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Occludin is a functional component of the tight junction

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SUMMARY

Occludin's role in mammalian tight junction activity was examined by 'labeling' the occludin pool with immunologically detectable chick occludin. This was accomplished by first transfecting MDCK cells with the *Lac* repressor gene. Hyg^R clones were then transfected with chick occludin cDNA inserted into a *Lac* operator construct. The resulting Hyg^R/Neo^R clones were plated on porous inserts and allowed to form tight junctions. Once steady state transepithelial electrical resistance was achieved, isopropyl-β-D-thiogalactoside was added to induce chick occludin expression. Confocal laser scanning microscopy of monolayers immunolabeled with Oc-2 monoclonal antibody revealed that chick occludin localized precisely to the preformed tight junctions. When sparse cultures were maintained in low Ca^{2+} medium, chick occludin and canine ZO-1 co-localized to punctate sites in the cytoplasm suggesting their association within the same vesicular structures. In low calcium medium both proteins also co-localized to contact sites between occasional cell pairs, where a prominent bar was formed at the plasma membrane. Chick occludin was detectable by western blot within two hours of adding isopropyl-β-D-thiogalactoside to monolayers that had previously achieved steady state transepithelial electrical resistance; this coincided with focal immunofluorescence staining for chick occludin at the cell membrane of some cells. A gradual rise in transepithelial electrical resistance, above control steady state values, began five hours after addition of the inducing agent reaching new steady state values, which were 30-40% above baseline, 31 hours later. Upon removal of isopropyl-β-D-thiogalactoside chick occludin expression declined slowly until it was no longer detected in western blots 72 hours later; transepithelial electrical resistance also returned to baseline values during this time. While densitometric analysis of western blots indicated that the presence of chick occludin had no detectable effect on E-cadherin or ZO-1 expression, the possibility cannot be excluded that ZO-1 might be a limiting factor in the expression of chick occludin at the cell surface. To test whether expression of chick occludin affected the process of tight junction assembly, monolayers in low Ca^{2+} medium were treated with isopropyl-β-D-thiogalactoside for 24 or 48 hours, before Ca^{2+} was added to stimulate tight junction assembly. Chick occludin did not alter the rate at which transepithelial electrical resistance developed, however, steady state values were 30-40% above control monolayers not supplemented with the inducing agent. By freeze fracture analysis, the number of parallel tight junction strands shifted from a mode of three in controls to four strands in cells expressing chick occludin and the mean width of the tight junction network increased from 175±11 nm to 248±16 nm. Two days after plating confluent monolayers that were induced to express chick occludin, mannitol flux was reduced to a variable degree relative to control monolayers. With continued incubation with the inducing agent, mannitol flux increased on day 11 to 50%, and TER rose to 45% above controls. Both of these changes were reversible upon removal of isopropyl-β-D-thiogalactoside. These data are consistent with the notion that occludin contributes to the electrical barrier function of the tight junction and possibly to the formation of aqueous pores within tight junction strands.

Key words: Occludin, Tight junction, Transfection, IPTG inducible expression, Transepithelial electrical resistance, Mannitol flux

INTRODUCTION

Tight junctions (TJ), the most apical component of the mammalian junctional complex, form a diffusion barrier that regulates the flux of ions and hydrophilic molecules through the paracellular pathway (Reuss, 1992). Structurally they form a continuous network of parallel, interconnected intramembrane strands that are arranged as multiple barriers in series (Schneeberger and Karnovsky, 1976; Schneeberger et al., 1978). Although initial studies suggested a direct, arithmetic relationship between the transepithelial electrical resistance (TER) and the number of parallel TJ strands (Claude and Goodenough, 1973), later studies based on physiological and morphological data indicated that the relationship between

TER and strand numbers was logarithmic. This led Claude (1978) to postulate that the TJ strands contain or form pores that fluctuate between an open or closed state. Exceptions to this relationship, however, make it difficult to infer permeability properties from strand counts (Martinez-Paloma and Erlij, 1975; Stevenson et al., 1988). For example, while there is a 30-fold difference in TER between strains I and II MDCK cells, the number of TJ strands, network density and quantity of ZO-1, a TJ associated protein, was the same (Stevenson et al., 1988). The composition of the TJ strands and the organization and disposition of the inferred aqueous pores also remains to be determined.

Tight junctions, in addition to providing a barrier in the paracellular pathway, also form a fence marking the boundary between apical and basolateral plasma membrane domains. In this capacity they act as a constraint against the global diffusion of proteins and lipids between the two domains (Nelson, 1992; Gumbiner, 1996). By separating the cell surface into these two physically and functionally distinct domains, they enable epithelial cell monolayers to carry out a variety of polarized functions (Schneeberger and Lynch, 1992; Gumbiner, 1993).

Some of the factors controlling the assembly of TJs (Citi, 1992; Citi and Denisenko, 1995; Howarth et al., 1994; Balda et al., 1993), and the role of cytoplasmic proteins and membrane lipids in regulating their structure and function have been determined (Tsukita et al., 1993; Lynch et al., 1993; Stankewich et al., 1996; Anderson and Van Itallie, 1995). In the search for protein(s) that constitute the TJ strands, a number of important TJ-associated proteins, including ZO-1 (Stevenson et al., 1986), cingulin (Citi et al., 1988), 7H6 (Zhong et al., 1993), ZO-2 (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994) have been identified. Based on their subcellular localization, however, none of them proved to be the biochemical equivalent of a TJ subunit. One protein, occludin (~65 kDa), first identified in preparations of avian liver junctional complexes, appears to have the biochemical characteristics expected of a TJ strand component (Furuse et al., 1993, 1994, 1996). Except for its immunolocalization at the TJ, however, there are no published data that relate occludin's expression to changes in TJ physiology.

In the present study, chick occludin cDNA was introduced into MDCK cells in a Lac inducible vector in order to: (1) determine whether occludin is a functional subunit of the tight junction, and (2) to facilitate studies on occludin's role in TJ biology by 'labeling' the occludin pool in mammalian cells. This was accomplished by stably transfecting MDCK cells with two vectors: one containing the *Lac* I repressor gene and the other containing chick occludin DNA driven by the Rous Sarcoma Virus LTR promoter containing a modified *Lac* operator sequence. Expression is induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to the medium. Such a model provides a means of controlling when and for how long chick occludin is expressed, as well as the relative quantity of chick occludin that is used to 'label' the mammalian pool. Use of this system with currently available monoclonal antibodies that recognize avian occludin provides new insights into occludin's role in the assembly, structure and function of mammalian TJs. The chick occludin marker, synthesized after addition of IPTG, responds physiologically as an integral component of the MDCK cell TJ with respect to both its barrier function and its subcellular distribution.

MATERIALS AND METHODS

Reagents

Reagents and their source are listed below. Dulbecco's modified Eagle's medium (DMEM), Ham's F12 nutrient mixture, Earle's balanced salt solution (EBSS), Dulbecco's PBS (DPBS) and individual components to make essential medium (without calcium, without phosphate) (LCM), T4 ligase and *Eco*RI (Gibco, Grand Island, NY). Newborn calf serum (BCS) (Hyclone Laboratories, Logan, UT). Trypsin (Worthington Diagnostic Systems, Freehold, NJ). *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), (ethylenedinitrilo)tetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA), dithiothreitol (DTT) (Sigma Chemical Co., St Louis, MO). Restriction endonuclease enzymes (*Not*I, *Bst*XI) (NE Biolabs, Beverly, MA). The Lac Switch inducible expression system (Stratagene, La Jolla, CA) (DuCoeur et al., 1992). Nunc tissue culture inserts, pore size 0.2 μm (Nunc, Inc. Naperville, IL) and Millicell HA inserts (Millipore Corp. Bedford, MA). Hygromycin B (Hyg) and Geneticin (Neo) (Boehringer-Mannheim, Indianapolis, IN).

Antibodies

Oc-2 rat anti-chick occludin hybridoma supernatant was supplied by M. Furuse. Monoclonal antibodies (mAbs) were generated from the following hybridomas: anti-ZO-1 (R26-4C, gift from D. A. Goodenough, and R40.76, gift from B. R. Stevenson), anti-E cad (RR-1, gift from B. M. Gumbiner), anti-E cad (3G8, gift from W. Gallin). Rabbit anti-ZO-1 (Zymed Laboratories, Inc. S. San Francisco, CA).

Cell culture

MDCK cells (CCL34) obtained from the American Type Tissue Culture Collection, Rockville, MD, were maintained in antibiotic-free DMEM/Ham's F-12 medium supplemented with 10% BCS and subcultured weekly at a split ratio of 1:6. The medium was changed at 2-3 day intervals and cultures were kept at 37°C in a humidified incubator with 95% air, 5% CO_2. Stock cultures were routinely monitored for the presence of mycoplasma.

Purification of chick occludin cDNA

Bluescript SK(–) phagemid containing full-length chick cDNA, provided by M. Furuse, was amplified in *E. coli* DH5α and purified by alkaline lysis and ethanol precipitation (Birnboim and Doly, 1979). After digestion with *Eco*RI, the 1,632 bp occludin fragment was separated from vector DNA by gel electrophoresis, and purified on a GenElute Spin Column (Supelco, Bellefonte, PA).

Ligation of conversion adaptors to chick occludin cDNA

The *Eco*RI cohesive ends of the occludin fragment were converted to *Not*I cohesive ends with *Eco*RI-*Xmn*I and *Not*I-*Xmn*I adaptors (NE Biolabs, Beverly, MA). The two adaptors were annealed to each other according to the manufacturer's protocol and then ligated to 2 μg of occludin (10:1 molar ratio). The ligation product was recovered by phenol/chloroform extraction and PEG/ethanol precipitation.

Insertion of chick occludin cDNA into the operator vector, pOPRSVI

Operator vector, pOPRSVICAT (Lac Switch System, Stratagene, La Jolla, CA) was cleaved with *Not*I and the adaptor-modified occludin was ligated to vector pOPRSVI. The resulting ligation product was transformed into competent *E. coli* XL-I Blue MR cells and plated on Ampicillin plates. Plasmid minipreps prepared from Amp^R colonies

were tested for direction of occludin insertion by *Bst*XI digestion. Desired clockwise insertion yielded a 2,202 bp and a 4,924 bp fragment. A large scale plasmid preparation of a clone with correctly inserted occludin was purified on a Qiagen Tip 2500 (Qiagen Inc. Chatsworth CA) according to the manufacturer's instructions.

Transfection of MDCK cells

Lac repressor vector

MDCK cells were first transfected with *Lac* repressor vector, p3′SS, by calcium phosphate co-precipitation (Chen and Okayama, 1988). Hyg^R cells were cloned and the presence of Lac repressor was confirmed by immunofluorescence using Lac repressor protein antiserum.

pOPRSVI/chick occludin

Lac repressor positive cells were transfected as above with the pOPRSVI/occludin construct and Hyg^R/Neo^R clones were selected. Positive clones were plated on collagen coated coverslips and incubated either with or without 5 mM IPTG for 16 hours. Monolayers were prepared for immunofluorescence as described below. Stock cultures and experimental monolayers were maintained in 150 μg/ml hygromycin and 200 μg/ml genetecin.

Confocal laser scanning microscopy (CLSM)

Transfected MDCK cells were plated either at confluence or at low cell density on 0.5 cm^2 NUNC tissue culture inserts (Nunc, Inc. Naperville, IL) in DMEM/F12 medium, 10% BCS, 1.8 mM $CaCl_2$ (NCM). After 2 hours, when the cells were adherent, medium on the confluent monolayers was changed to NCM ± 5 mM IPTG. Alternatively, after rinsing the sparse cultures with Dulbecco's phosphate buffered saline (DPBS), they were maintained in LCM, 1% BCS ± 5 mM IPTG. After 24 or 48 hours, monolayers were fixed in 1% formaldehyde in phosphate buffered saline (PBS) for 5-10 minutes at room temperature (RT). This and all subsequent steps were followed by three washes in PBS, 2% normal goat serum. Monolayers were permeabilized with 0.2% Triton X-100 (TX-100) and immunolabeled with Oc-2 mAb culture supernatant (diluted 1:5), followed by FITC-conjugated goat anti-rat IgG. Dual immunolabeling for chick occludin and canine ZO-1 was conducted on sparse cultures maintained in LCM. Chick occludin was immunolabeled with Oc-2 mAb followed by FITC-conjugated goat anti-rat IgG. ZO-1 was reacted with polyclonal rabbit anti-ZO-1 followed by CY3-conjugated goat anti-rabbit IgG. Quenching was minimized with 2% *n*-propylgallate in 50% glycerol/50 mM Tris-HCl, pH 8.0, mounting medium.

Monolayers were examined using a Sarastro 2000 confocal scanning microscope (Molecular Dynamics, Sunnyvale, CA) fitted with a 25 mW argon-ion laser. Fields were selected at random and the cells were brought into focus under bright-field conditions. The operating conditions of the microscope were set to a laser power of 18.5 mW; the excitation and emisssion were set to 488 nm and >510 nm, respectively. A 50 μm pinhole aperture was placed in front of the photomultiplier tube set to 530 volts. In cross sectional scans (*x*-*z*) images were collected at a 0.17 μm pixel size, and step increments in the *z*-axis were at 0.17 μm. Cross section scans and *x*-*y* optical sections are presented without image filtering or processing. Fluorescent pixel intensities were assigned values to a gray look-up table with black being zero and white (max. intensity) set to 255.

Determination of transepithelial electrical resitance (TER)

For repeated, sequential TER measurements on individual monolayers on Millicell HA inserts (0.6 cm^2), a Millicell-ERS epithelial volt-ohmmeter (World Precision Instruments, New Haven, CT) was utilized under temperature controlled conditions at 37°C with electrodes reproducibly placed. TER values were calculated by subtracting the contribution of the bare filter and medium and multiplying by the surface area of the filter.

Mannitol flux measurements

The net flux of [^{3}H]mannitol was measured either from the basolateral to the apical compartment or vice versa on monolayers supplemented with or without IPTG on Millicell HA inserts (0.6 cm^2) (Rochat et al., 1988). DMEM/F12, 10% BCS, 1% P/S (1 ml) was added to the well and 0.5 ml of the same medium supplemented with 1 mM mannitol and 4 μCi/ml of [^{3}H]mannitol was added to the insert for apical to basolateral measurements. In measurements of mannitol flux in the basolateral to apical direction, carrier mannitol and [^{3}H]mannitol at the same concentration as above were placed in the opposite compartment. At 0, 30, 60 and 120 minutes, 100 μl samples were removed from the recipient compartment and the same volume of medium ± IPTG was replenished. At 30 and 60 minutes, 10 μl samples were obtained from the donor compartment to determine the dilution of label. After adding Ultima-Gold fluor (Packard Instruments Co, Meriden, CT), the samples were counted in a Packard 2200 CA liquid scintillation counter.

Plasma membrane preparation

For western blot analysis a crude membrane fraction was prepared (Stevenson et al., 1986). Briefly, monolayers were rinsed in PBS and the cells were scraped in 2 ml of buffer II, pH 7.4 (10 mM imidazole, 4 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM DTT, 0.1 mM TI U/ml aprotinin, 2.5 mg/ml leupeptin, 0.5 μg/ml pepstatin, 30 μg/ml soybean trypsin inhibitor, 70 μg/ml TPCK). All subsequent steps were conducted at 4°C. After centrifugation for 8 minutes at 400 ***g***, cells were resuspended in 6 ml ice-cold buffer II and disrupted with 8-12 strokes in a Dounce homogenizer. The homogenate was centrifuged at 8,000 ***g*** for 10 minutes. The supernatant was harvested and centrifuged for 1 hour at 100,000 ***g***. The resulting membrane pellet was solubilized in 1% SDS, 5 mM EDTA, 2.5 mM EGTA in 15 mM Tris-HCl buffer, pH 7.5, containing the above indicated protease inhibitors.

SDS-PAGE and western blot analysis

Protein samples were heated in SDS sample buffer with 4% 2-mercaptoethanol at 100°C for 10 minutes, separated by SDS 7.5% PAGE (Laemmli, 1970) and transferred electrophoretically in 25 mM Tris, 192 mM glycine, 20% methanol, 0.2% SDS to nitrocellulose membranes at 250 mAmp, 105 V, at 4°C for 3 hours (Towbin et al., 1979). Nonspecific protein binding was blocked with TBS-Blotto (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5% nonfat dry milk, 0.05% Tween-20) for 1 hour at RT. Individual lanes of the membrane were cut and incubated with one of the following hybridoma supernatants diluted in TBS-Blotto: Oc-2 (anti-chick occludin; 1:50), R40-76 (anti-ZO-1; 1:5) or purified RR1/3G8 (anti-E-cadherin) in PBS (1.3 μg/ml each) for 1 hour at RT. The blots were then reacted with HRP tagged anti-mouse IgG (for E-cad) or HRP tagged anti-rat IgG for ZO-1 and chick occludin and detected with a chemiluminescence detection system (ECL, Amersham, Corp., Arlington Heights, IL). Biotinylated molecular mass standards used were, 205 kDa (myosin), 116 kDa (β-galactosidase), 97 kDa (phosphorylase b), 58 kDa (catalase), 40 kDa (alcohol dehydrogenase) and 29 kDa (carbonic anhydrase) (Sigma Chemical Co.).

Freeze fracture

Transfected MDCK cells were plated at confluence in each of two 10 cm diameter tissue culture plates in DMEM/F12 medium, 10% BCS. After 48 hours, medium containing 5 mM IPTG was added to one and medium with vehicle alone to the second. Monolayers were fixed, 48 hours later, in 2% glutaraldehyde in 0.1 M cacodylate, 0.03% sucrose for 30 minutes at RT. After rinsing with 0.15 M cacodylate buffer, pH 7.3, they were scraped and stored in the same buffer. The sheets of cells were infiltrated with 25% glycerol, frozen in liquid nitrogen slush, produced under vacuum, and freeze fractured in a Balzers 400 freeze fracture unit (Balzers, Liechtenstein). Replicas were cleaned with sodium hypochlorite, washed in distilled water, placed on

Formvar coated grids and examined in a Philips 301 electron microscope.

Morphometry

In a given replica, every grid square was examined and all images of TJs were photographed. An equal total length of TJs was measured in both IPTG treated and control monolayers requiring at least 36 micrographs for each category. The number of parallel strands was measured, as described (Lynch et al., 1995), on micrographs at a final magnification of ×47,500 by two individuals who did not know the source of the replicas. The width of the TJ network was measured at three equidistant points on each micrograph. The morphometric data was analyzed as a parallel electrical circuit as described (Madara and Dharmsathaphorn, 1985).

RESULTS

Culture characteristics of occludin transfected clones

Five clones were successfully transfected with both plasmids. By phase microscopy, the cell morphology of each of the five clones was uniform, however, the growth characteristics varied among the clones. Those forming tightly packed monolayers tended to achieve confluence more rapidly than clones composed of large flat cells. Clones also varied in the strength of their adhesion to the substratum; some detached readily in the presence of trypsin while others required lengthy incubations. All experiments reported in the present study were conducted with the clone designated as A* which, from a 1:6 split ratio, reached confluence within four days and formed uniform, tightly packed monolayers that developed domes. Monolayers of the A* clone were readily detached from the substratum upon incubation with trypsin.

Confocal laser scanning microscopy

At 24 and 48 hours after adding 5 mM IPTG, chick occludin was detected by immunofluorescence staining as a fine line encircling the perimeter of each cell in the *x-y* (en face) plane (Fig. 1a). Higher levels of occludin were observed at the points of contact between three cells; these account for the two very bright foci of staining seen in the right half of Fig. 1a′. In this *x-z* (lateral) plane, chick occludin formed a discrete focus of staining near the apical end of the lateral plasma membrane domain (Fig. 1a′). In the absence of IPTG, there was no staining for chick occludin (Fig. 1b and b′), confirming previous observations that Oc-2 mAb does not recognized canine occludin (Furuse et al., 1993). When transfected cells were plated at subconfluent densities in LCM, chick occludin localized to an area in the cytoplasm close to the nucleus and to punctate sites in the cytoplasm suggestive of cytoplasmic vesicles, some of which were close to the plasma membrane (Fig. 2). In dual labeling experiments with sparse cultures in LCM, chick occludin co-localized with canine ZO-1 to the same punctate areas in the cytoplasm (Fig. 3a,b,c). That some punctate sites stained for ZO-1 alone, suggests the possibility that some ZO-1 may not have been associated with occludin or, alternatively, been associated solely with canine occludin. To distinguish between these possibilities will require the use of immunological reagents specific for mammalian occludin. At sites of contact between pairs of cells, there was intense co-localization of both chick occludin and canine ZO-1 (Fig. 3d,e,f), in a pattern similar to that previously reported for ZO-1 in MDCK cells cultured in low Ca^{2+} medium (Howarth et al., 1994).

Expression of chick occludin increases transepithelial electrical resistance (TER)

To assess TJ assembly, cells were first plated on Millicell HA inserts (0.6 cm^2) in LCM for 24 hours, then switched to NCM to initiate the process and TER measured at timed intervals thereafter. MDCK cells transfected with the *Lac* repressor construct (p3′SS) alone, required more time to achieve peak TER, following Ca^{2+} addition, than nontransfected MDCK cells (~60 vs 12 hours). After transfecting these cells a second time with the pOPRSV1/chick occludin cDNA construct, the time required to achieve peak TER values lengthened further

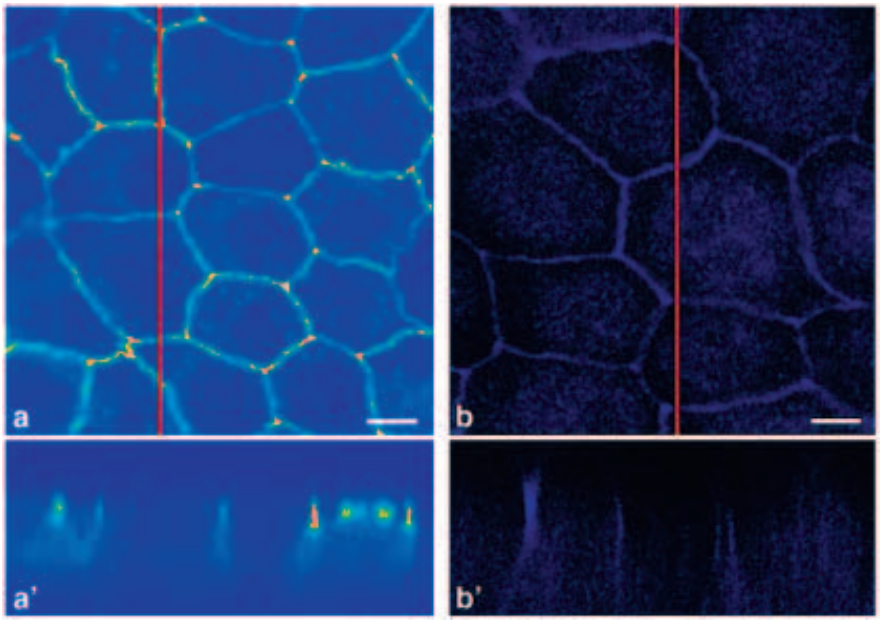

Fig. 1. (a,a′) MDCK cells transfected with chick occludin were incubated in NCM with 5 mM IPTG for 48 hours, fixed and immunostained for chick occludin utilizing Oc-2 mAb. Pseudo-color images show intense staining at the perimeter of all cells in the *x-y* plane (a) and in the *x-z* plane (a′) the staining is concentrated near the apical end of the paracellular space. (b,b′) Chick occludin transfected MDCK cells, not supplemented with IPTG and immunostained as in a. Note the absence of chick occludin expression. The red line in a and b indicates the plane from which the *x-z* images were optically reconstructed. Bars, 5 μm.

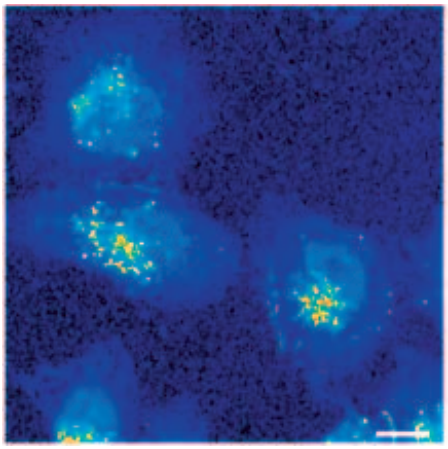

Fig. 2. A sparse culture of chick occludin transfected MDCK cells was cultured in LCM for 48 hours with 5 mM IPTG. The cells were then fixed and immunostained for chick occludin. Note the intense staining in the paranuclear area, as well as in a number of punctate sites close to the plasma membrane. In the right lower corner a pair of cells shows linear staining for chick occludin at the site of membrane contact. Bar, 10 μm.

to between 80-90 hours following addition of Ca^{2+}. In the absence of IPTG, the mean steady state TER ± s.e.m. of the A* clone was 182±9 Ω·cm^2.

To determine the effect of chick occludin expression on TER, two types of experiments were conducted. In the first, chick occludin transfected cells were plated at confluence on two triplicate sets of Millicell HA inserts in NCM supplemented with 500 U/ml penicillin, 0.5 mg/ml streptomycin. Two hours later, nonadherent cells were removed by washing with DPBS and 5 mM IPTG in LCM was added to the first set of inserts and the second set received LCM alone. After 24 or 48 hours, medium was changed to NCM in both sets of monolayers ('Ca^{2+} switch'); IPTG supplementation was continued in the first set. All subsequent, sequential TER measurements were made at timed intervals at 37°C on the same set of monolayers until steady state values were achieved; the culture plates were returned to the incubator between measurements. These assays were repeated at least three times.

Addition of 1.8 mM Ca^{2+} to transfected monolayers preincubated for 24 or 48 hours with 5 mM IPTG triggered a rise in TER that was similar in rate to that of transfected monolayers, not treated with IPTG. However, the peak and steady

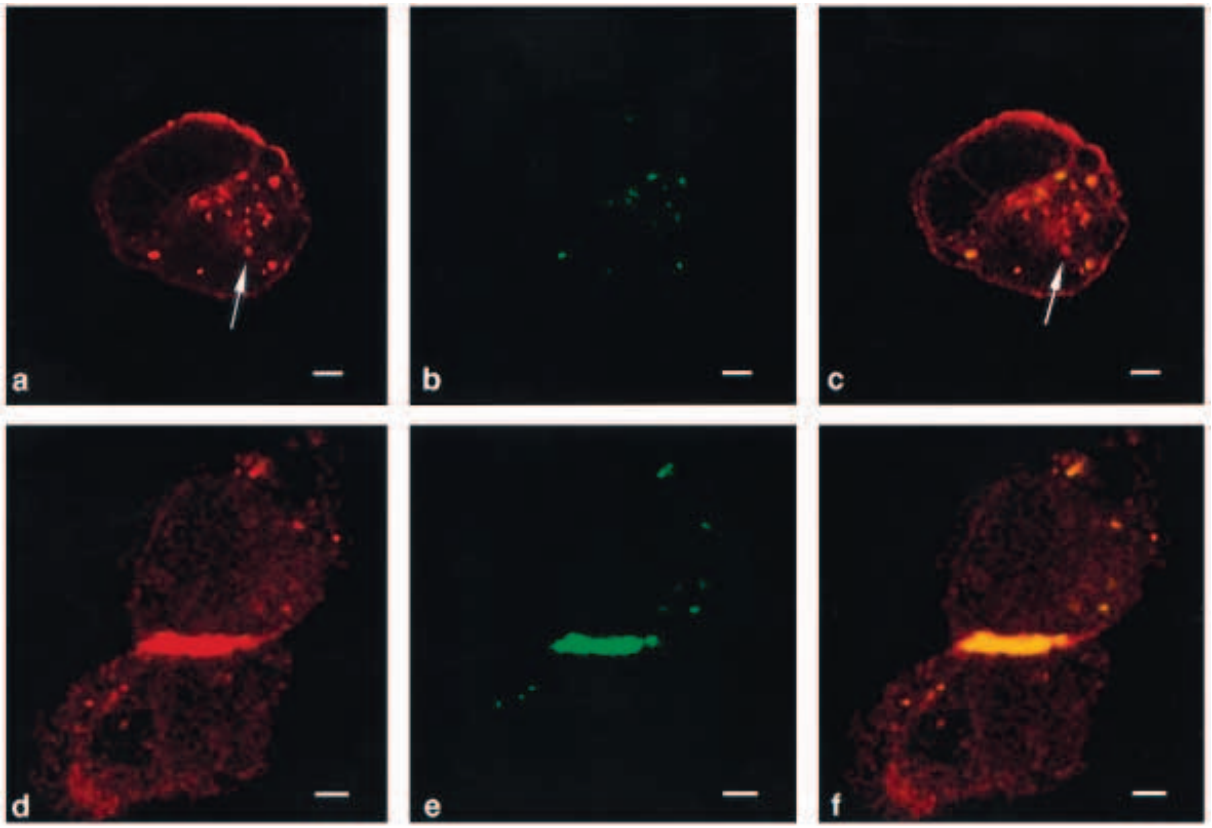

Fig. 3. (a,b,c) A sparse culture of chick occludin transfected MDCK cells was maintained in LCM and treated with 5 mM IPTG for 48 hours. Cells were double immunostained for chick occludin with OC-2 mAb followed by FITC labeled goat anti rat IgG and for canine ZO-1 with polyclonal rabbit anti-ZO-1 followed by CY3 labeled goat anti rabbit IgG. In a ZO-1 (red) is localized to punctate cytoplasmic structures. In b chick occludin (green) is localized to similar structures. In c the images in a and b are superimposed (yellow) and show considerable overlapping colocalization. Note that in c, while there are no green punctate sites, there are some sites that stain red (arrow in a and c) indicating that not all ZO-1 sites have associated with them chick occludin and suggest the possiblity that they are either occupied by ZO-1 alone or that they have canine occludin associated with them. (d,e,f) A pair of cells from the same preparation as in a-c, indicating that some cell-cell contacts occasionally occur even in LCM. In d ZO-1 (red) is localized to a broad bar at the membrane contact site. In e chick occludin (green) localizes to the same structure. In f the two images in e and f are optically superimposed and show precise colocalization of the two proteins at the site of contact. As in c, while there are no green staining sites in f there are some sites that stain only for ZO-1 (red). Bar, 20 μm.

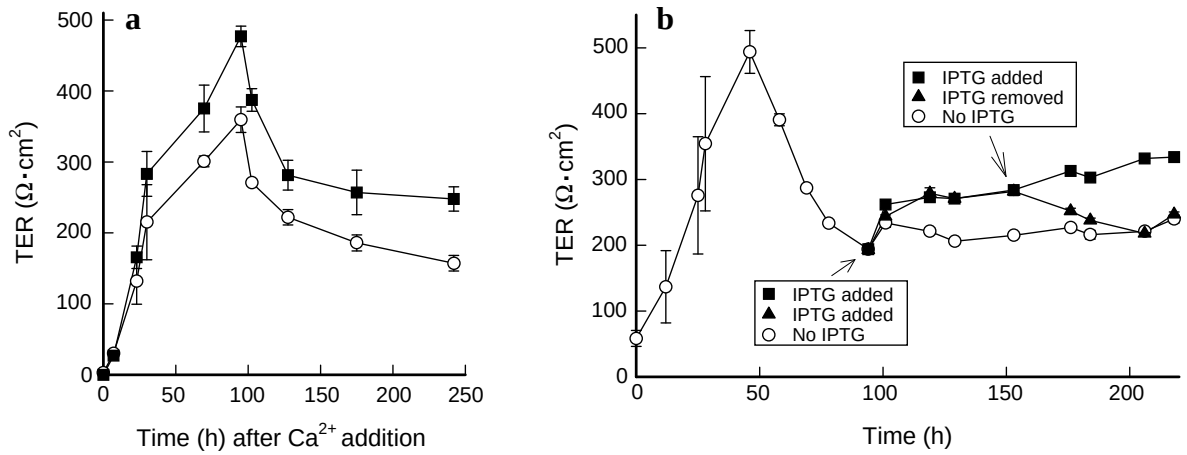

Fig. 4. (a) Chick occludin transfected MDCK cells were incubated for 48 hours either with (■) or without (○) 5 mM IPTG in LCM. The medium was then changed to NCM and IPTG supplementation continued in the monolayers induced to express chick occludin. TER was monitored at timed intervals. While the rate of Ca^{2+} induced TER development was similar in both sets of monolayers, peak and steady state TER of the monolayers expressing chick occludin is approximately 30% higher than those not incubated with IPTG. (b) Three triplicate sets of chick occludin transfected MDCK cells were incubated for 96 hours in NCM. At T_{96} 5 mM IPTG in NCM was added to the first two of three sets and TER measured at timed intervals. TER rose between 30-40% above control levels in the IPTG treated monolayers. All inserts were rinsed twice with medium and fresh IPTG was added to the first set. Fresh medium without IPTG was added to the second set and to the third control set and TER measurements were continued. TER returned to control levels 55 hours after removal of IPTG, while those maintained in the presence of IPTG continued to maintain an elevated TER. It should be noted that while TER measurements were made at more frequent intervals than are indicated in the graph, for clarity of presentation not all values are shown. Their omission does not change the results as shown.

state TER achieved by chick occludin expressing monolayers were 32±8% higher than controls (242 vs 176 $\Omega \cdot cm^2$ at steady state) (Fig. 4a). The magnitude of this increase in TER was similar in all five IPTG inducible clones (data not shown). Addition of IPTG to nontransfected MDCK cells or to MDCK cells transfected with the *Lac* repressor construct alone, had no effect on TER measured over 48 hours.

In the second experiment, chick occludin transfected cells were plated at confluence in three sets of triplicate Millicell HA inserts in NCM and TER was measured at timed intervals. When TER reached steady state values, 5 mM IPTG was added to the first two sets and vehicle alone to the third set; TER measurements were continued until a new steady state value was reached. One set of monolayers was then rinsed free of IPTG and fresh NCM, without IPTG, added. The second set was rinsed and fresh NCM with 5 mM IPTG added. The last set was rinsed and the medium changed to fresh NCM. TER measurements were continued for an additional three days.

After plating at confluence, approximately 94 hours were required before steady state TER was achieved (Fig. 4b). In transfected MDCK cells whether IPTG was present or not, TER initially rose to a level that was well above the final steady state value before declining to steady state TER, a phenomenon routinely observed in nontransfected MDCK cells (Cereijido et al., 1983). Within 5 hours of adding 5 mM IPTG, TER began to rise and a new steady state was reached approximately 24 hours later. At this point the TER of the IPTG treated monolayers was 31% above that of the untreated controls (263±4 vs 201±2 $\Omega \cdot cm^2$). Following the addition of fresh IPTG, TER rose to 40% above baseline values and was maintained at this level in the presence of the inducing agent (Fig. 4b). Within 25 hours of removing IPTG, TER began to decline and reached control levels by 55 hours.

Expression of chick occludin and its effect on [^{3}H]mannitol flux

Net mannitol flux measurements were conducted in order to assess the effect of chick occludin expression on the permeability of the paracellular pathway to small water soluble solutes. Two types of protocols were utilized: in the first, freshly plated monolayers, in quadruplicate, were incubated with or without IPTG; media changes ± IPTG were made daily. Net mannitol flux was then measured on days 2, 3, 4, 7 and 11. TER was monitored throughout this period. In the second, three triplicate sets of monolayers were plated and when steady state TER was achieved at day four, IPTG was added to two triplicate sets; over the next three days, IPTG was administered daily with a fresh medium change. Control monolayers received a medium change at the same time. TER was monitored daily. On day seven, IPTG was removed from one experimental set and IPTG supplementation continued on the second set for another three days. Mannitol flux was measured on day ten.

Although absolute values varied between experiments, in the first two to three days after plating in the presence of IPTG, the mannitol flux in chick occludin expressing monolayers was lower or similar to controls. As the time in culture with IPTG lengthened, however, mannitol flux across chick occludin expressing monolayers progressively increased above those of controls (Table 1). Interestingly, TER values also continued to rise, indicating that the observed rise in mannitol flux was not due to a lifting of the monolayers from the substratum or to defects in the monolayers themselves. By day 11 both TER and mannitol flux were 47% and 46%, respectively, higher than controls (Table 1). These changes in mannitol flux and TER were readily reversed when IPTG was removed from the monolayers and chick occludin ceased to be expressed (Table 2).

Table 1. Net mannitol flux determination on chick occludin transfected MDCK cells

Time in culture (days)	IPTG (nM)	Number of doses of IPTG	Final TER* ($\Omega \cdot cm^2$)	% diff. of TER	Mannitol flux* ($nMol/cm^2$ per hour)	% diff. of manfl.
2	0	0	461±12	28	0.854±0.12	–7
2	5	2	592±19		0.790±0.10	
3	0	0	262±6	29	1.021±0.10	1
3	5	3	340±17		1.029±0.10	
4	0	0	221±4	26	1.110±0.20	12
4	5	4	279±17		1.248±0.30	
7	0	0	204±5	31	0.990±0.18	32
7	5	7	268±5		1.303±0.18	
11	0	0	216±1	47	1.078±0.02	46
11	5	11	318±10		1.573±0.48	

*****Values are mean ± s.d. of four determinations each.

Table 2. Alterations in mannitol flux in chick occludin transfected MDCK cells are reversible upon withdrawal of IPTG

Time in culture (days)	IPTG (nM)	Number of doses of IPTG	Final TER* ($\Omega \cdot cm^2$)	Mannitol flux* ($nMol/cm^2$ per hour)
10	0	0	187±7	0.731±0.12
10†	5	3‡	188±11	0.750±0.06
10	5	5	215±7	1.104±0.12

*****Values are mean ± s.d. of four determinations each.
†All monolayers were cultured for 5 days, before daily doses of 5 mM IPTG were applied.
‡IPTG was not present during the last three days of incubation.

Freeze fracture analysis of monolayers expressing chick occludin

Cell monolayers were incubated with or without IPTG for 48 hours in NCM. The cells were then processed for freeze fracture. Induction with IPTG for 48 hours resulted in a shift in the mean number of parallel TJ strands from 3.65±1.23 in non-induced monolayers to 4.29±0.93 in monolayers incubated with IPTG. When the data were plotted as a histogram the mode shifted from 3 strands in controls to 4 strands in monolayers induced to express chick occludin (Fig. 5), the mode being defined as the class of observations (strand numbers) that occurs most frequently (Ipsen and Feigl, 1970). In addition to a slight increase in the number of parallel strands, the network formed appeared to be somewhat more complex than those of controls (Fig. 6a,b,c), although this was not quantified. The prevalence of short segments of strands that appeared as closely spaced doublets was increased by 23% in IPTG treated monolayers (Fig. 6a,b,c). The increase in the number of parallel strands was associated with a 42% increase in the mean width ± s.e.m. of the TJ belt from 175±11 nm in controls (–IPTG) to 248±16 nm in IPTG induced monolayers.

When the morphometric data of the tight junction strand frequency were analyzed as a parallel electrical circuit (Table 3), the calculated TER for IPTG induced monolayers was 223 $\Omega \cdot cm^2$, a value which is approximately 47% higher than the calculated TER, 152 $\Omega \cdot cm^2$, of the noninduced monolayers. Although the calculated values are lower, they are within the range of measured TER values.

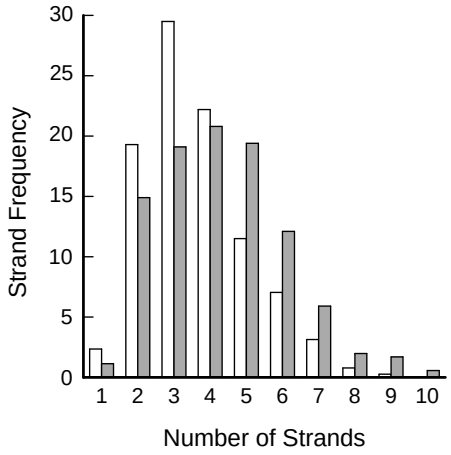

Fig. 5. The number of parallel strands were counted in freeze fracture replicas of tight junctions in chick occludin transfected cells incubated for 48 hours with or without 5 mM IPTG in NCM. In control, noninduced transfected cells, the mode of the strand counts is 3 (white columns), whereas in monolayers induced by IPTG to express chick occludin the strand count mode shifts to 4 (filled columns).

Expression of chick occludin and membrane associated ZO-1 and E-cadherin

Since E-cadherin may indirectly participate in TJ assembly (Gumbiner et al., 1988) and it has been suggested that ZO-1 may play a role in targeting occludin to the cell surface (Furuse et al., 1994), we determined whether the induction of chick occludin expression altered the amount of E-cadherin and ZO-1 at the cell surface. Two sets of transfected MDCK cell monolayers were plated at confluence in NCM in Petri dishes (10 cm in diameter). The first set was supplemented for 48 hours with 5 mM IPTG, while the second received vehicle alone. After preparing crude plasma membrane fractions, equal amounts of protein were applied to 7.5% SDS gels and analyzed by western blot. Expression of the avian protein had no detectable effect on the amount of E-cadherin or ZO-1, as determined by densitometry (Fig. 7). Furthermore, chick occludin was not detected in the absence of IPTG.

Correlation of chick occludin expression and TER

To relate the expression of chick occludin to the measured

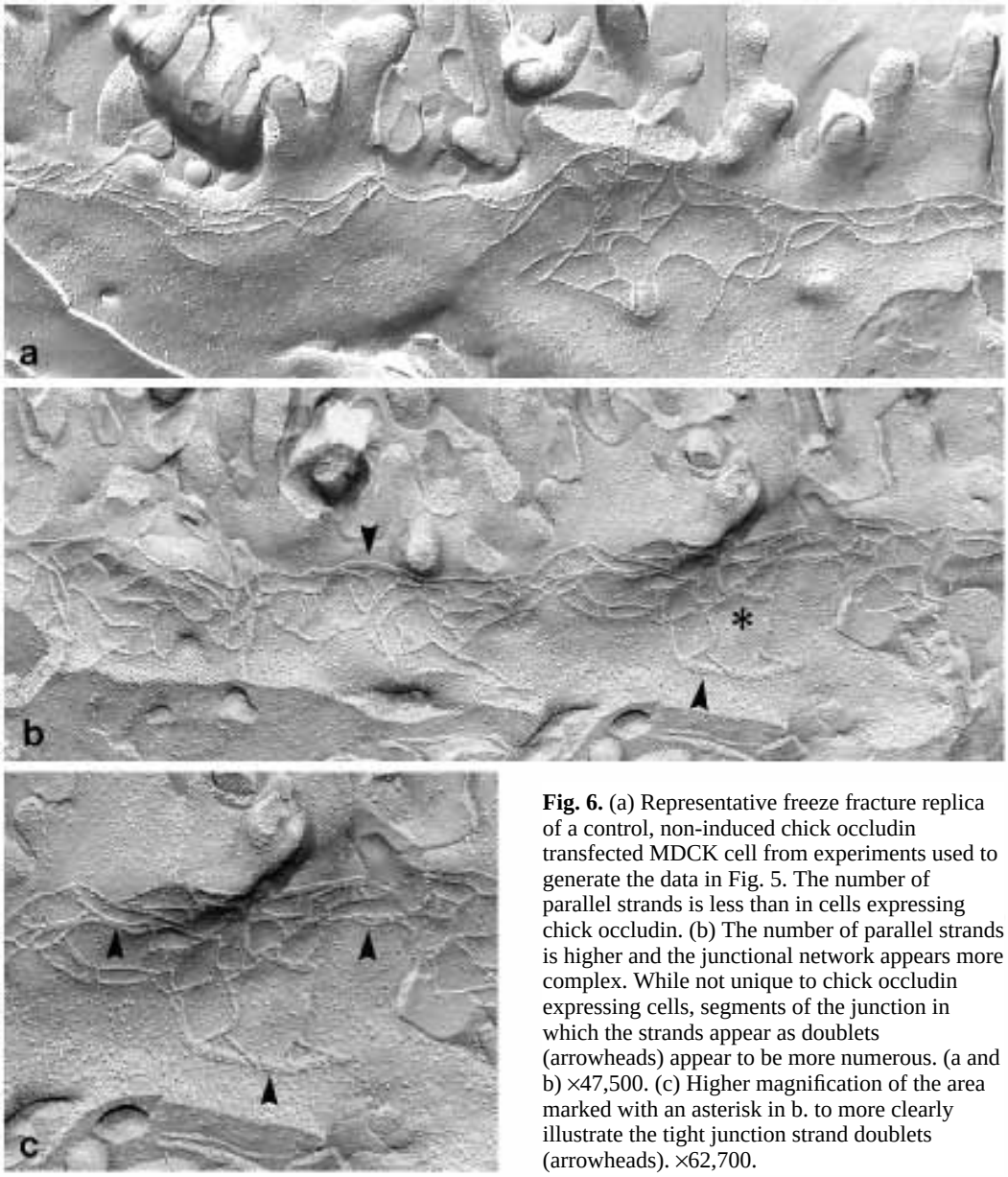

Fig. 6. (a) Representative freeze fracture replica of a control, non-induced chick occludin transfected MDCK cell from experiments used to generate the data in Fig. 5. The number of parallel strands is less than in cells expressing chick occludin. (b) The number of parallel strands is higher and the junctional network appears more complex. While not unique to chick occludin expressing cells, segments of the junction in which the strands appear as doublets (arrowheads) appear to be more numerous. (a and b) ×47,500. (c) Higher magnification of the area marked with an asterisk in b. to more clearly illustrate the tight junction strand doublets (arrowheads). ×62,700.

TER, a set of confluent monolayers plated in 10 cm in diameter Petri dishes in NCM was supplemented with IPTG for timed intervals before harvest. A second set of monolayers was supplemented with IPTG for 48 hours; the IPTG was then removed and after rinsing, the monolayers were cultured for another 24 to 72 hours before harvesting. Crude plasma membrane fractions were prepared from all the monolayers and equal amounts of protein were analyzed by SDS 7.5% PAGE. After electrophoretic transfer, chick occludin was detected on western blots and quantified by densitometry. Sequential TER measurements were made on parallel monolayers in Millicell HA inserts (0.6 cm^2). Monolayers plated on collagen coated coverslips were stained for chick occludin at parallel time points and examined by fluorescence microscopy.

Prior to addition of IPTG, no chick occludin was observed either on western blots (Fig. 8) or by immunofluorescence (Fig. 9a). The avian protein was first detected on western blots two hours after addition of IPTG (Fig. 8) which coincided with sparse, focal segments of linear staining at the plasma membrane of some cells (Fig. 9b). Five hours after addition of IPTG when TER began to increase (Fig. 4b), chick occludin was readily detected by western blot (Fig. 8) and a faint continuous, circumferential rim of staining was observed at the plasma membrane by 4 hours (Fig. 9c). The intensity of staining increased between 8 (Fig. 9d) and 24 hours (Fig. 9e) of IPTG supplementation. At present we have no plausible explanation for the apparent increased expression of chick occludin observed by western blot at 8 hours (Fig. 8) particularly since the amount of protein added to each lane was virtually identical. The level of chick occludin expression decreased by 24 hours after removal of IPTG (72nd hour of the experiment). TER fell to control values 55 hours after removal of IPTG (Fig. 4b) and chick occludin was no longer detectable by western blot at 72 hours after removal of the inducer (120th hour of the experiment). Chick occludin

Table 3. Analysis of parallel tight junction strand frequency as a parallel electrical circuit*

IPTG (nM)	Number of strands	Frequency	Resistance† ($\Omega \cdot cm^2$)	Frequency (F)×1/R† ($\times 10^{-5}$)	Total (F)($1/R_T$)	R_T ($\Omega \cdot cm^2$)
0	1	9	9.00×10^3	100.000		
	2	74	2.50×10^4	296.000		
	3	113	6.25×10^4	180.000		
	4	85	1.25×10^5	68.000		
	5	44	4.00×10^5	11.000		
	6	27	7.70×10^5	3.500		
	7	12	1.60×10^6	0.800		
	8	3	5.50×10^6	0.050		
	9	1	1.00×10^7	0.010		
	10	0	5.00×10^7	0.000		
					0.0066	151.5
5	1	4	9.00×10^3	44.000		
	2	53	2.50×10^4	212.000		
	3	68	6.25×10^4	108.800		
	4	74	1.25×10^5	59.200		
	5	69	4.00×10^5	17.250		
	6	43	7.70×10^5	5.580		
	7	21	1.60×10^6	1.400		
	8	7	5.50×10^6	0.130		
	9	6	1.00×10^7	0.060		
	10	2	5.00×10^7	0.004		
					0.0045	222.8

*The data used in these calculations are the same as those shown in Fig. 5.
†The resistance values were obtained from Fig. 4 of Claude (1978) and the calculations were similar to those discussed by Claude (1978).

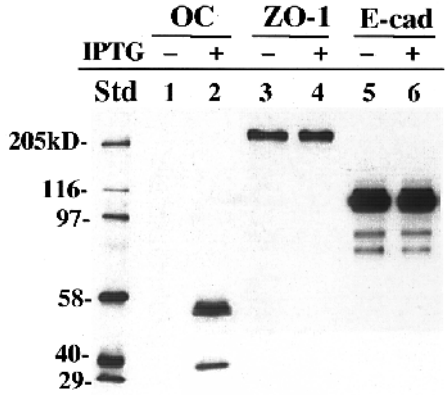

Fig. 7. Western blot analysis of a crude plasma membrane preparation of: (lanes 1,3,5) non-induced chick occludin transfected MDCK cells; (lanes 2,4,6) IPTG induced chick occludin transfected MDCK cells. Equal amounts of protein were separated on a 7.5% SDS gel and the electrophoretically transferred proteins were probed for chick occludin (lanes 1,2), ZO-1 (lanes 3,4), or E-cadherin (lanes 5,6). Densitometric measurements indicate that the amount of ZO-1 and E-cadherin detected was similar regardless of whether chick occludin was expressed (lane 2) or not (lane 1).

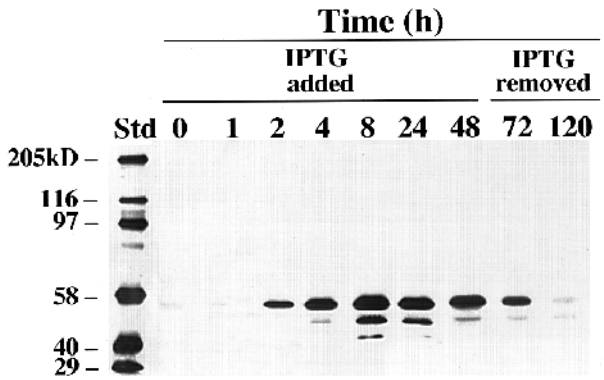

Fig. 8. Western blot analysis of chick occludin transfected MDCK cells harvested at timed intervals after addition and following removal of 5 mM IPTG. Chick occludin was first detected within 2 hours of addition of IPTG. It was no longer detectable 72 hours after removal of the inducing agent, a time when TER had returned to baseline values (Fig. 4b). Western blot analysis of ZO-1 in the same preparation showed a constant amount of the protein regardless of the presence or absence of IPTG (data not shown).

expression preceded the development of a measurable TER and a rising TER (Fig. 10) correlated with the development of a continuous, circumferential immunofluorescence staining pattern for chick occludin (Fig. 9). Both TER and chick occludin levels on western blots fell in parallel after removal of IPTG; trace amounts of the protein were still detectable on western blots when TER was at control levels.

DISCUSSION

With the identification of chick occludin, a ~65 kDa integral membrane protein that localizes precisely to the TJ (Furuse et al., 1993, 1994; Fujimoto, 1995) and the cloning of the mammalian occludin gene (Ando-Akatsuka et al., 1996), the goal of identifying an important component of the TJ strands observed by freeze fracture techniques has, in part, been achieved. In a recent study, the use of recombinant baculovirus infection to overexpress chick occludin in insect cells resulted in the formation of cytoplasmic multi-lamellar structures into which chick occludin was incorporated (Furuse et al., 1996). The outer leaflets of the apposed lamellar membranes appeared fused in a manner similar to that observed at TJ contact sites in mammalian cells. By freeze fracture immunolabeling, the protein appeared as short, intramembrane rods (Furuse et al.,

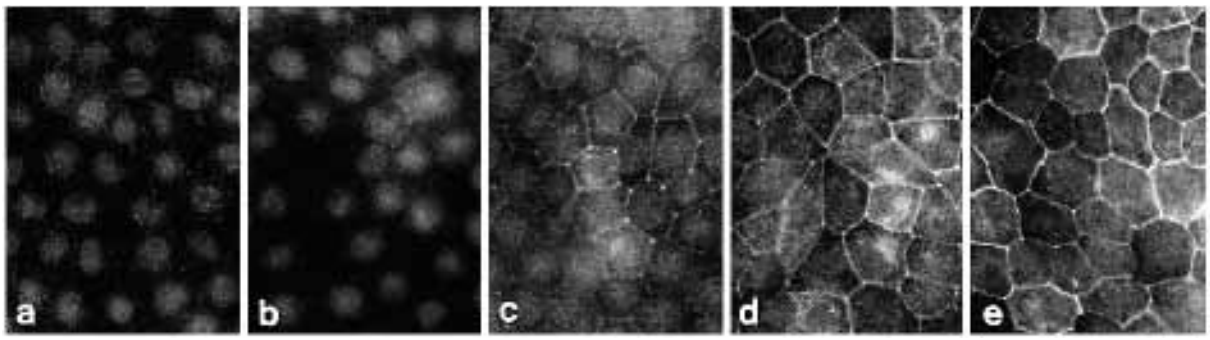

Fig. 9. Monolayers of chick occludin transfected MDCK cells cultured in NCM, fixed and stained for chick occludin with Oc-2 mAb at (a) 0, (b) 2, (c) 4, (d) 8 and (e) 24 h after addition of IPTG. (a) In the absence of IPTG there is no staining for chick occludin. (b) At 2 hours after addition of IPTG a few foci of faint linear staining is detected around the perimeter of some cells. (c) By 4 hours after addition of IPTG the staining at the perimeter of all cells is faint but continuous and the tricellular areas show bright punctate staining. The staining for chick occludin continues to increase in intensity between 8 (d) and 24 hours (c). ×250.

1996) that did not, however, form the typical network of TJ strands. That the presence of chick occludin was associated with sites of membrane 'fusion' suggests that the extracellular segments of the molecule appear to have an adhesive function. However, except for the latter study (Furuse et al., 1996) and the transient expression of chick occludin in mammalian cells (Furuse et al., 1993), no data were available concerning occludin's contribution to the barrier activities of the TJ in mammalian cells.

Time sequence of chick occludin expression

The present study was undertaken to examine occludin's contribution to TJ function. To accomplish this, advantage was taken of the Lac inducible expression system to stably transfect MDCK cells with chick occludin cDNA. This system increases expression of the protein of interest by as much as 14-fold (DuCoeur et al., 1992) and provides a means to control the timing of its expression. When induction was initiated in monolayers with intact TJ of canine origin, chick occludin began to appear in focal, linear segments of the plasma membrane two hours after addition of IPTG, the earliest time point that the protein was detectable by western blot. Continuous, fine linear staining at the plasma membrane was not observed, however, until four hours, close to the time when TER first began to rise above the baseline values of the canine TJ. A new steady state TER was achieved by 24 hours, which coincided with the accumulation of chick occludin, demonstrated by western blot, and its appearance as a distinct, continuous, immunofluorescent line localized precisely at the TJ, confirming the initial observations (Furuse et al., 1993). Within 24 hours of removing IPTG, TER began to decline and reached baseline values by 55 hours; chick occludin was no longer detected on western blots 72 hours after removal of IPTG. These observations suggest that, at least for the transfected avian occludin, there is active turnover of the protein at the TJ. Because of the relatively low homology (50%) between avian and mammalian occludin (Ando-Akatsuka et al., 1996), however, it is possible that interactions between avian and canine occludin as well as between avian occludin and canine para-junctional proteins (ZO-1, ZO-2) are less than optimal and might lead to a more rapid turnover of the transfected protein. Determination of the turnover rate of the native tight junction protein(s) awaits further study. While the immunolabeling data reported by Balda et al. (1996) for MDCK cells transfected with full length chick occludin cDNA are similar to those reported here, they employed a butyrate induced expression system which enabled them to achieve higher levels of expression and a more than threefold increase in TER.

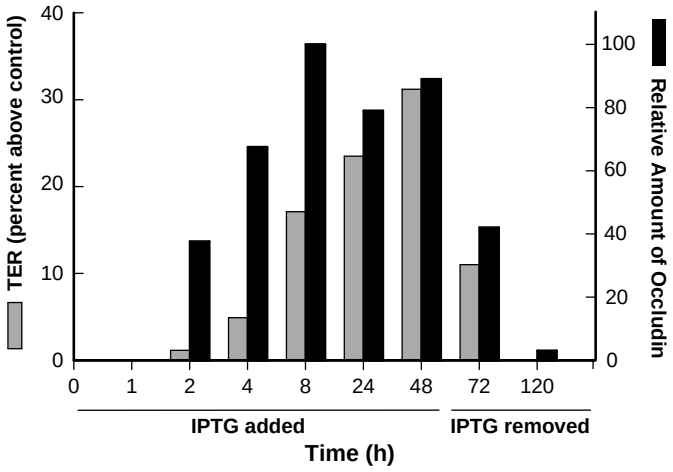

Fig. 10. Graph relating densitometric values of chick occludin detected by western blot and % increase in TER above control values, at intervals after addition and subsequent removal of IPTG.

Interaction of occludin with ZO-1

Data obtained with truncated forms of chick occludin suggested that the terminal 150 amino acids of the carboxy terminus are required for the binding of ZO-1 as well as the targeting of occludin to the cell membrane (Furuse et al., 1994). Recent data reported by Balda et al. (1996), however, clearly showed that truncated chick occludin lacking the carboxy terminus, and presumably not bound to ZO-1, localized efficiently to the TJ. However, unlike full length chick occludin, expression of the truncated form resulted in a discontinuous staining pattern by immunofluorescence. In these studies, the truncated avian protein may have been transported to the cell surface bound or complexed to native occludin. That ZO-1 may interact or at least be associated with occludin before the latter is incorporated into the TJ, is suggested by the observation that in cells maintained in low

calcium medium, chick occludin and canine ZO-1 co-localized to a paranuclear area and to scattered punctate sites in the cytoplasm, suggesting their co-localization in cytoplasmic vesicles. A few punctate sites, however, were labeled with canine ZO-1 alone, raising the possibility that they contained only ZO-1 or, alternatively, that at these sites ZO-1 was associated exclusively with canine occludin. Co-localization, does not imply that the two proteins are necessarily physically linked, but does suggest that they may be transported by the same vesicle population to the cell surface. Further studies will be required to determine precisely at what time point ZO-1, as well as other parajunctional proteins (ZO-2, ZO-3 and cingulin) become physically linked to occludin.

Evidence that occludin is a component of the TJ strand

The induced expression of chick occludin was associated with a 30-40% increase in TER and a 15% increase in the mean number of parallel TJ strands, as measured on freeze fracture replicas. The freeze fracture observations together with the immunofluorescence data, suggest that avian occludin was targeted to and incorporated into the structural elements of the canine TJ, although in the absence of freeze fracture immunolabel data, its precise location is unclear. In immunogold labeled freeze fracture replicas of chick hepatocytes and endothelium, however, occludin was precisely aligned with the TJ strands (Fujimoto, 1995). It is of interest that, while overexpression of chick occludin in insect cells provided evidence that occludin is an integral membrane protein with apparent adhesive properties, it did not result in the formation of the typical network of TJ strands (Furuse et al., 1996). This may have been due, in part, to a lack of avian ZO-1 in these cells which, by itself, did not interfere with occludin's ability to form homotypic adhesion sites. However, the possibility cannot be excluded that occludin may not be the sole component of the TJ strand.

If ZO-1 is involved in the correct targeting of occludin to the TJ, then the relatively modest expression of chick occludin achieved in the present study apparently did not outstrip the supply of canine ZO-1. Indeed, by western blot analysis alterations in canine ZO-1 levels were not observed at various times after the induction of chick occludin (data not shown). In their recent study, Balda et al. (1996) reported that, similar to our observations, MDCK cells transfected with full length chick occludin achieved an increase in TER and a modest increase in the number of parallel TJ strands (3.8±1.9 without and 4.7±2.6 with butyrate). However, the apparent relationship between TER and the number of TJ strands was not maintained when the N terminus of chick occludin was tagged with hemagglutinin or the carboxy terminus of the molecule was truncated.

The TJ as an electrical and permeability barrier

Induction of chick occludin expression by IPTG was associated consistently with a 30-40% increase in TER, and this increase was readily reversed upon removal of the inducing agent. It was, therefore, anticipated that if the expression of chick occludin made the paracellular barrier electrically tighter it should follow that the flux of small water soluble molecules such as mannitol through this pathway should be reduced. In the first two to three days after plating and exposure to IPTG, the net mannitol flux in chick occludin expressing monolayers was slightly less or the same and the TER was greater than in non-induced controls. However, as the time in culture and exposure to IPTG lengthened, both the net mannitol flux and the TER in fact were greater than in the chick occludin expressing monolayers than in controls. That this was not due to a defect in the monolayers or a toxic effect of IPTG on the cells was shown by the fact that both TER and mannitol flux returned to control levels after removal of IPTG.

How can these apparently contradictory results be reconciled with the TJ model originally proposed by Claude (1978) and later modified by Cereijido et al. (1989)? In this model, the TJ is viewed as a network of interconnected strands that act as though they contain aqueous pores that fluctuate between an open or closed state. Hydrophilic tracers would gain access to a compartment enclosed by the strands only if a given aqueous pore is in an open state and would be prevented from doing so if the pore or pores are in a closed state. The time required for a small hydrophilic molecule to traverse the TJ through the compartments in the TJ network would be considerably greater than that required for the instantaneous electrical pulse used to measure TER. The latter, therefore, is a measure of the open or closed state of pores within TJ strands at any instant in time.

Our observation that TER remains elevated in chick occludin expressing cells, while the mannitol flux is initially slightly lower or the same, but over time becomes higher than that of monolayers expressing only native occludin, suggests the following possibilities. The interaction of occludin with ZO-1 may regulate occludin's activity as a sealing element. At low levels of chick occludin expression, such as those achieved in our preparations, there may be sufficient ZO-1 available initially to interact with chick occludin, enabling the induced protein to perform primarily as a seal, and therefore producing an elevated TER and a slightly reduced mannitol flux. This is supported by the observation that within the first 24-48 hours of incubation in LCM, chick occludin always co-localized to the same punctate sites in the cytoplasm as ZO-1 and was not observed to localize alone at these sites. The latter might occur if the induced chick occludin had outstripped the supply of ZO-1. It is possible that as the time of exposure to the inducing agent increased, the supply of ZO-1 becomes insufficient so that a certain proportion of chick occludin is no longer associated with ZO-1, resulting in a channel forming rather than a sealing property of occludin. This would be reflected in an elevated TER and an increased mannitol flux. Support for this possibility comes from the observations reported by Balda et al. (1996) in which overexpression of carboxy terminus truncated chick occludin resulted in a marked increase in both TER and mannitol flux. At present it is unclear to what extent the interaction of the various parajunctional proteins (ZO-1, ZO-2, ZO-3 and cingulin) with occludin has on the latter's conformation and function. It is tempting to speculate that the activity of cytoskeletal elements might control the level of TJ sealing and if this occurred intermittently it might account for the fluctuating pores predicted by Claude (1978). The possibility cannot be excluded that occludin may not be the sole subunit of the TJ. One might speculate that occludin, with its unique extracellular loops rich in tyrosine and glycyine residues (Furuse et al., 1993), would primarily have a sealing function. With maturation of the TJ, and to account for the increase in mannitol flux, a second, aqueous pore forming protein might be inserted into the tight junction strands.

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