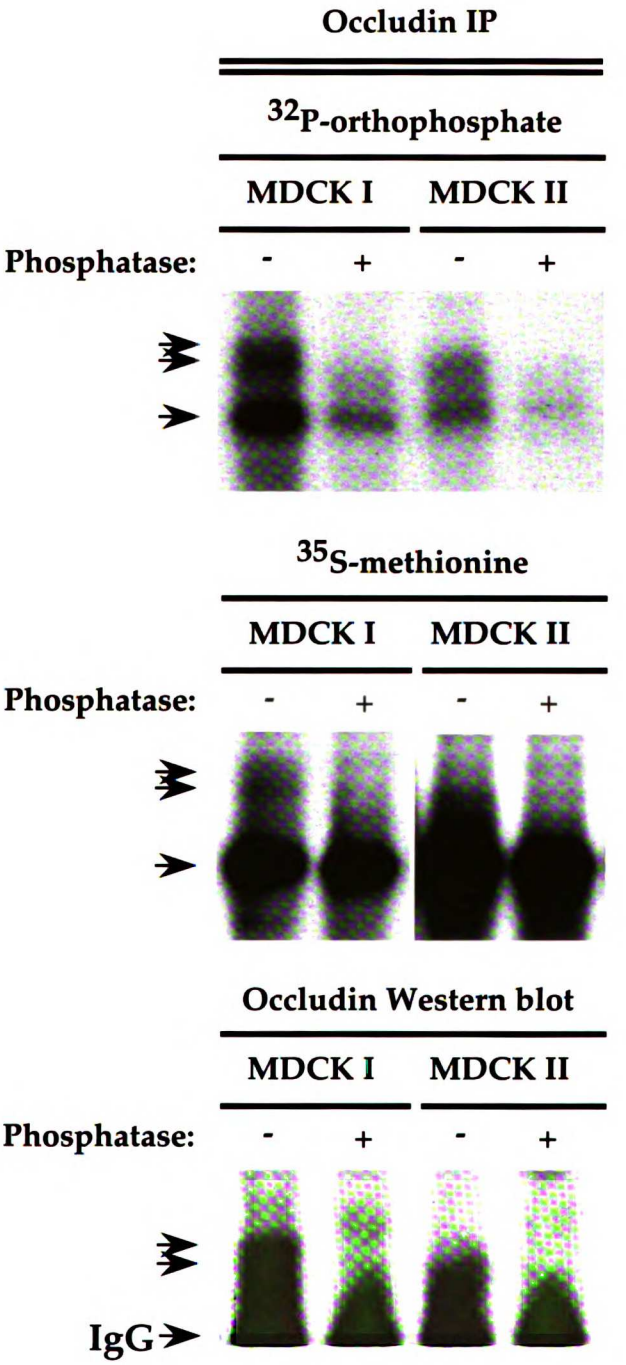
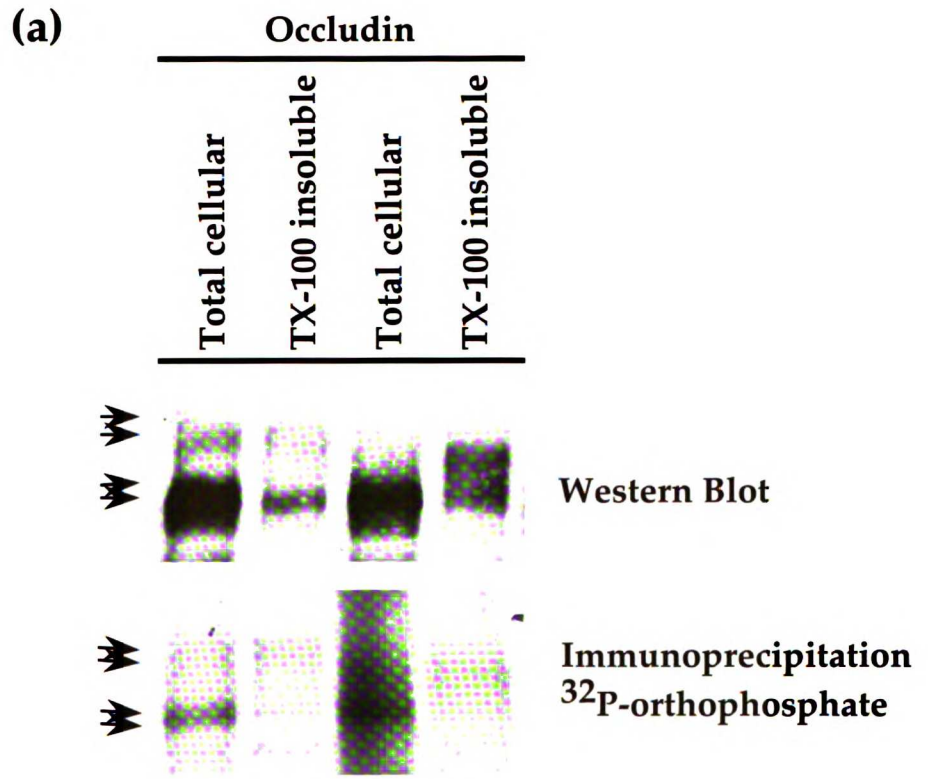

Occludin IP
32P-orthophosphate
MDCK I
MDCK II
Phosphatase:
- + - +
35S-methionine
MDCK I
MDCK II
Phosphatase:
- + - +
Occludin Western blot
MDCK I
MDCK II
Phosphatase:
- + - +
IgG

Fig. 2.3. The high MW forms of occludin are Triton X-100 (TX-100) insoluble whereas the low MW forms are mostly TX-100 soluble. (a) Antigen blot analyses of total cell lysate and TX-100 insoluble fractions of MDCK cells. The high MW forms and a small fraction of the low HW forms of occludin were TX-100 insoluble. The majority of the low MW forms of occludin were TX-100 solublized. Autoradiography of ^{32}P-occludin immunoprecipitates from intact or TX-100 extracted cells were performed in parallel to confirmed that all the hyperphosphorylated high MW forms of occudin were TX-100 insoluble. (b) Indirect immunofluorescence microscopy for occludin showed that the TX-100 insoluble pool of occludin represented the junction localized species of occludin.


(a)
Occludin
Total cellular
TX-100 insoluble
Total cellular
TX-100 insoluble
Western Blot
Immunoprecipitation
32P-orthophosphate

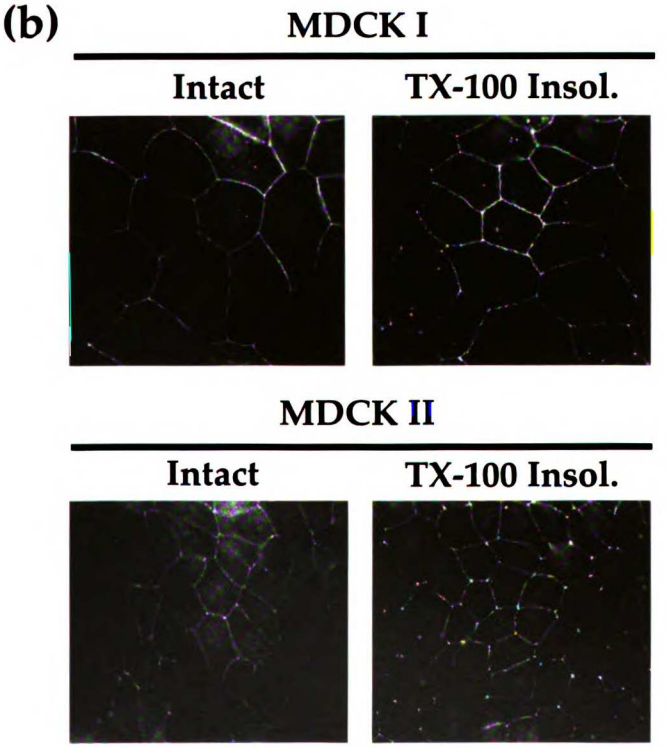
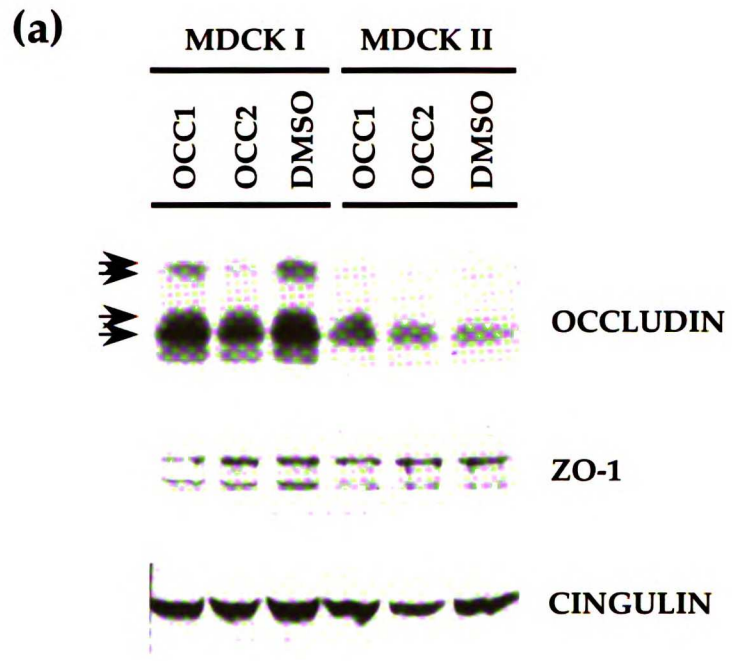

(b)
MDCK I
Intact
TX-100 Insol.
MDCK II
Intact
TX-100 Insol.

Fig. 2.4. Depletion of junctional occludin by OCC2 peptide correlates with a substantial decrease in the high MW forms of occludin but only a slight reduction in the low MW forms. (a) Antigen blot analyses of total cell lysates of OCC1 (control peptide), OCC2, and DMSO treated MDCK I and II cells. OCC2 peptide decreased the high MW forms and to a lesser extent the low MW forms of occludin without altering the levels of ZO-2 and cingulin. (b) Indirect immunofluorescence microscopy for occludin of OCC1 and OCC2 treated MDCK cells. OCC2 greatly depleted occludin from the tight junction in both MDCK I and II cells.


(a)
MDCK I
MDCK II
OCC1
OCC2
DMSO
OCC1
OCC2
DMSO
OCCLUDIN
ZO-1
CINGULIN

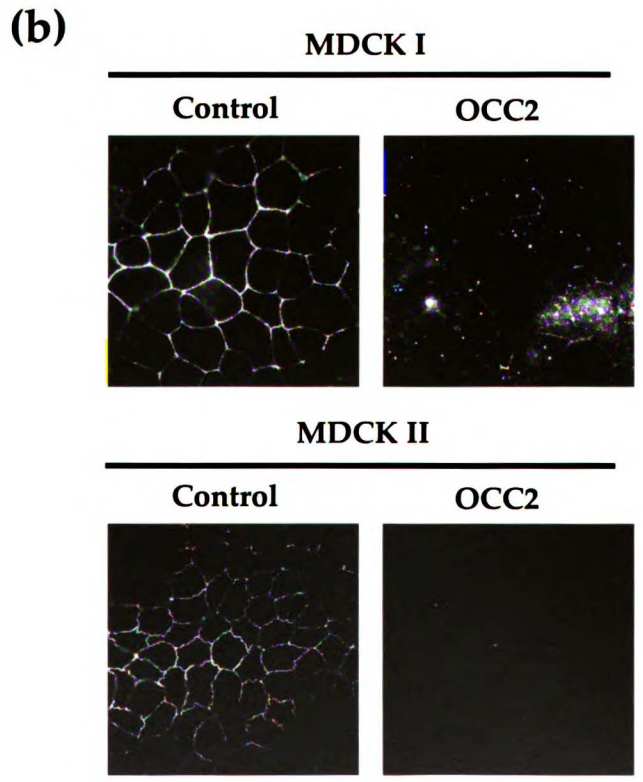

(b)
MDCK I
Control
OCC2
MDCK II
Control
OCC2

Fig. 2.5. A hypothetical model for the regulation of occludin localization/function by phosphorylation. Two main pathways could be used; one represents recruiting occludin from the plasma membrane while the other pathway represents recruiting occludin from intracellular vesicles. Three possible mechanisms could be involved in recruiting occludin from the plasma membrane. Phosphorylation of occludin could stabilize the assembly of occludin in the formation of a continuous barrier in the plane of the plasma membrane, an event that resembles the polymerization of occludin (A). Alternatively, phosphorylation of occludin could stabilize binding of occludin to its extracellular binding protein to form the functional seal of the tight junction (B). Furthermore, phosphorylation of occludin could stabilize interaction of occludin cytoplasmic domain to cytoplasmic tight junction proteins such as ZO-1 and ZO-2 to allow incorporation of occludin into the tight junction (C). The second pathway that involves recruiting occludin from intracellular vesicles represents a mechanism similar to docking of occludin containing vesicles to the tight junction, perhaps via interaction with the cytoplasmic proteins of the tight junction (D). Each or a combination of the above possible mechanisms could allow regulation of occludin in the formation of the tight junction.

Hypothetical Model for Occludin Regulation by Phosphorylation

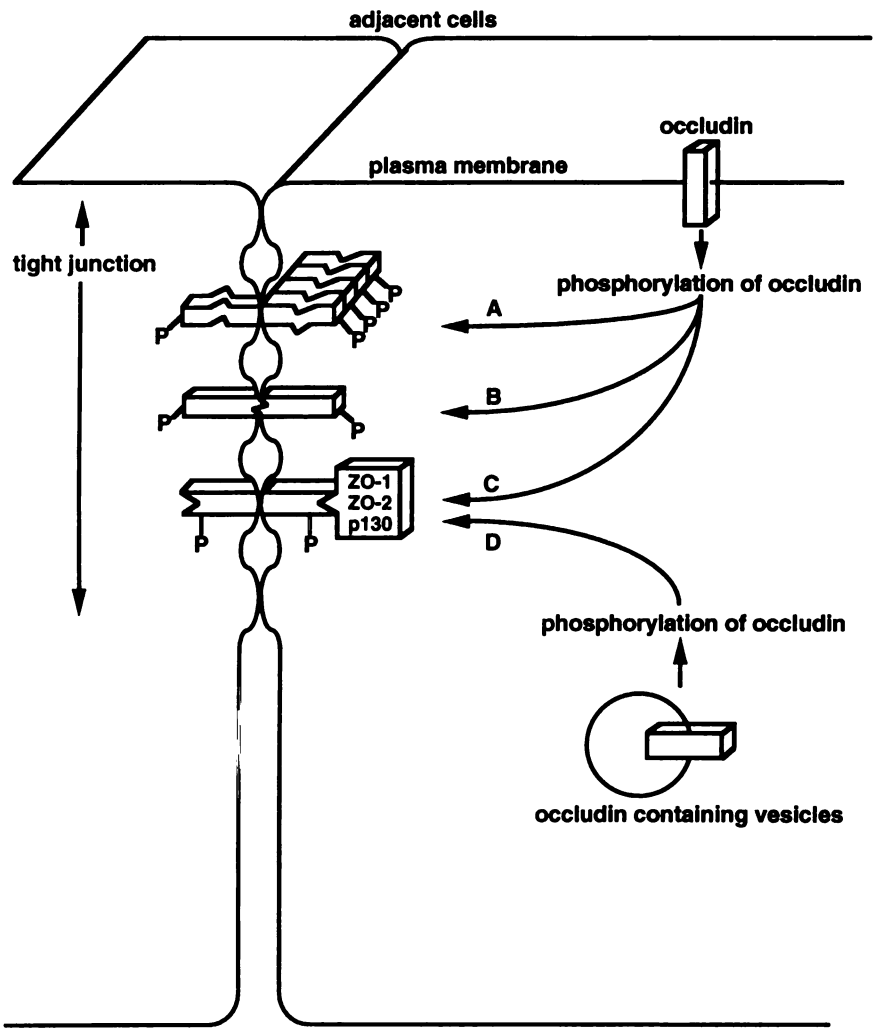
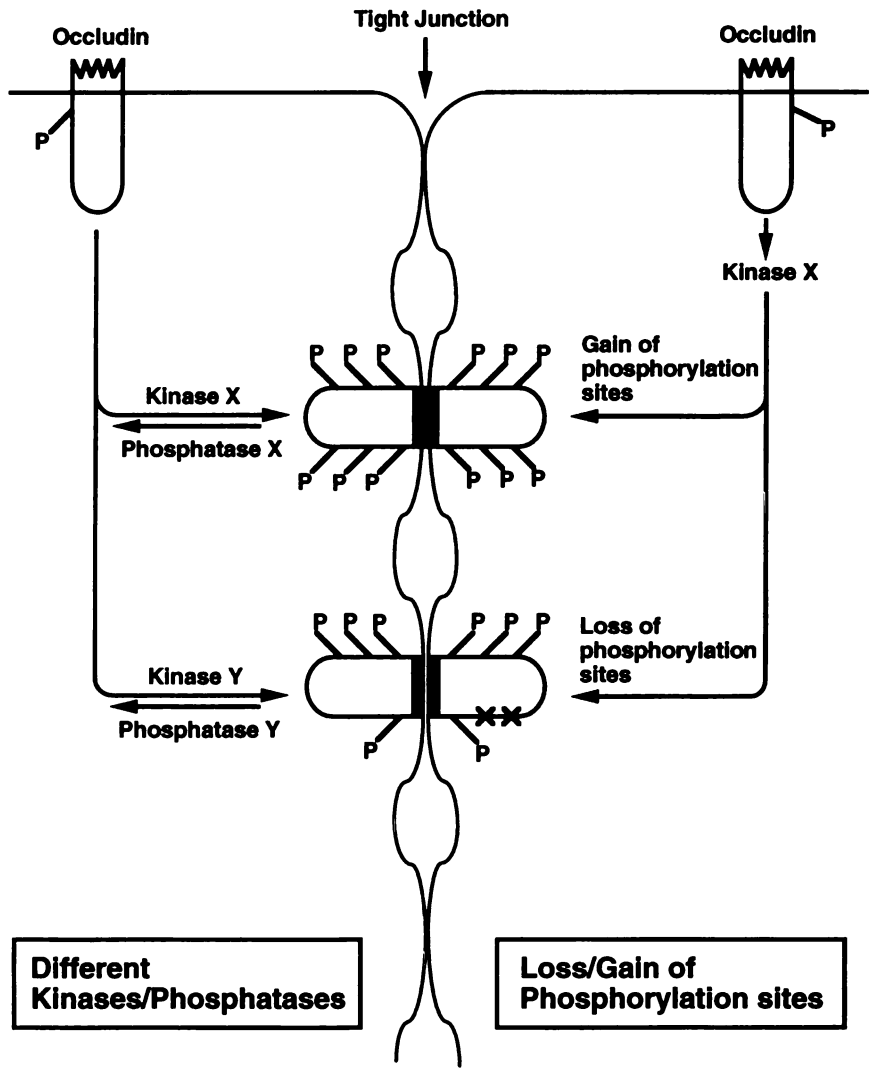


Fig. 2.6. A hypothetical model for the regulation of the tight junction permeability properties via differential phosphorylation of occludin. The difference in hyperphosphorylation of occludin in MDCK I and II cells could reflect the presence of different occludin kinases and phosphatases in the two strains of MDCK cells. Alternatively, the difference in TER of MDCK I and II cells could be due to a gain or a loss of phosphorylation sites that influenced TER, assuming that both strains of cells have the same occludin kinases and phosphatases.

Hypothetical Model for Tight Junction Regulation by Occludin



SUMMARY

The role of occludin in the tight junction permeability barrier was studied using synthetic peptides as potential competitive inhibitors of endogenous occludin function. Treatment of *Xenopus* kidney epithelial A6 cells with OCC2, a peptide corresponding to the second extracellular domain of chick occludin, caused a reversible and dose dependent perturbation of the tight junction permeability barrier. This perturbation of the tight junction barrier consistently correlated with a selective depletion of total cellular content and junction localization of occludin. However, epithelial cell morphology and the cytoplasmic components of the tight junction were not altered by the peptide. These results suggested that the localization of occludin at the tight junction is essential for the formation of a proper permeability barrier, indicating a fundamental role of occludin in sealing the paracellular pathway. Furthermore, the study of occludin biochemistry in Madin-Darby canine cells (MDCK) suggested that phosphorylation of occludin might have a role in regulating occludin localization/function. The different phosphorylated forms of occludin found in the two strains of MDCK cells (I and II) that have different transepithelial electrical resistance (TER) suggested a potential role of occludin in dictating the tight junction permeability properties.

DISCUSSION

This work demonstrates for the first time that a protein is functionally involved in the formation of the tight junction permeability barrier. Historically, before its characterization at the molecular level, the tight junction has been indicated by its distinct morphologies of membrane contacts and intramembrane fibrils at the level of electron microscopy (Gumbiner, 1987). The apparent close membrane contacts of the tight junction that is seen in transmission EM of thin sections has led to the hypothesis that the tight junction is formed by fusions of the outer leaflets of the plasma membranes from adjacent cells (Kachar & Reese, 1982). Nevertheless, it has not been clear whether the sealing elements of the tight junction are made up of lipids or proteins. Many tight junction proteins were identified but their functions in the formation of the tight junction seal have not been demonstrated. The integral membrane protein of the tight junction, occludin, has been shown to be localized to the membrane contact sites and to the intramembrane fibrils as seen in transmission EM of freeze fracture replicas (Fujimoto, 1995; Furuse et al., 1993). Yet, the function of occludin in the formation of the tight junction has been difficult to elucidate. Stable expression of chick occludin in the Chinese hamster ovary (CHO) cells was unable to constitute functional tight junctions when assessed by measurements of transepithelial electrical resistance (TER) and paracellular tracer flux (V. Wong, unpublished observations). However, in this study, by employing synthetic peptides as potential competitors for endogenous occludin function, the role of occludin in the formation of the tight junction permeability barrier has been indicated.

A simple model to describe how occludin might participate in the formation of the tight junction involves the binding of occludin to its extracellular binding protein, possibly occludin itself, to seal off the

intercellular space. In addition, occludin would also polymerize in the plane of the plasma membrane to completely circumscribe the apecies of cells, a delineation that resembles the arrangements of intramembrane fibrils. Interestingly, a stretch of 13 a.a. at the C-terminus of the second extracellular domain of occludin is highly conserved (completely identical between mouse, human, and dog; 11 a.a. identical between chick, rat-kangaroo, and the mammlian species), suggesting that the second extracellular domain is functional significant, perhaps involved in protein-protein interactions. Moreover, expression of chick occludin in CHO cells showed that occludin is enriched at cell boundaries, implying that occludin might have homophilic or self-polymerizing activities at cell-cell contacts (V. Wong, unpublished observations). Furthermore, assessment of binding activities of the second extracellular domain of occludin using OCC2 peptide showed that OCC2 coupled beads would form large aggregates (V. Wong, unpublished observations). The aggregation of OCC2 coupled beads could be competed by excess free OCC2 but not OCC2(S), the scrambled OCC2 peptide, demonstrating that the binding events were specific to the sequence of the OCC2 peptide. Although it is not clear whether the aggregation of beads represents a homphilic binding event or a polymerization event, the results support the hypothesis that occludin binds to itself. Future experiments would be necessary to address how occludin might form the paracellular barrier.

The tight junction impedes the movement of macromolecules but selectively allows various small ions to pass through (Kottra & Fromter, 1983), a characteristics that is also shared by conventional cation channels. Thus, it is possible that occludin is the 'channel' protein of the tight junction. There are noteworthy similarities between the tight junction and the conventional cation channels. Firstly, they both can be blocked by positively

charged ions, such as barium (Kobu, Baldwin, Jan & Jan, 1993; Kottra & Fromter, 1990). Secondly, their estimated pore diameters are similar (e.g. ~6.4 Å for the retinal ATP-gated channel and ~4-11 Å for tight junctions) (Goulding, Tibbs, Liu & Siegelbaum, 1993; Madara & Dharmsathaphorn, 1985). Most imortantly, they both are cation selective (e.g. the permeability of K^+/Na^+ to Cl^- is >10 in MDCK cells) (Cereijido, Meza & Martinez-Palomo, 1981). However, unlike the cation channels that is formed in the plane of the plasma membrane, the tight junction is constituted when the plasma membranes of adjacent cells contact each other. Therefore, the tight junction 'channel' would be predicted to be formed by the extracellular domains of proteins. Interestingly, the first extracellular domain of occludin contains multiple conserved glycine-tyrosine-glycine (GYG) motifs; this GYG motif is also conserved in potassium channels and has been implicated to be responsible for ion selectivity of the potassium channels. In addition, the first extracellular domain also has an exceptional high percentage of tyrosine residues (25%); these tyrosine resudues would allow multiple 'cation-π' interactions, a mechanism involves favorable interactions between cations and the electron dense ring of tyrosine. Hence, it appears that the first extracellular domain of occludin would accomodate the cation selectivity of the tight junction and might participate in the formation of the tight junction 'channel'. Consequently, occludins that have different first extracellular domains might give rise to tight junction 'channels' that have distinct ion selectivities.

Occludin, being the potential channel protein of the tight junction, might also have a role in the regulation of the permeability properties within an epithelium or an endothelium. In this study, different hyperphosphorylated forms of occludin were found in the two strains of MDCK cells that have very

different transepithelial electrical resistance. These hyperphosphorylated forms represented the junction localized and functional forms of occludin, and therefore, were the structural components for the formation of the physical tight junction seal. Therefore, it seems possible that the different hyperphosphorylated forms of occludin that are found in the two strains of MDCK cells might form tight junction seals that had distinct permeability properties. In that case, differential phosphorylation of occludin would be a mechanism by which the tight junction permeability properties is regulated within the same epithelium or endothelium. Nevertheless, future experiments are required to address the direct relationship between occludin phopshporylation and the tight junction channel properties.

Furthermore, the potential regulation of the tight junction barrier via occludin localization/delocalization that has been indicated in this study (see chapter I) might provide insight into the understanding of the dynamics of the tight junction. Occludin was selectively depleted from the tight junction by the OCC2 peptide in the abscence of detectable changes in the levels and localization of other known tight junction proteins, suggesting that occludin might be regulated separately. Instead of assembling and disassembling the whole tight junction complex, the permeability barrier would be regulated by depleting and enriching occludin at the tight junction. The localization of occludin at the tight junction could be accomplished by degradation/resynthesis of occludin or by assembly/disassembly of occludin. Interestingly, in MDCK cells, hyperhposphorylation of occludin correlated with its localization/function at the tight junction, suggesting that phosphorylation might be a mechanism for the regulation of occludin. The potential rapid mean of regulation that is provided by phosphorylation is consistent with the observations that the tight junction seal could be opened

and resealed within a short time (~1 hour) during dynamic physiological processes such as leukocyte transmigration across an endothelium. Since the phosphorylation of occludin would allow multiple regulatory mechanisms, it is tempting to speculate that the formation of the tight junction barrier could also be regulated at by multiple pathways via the control of occludin phosphorylation.

The role of occludin in the formation of the characteristic tight junction membrane contact sites and intramembrane fibrils have not been thoroughly tested in this study. However, the depletion of occludin at the tight junction by OCC2 peptide did not results in any detectable change in epithelial cell morphology. In addition, preliminary results, as assessed by transmission EM of thin sections, suggested that the the tight junction membrane contacts was also not detectably altered after OCC2 treatment (V. Wong, unpublished observations). Therefore, it seems possible that occludin is not required for the formation of the tight junction physical ultrastructures, but simply functions as a channel/permeability seal. Perhaps, other unidentified proteins or lipids are responsible for the organization of the membrane contacts and intramembrane fibrilar structures. Future experiments employing OCC2 peptide, immunogold labeling, and quantitative EM analysis might be able to acknowledge whether occludin is important for the formation of these characteristics tight junction ultrastructures.

The role of occludin in the maintenance of the polarity of the plasma membrane was also not studied in this work. Nevertheless, the synthetic peptides would provide a specific mean to further investigate the many potential functions of occludin.

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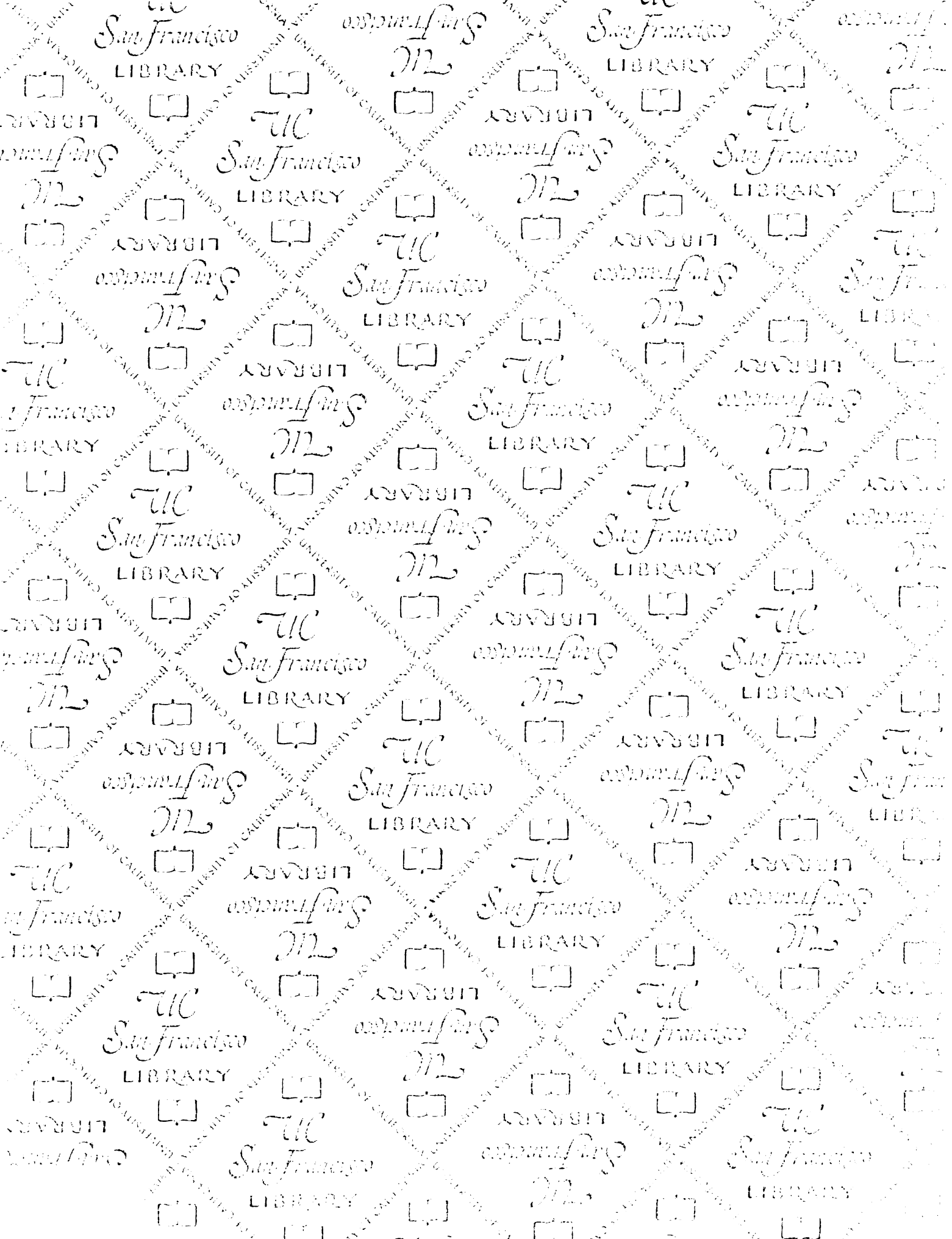
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