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Redistribution and Phosphorylation of Occludin During Opening and Resealing of Tight Junctions in Cultured Epithelial Cells

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Abstract. We studied the expression, distribution, and phosphorylation of the tight junction (TJ) protein occludin in confluent MDCK cell monolayers following three procedures for opening and resealing of TJs. When Ca^{2+} is transiently removed from the culture medium, the TJs open and the cells separate from each other, but the occludin band around each cell is retained. When Ca^{2+} is reintroduced, the TJs reseal. When the monolayers are exposed to prolonged Ca^{2+} starvation the cells maintain contact, but occludin disappears from the cell borders and can be detected only in a cytoplasmic compartment. When Ca^{2+} is reintroduced, new TJs are assembled and the transepithelial electrical resistance (TER) is reestablished in about 20 hr. Monolayers treated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) show a different pattern of TJ opening: the cell-cell contact is maintained but the TJ strand network, as seen in freeze-fracture replicas, becomes discontinuous. Occludin is still localized at the cell periphery, but in a pattern of distribution that matches the discontinuous TJ. These TJs do not reseal even 24 hr after removal of the TPA. Western blot analysis showed that the 62–65 kD double band of occludin did not change with these treatments. However, in vivo phosphorylation analysis showed that the TPA treatment reduced the phosphorylation levels of occludin, while the prolonged Ca^{2+} starvation completely dephosphorylated the two occludin bands. In addition, a highly phosphorylated 71 kD band that immunoprecipitates with occludin is not present when TJ is opened by the Ca^{2+} removal. Phosphoaminoacid analysis showed that the 62–65 kD occludin bands are phosphorylated on serine and threonine, while the 71 kD band was phosphorylated exclusively on serine. Our results provide further evidence that phosphorylation of occludin is an important step in regulating TJ formation and permeability.

Key words: Tight junction — Occludin — ZO-1 — Transepithelial resistance

Introduction

The tight junction (TJ), the most apical component of the mammalian junctional complex in epithelial cells, forms a diffusion barrier that regulates the flux of hydrophilic molecules through the paracellular pathway (Fromter & Diamond, 1972). Structurally, the TJ forms a continuous network of parallel, interconnected intramembrane strands, presumably rows of transmembrane proteins with associated cytoplasmic components (Staehelin, 1973; Lane, Reese & Kachar, 1992; Anderson & van Itallie, 1995; Denker & Nigam, 1998). Although the function of the TJ has been well characterized, the molecular composition of the TJ strands (Kachar & Reese, 1982; Pinto da Silva & Kachar, 1982; Gumbiner, 1987; Simons, 1990; Saitou et al., 1998; Furuse et al., 1998*b*) and the mechanism of strand formation (Gumbiner, 1987; Kachar & Pinto da Silva, 1991; Jaeger et al., 1997; Denker & Nigam, 1998) are still not well understood.

TJs are highly dynamic structures regarding their morphological aspects and physiological behavior, the latter characterized by the degree of sealing the TJs impose on the paracellular pathway. The TJs respond to a series of physiological, pathological and pharmacological challenges (reviewed by Anderson & van Itallie, 1995). However, the mechanisms involved in the control of the TJ seal are not understood. Several studies, most of which carried out with cultured monolayers, indicate that complex cellular signaling mechanisms are

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involved. The sealing capacity of TJs in natural epithelia as well as in cultured monolayers depends not only on the length and number of tight junction strands (Claude, 1978) but also on other factors including the phosphorylation state of junctional components (Stevenson et al., 1986). For example, studies have shown that TJ permeability can be regulated by protein kinase activation (Jacobson, 1979; Duffey et al., 1981; Ojakian et al., 1981; Balda et al., 1991; Ellis, Scheneeberger & Rabito, 1992; Mullin et al., 1992). In particular, 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter and known activator of protein kinase C, has been shown to disrupt TJ (Ojakian et al., 1981; Mullin et al., 1992) and to inhibit TJ formation (Balda et al., 1991).

Several TJ-associated proteins have been identified, and they include ZO-1 (Stevenson et al., 1986), cingulin (Citi et al., 1988), ZO-2 (Gumbiner et al., 1991), 7H6 (Zhong et al., 1993), occludin (Furuse et al., 1993) and more recently claudin-1 and claudin-2 (Furuse et al., 1998*a*). Occludin and claudin-1 and claudin-2 are integral membrane proteins localized at the points of membrane-membrane interaction of the TJ (Furuse et al., 1993; 1998*a, b*). It was initially suggested that occludin forms the intramembrane strand component of the TJs (Furuse et al., 1994; Balda et al., 1996; McCarthy et al., 1996; Hirase et al., 1997; van Ittalie & Anderson, 1997). Several reports have directly implicated occludin in the formation and resealing of TJs (Balda et al., 1996; McCarthy et al., 1996; Lacaz-Vieira et al., 1999). Occludin is a highly phosphorylated protein (Cordenonsi et al., 1997; Farshori & Kachar, 1997; Sakakibara et al., 1997; Wong, 1997). It has also been shown that highly phosphorylated occludin is selectively concentrated at TJs while nonphosphorylated or less phosphorylated occludin is distributed in the basolateral membrane and in the cytoplasm (Sakakibara et al., 1997), suggesting that occludin phosphorylation is an important step in TJ assembly. However, recently it has also been shown that occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing TJs, indicating that other TJ integral membrane proteins may recruit ZO-1 and function as a barrier without occludin (Saitou et al., 1998).

To study the role of occludin and occludin phosphorylation in TJ formation and in the regulation of TJ permeability, we compared the expression, distribution and phosphorylation of occludin in MDCK cell monolayers following three different procedures for opening and resealing of TJs.

Materials and Methods

Cell Culture

MDCK cells obtained from American Type Culture Collection (Rockville, MD) were plated on Transwell filters (Costar) or on glass coverslips and cultured at 37°C and 5% CO_2 in minimum essential medium (MEM) with Earl's salt (GibcoBRL) supplemented with 10% fetal bovine serum and with antibiotic-antimycotic solution (GibcoBRL).

TER

Equal number of MDCK cells were plated on polycarbonate 24 mm Transwell filters and allowed to form a confluent monolayer for several days. TER was measured using an epithelial voltohmmeter (World Precision Instruments, New Haven, CT) operating at a constant current of 20 μA. The electrical resistance of the filter and the media was subtracted from the measured values. Results are expressed as percent change in TER as compared to the pretreatment level.

Disruption and Recovery of TJs

The TJs were opened by three different procedures: (i) a rapid Ca^{2+} switch, by replacing, for 10–15 min, the normal Ca^{2+} culture medium with a Ca^{2+}-free medium containing 2.5 mM EGTA; (ii) a prolonged Ca^{2+} removal and Ca^{2+} switch, in which the monolayers were incubated overnight in a Ca^{2+}-free medium and then allowed to recover in a normal Ca^{2+} medium (Ca^{2+} Switch) for several hr (Gonzalez-Mariscal et al., 1990); and (iii) treatment with 1–2 μM TPA for 30, 60, or 90 min. Following TJ opening treatments, the monolayers were rinsed and allowed to recover for 5–24 hr in the normal Ca^{2+} culture medium.

Antibodies

We used two different affinity purified anti-occludin polyclonal antibodies, one commercially available from Zymed Lab (San Francisco, CA) and one custom made against the synthetic peptide QELPATSPADDFRQPRYSS corresponding to amino acids 351 to 369 of the cytosolic domain of the dog occludin sequence (Ando-Akatsuka et al., 1996). Immune sera were affinity purified against the immunizing peptide. Affinity purified rat anti-ZO-1 was obtained from Chemicon (Temecula, California), while fluorescein-conjugated or Texas red-conjugated secondary antibodies were obtained from Amersham (Arlington Heights, IL).

Immunocytochemistry

Cells grown on coverslips were rinsed in PBS, fixed, and permeabilized with 1% Triton X-100 in 2% paraformaldehyde, rinsed in PBS, and incubated in blocking solution (3% BSA and 3% normal goat serum) for 30 min. After blocking, the cells were incubated with primary antibody (1:50 dilution in PBS) for 60 min at 37°C. Following primary antibody incubations, coverslips were washed in PBS and incubated with FITC–labeled donkey anti-rabbit antibody (Amersham). Cells were again rinsed with PBS three times and mounted in ProLong mounting medium (Molecular Probes, Eugene, OR). For double labeling, cells were incubated with a fluorescein-labeled donkey anti-rabbit antibody against the occludin antibodies, followed by Texas-Red labeled sheep anti-rat antibody against ZO-1. Slides were viewed with a Zeiss Axiophot using a 100X Plan-Neofluar 1.3 N.A objective. Double

labeling was observed with the XF53 dual excitation filter (Omega, Brattleboro, Vermont). As negative controls we pre-adsorbed the primary antibody with an excess of the immunizing peptide or used the secondary antibody alone.

Freeze-fracture Electron Microscopy

MDCK cells were grown to confluency in T75 tissue culture flasks. The day of the experiment cells were rinsed in PBS and fixed in 2% glutaraldehyde in PBS. Following fixation cells were gently rinsed in PBS, scraped and pelleted in an eppendorf tube. The cell pellet was incubated in 30% glycerol in PBS, mounted and frozen in liquid Freon 22 for conventional freeze-fracture. Samples were freeze-fractured at −110°C in a Balzers 301, shadowed with platinum-carbon and observed in a Zeiss 902 TEM.

Gel Electrophoresis and Western Blotting

MDCK cells were scraped from the bottom of the T75 tissue culture flasks, centrifuged at 1000 rpm, washed twice in PBS and sonicated in 500 μl of boiling stop buffer (20 mM Tris with 0.5% SDS). After sonication, 4× sample buffer (200 mM Tris, pH 7.0; 8% SDS, 0.02% bromophenol blue, 40% β-mercaptoethanol plus 20% glycerol) was added and the extracts were electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Following the incubation in the blocking buffer (5% dry milk in PBS), the nitrocellulose membrane was incubated for 2 hr with the primary antibody, washed with 0.3% Tween in PBS and incubated with peroxidase-conjugated secondary antibody for 1 hr. Finally, the membrane was washed 3 times and developed with peroxidase substrate (Sigma DAB tablet with metal enhancer) or with ECL reagents (Amersham).

Phosphorylation and Immunoprecipitation

MDCK cells were grown to confluency in T75 flasks and were washed and incubated for 1 hr in phosphate-free DMEM (Cellgro) containing 0.2% BSA. The cells were then metabolically labeled by adding 100 μCi/ml ^{32}P (NEN-Dupont) to each flask for 3–4 hr. At this point some cells were treated with 2.5 mM EGTA (in Ca^{2+}-free medium) for 15 min, or with 2 μM TPA for 90 min.

For prolonged Ca^{2+} removal and Ca^{2+} switch experiments cells were grown to confluence in T75 flasks. A day before the experiment confluent cells from one flask were trypsinized and re-plated in the same flask in normal culture medium for 90 min. Then, the cells were gently washed in Ca^{2+}-free medium containing 2% FBS (serum was dialyzed extensively in Ca^{2+}-free medium) and incubated for 20 hr. The next day, these cells were metabolically labeled in Ca^{2+} and phosphate free medium as mentioned above. Following treatment, flasks were rinsed in warm PBS to remove unincorporated ^{32}P. Finally, the reaction was stopped by adding 8 ml of boiling stop buffer (20 mM Tris, 0.5% SDS) to each flask. Cells extracts were removed from the flasks and passed twice through a syringe with a 23-gauge needle to obtain membrane-free cell extracts.

The cell extracts were transferred to 100 mM Tris-HCl, pH 7.8, containing 0.3 M NaCl, 4 mM EDTA and 2% Triton X-100 in order to inhibit the SDS action. Then, extracts were centrifuged at 2000 rpm for 5 min to remove any cell debris. The samples were pre-incubated for 30 min in the presence of protein G agarose beads (50 μl of a 10% wt./vol. mixture, GibcoBRL). The beads were pelleted by low speed centrifugation (1,000 rpm for 5 min) and the supernatant was collected. Then, 15 μl of affinity purified anti-occludin antibody was added to each supernatant fraction and samples were left overnight on a shaker at room temperature. Following primary antibody incubation, protein G agarose beads were added to the supernatant. After 3 hr of incubation, the beads were pelleted by centrifugation and washed (three times, 15 min each) in wash buffer (10 mM Tris, pH 7.8, 0.2% SDS, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA). Following the final wash, the beads were pelleted and resuspended in 4× sample buffer. Equal volumes of these immunoprecipitates were electrophoresed on a 10% polyacrylamide gel. The gels were stained with Coomassie Blue, destained in 10% acetic acid and dried on a gel drier (Savant). The dried gels were exposed to Kodak X-OMAT film to obtain autoradiograms of immunoprecipitated occludin. Some of the immunoprecipitated samples were transferred to a nitrocellulose membrane for Western blot analysis. All phosphorylation experiments were repeated at least three times.

Phosphoamino-acid Analysis

Phosphoamino-acid analysis was performed on phosphorylated occludin which had been immunoprecipitated from the confluent monolayers. First, the immunoprecipitated samples were electrophoresed (10% acrylamide gel) and transferred to immobilon-P membrane (Millipore). Individual slices of the immobilon-P membrane were placed in a 1.5 ml screw cap tube and incubated with 15 μl methanol and 150 μl of 6 N HCl. Samples were heated at 110°C for 2 hr, lyophilized, dissolved in water and lyophilized again. To these hydrolyzed and lyophilized samples 6 μl of 2 mg/ml combined standards (phospho-serine, -threonine and -tyrosine) were added. Samples were now spotted on a plate in 1 μl increments for a total of 4 μl. The plate was placed in an E-C apparatus Isoelectric Focusing System (Biotechnology) and samples were run at 800 volts for 90 min. Then, the plate was dried and sprayed with 0.25% ninhydrin in acetone in the hood. The plate was vacuum dried for 1 hr and placed in a phosphorimager cassette (Molecular Dynamics, Sunnyvale, California) for 4–5 days and images were obtained by using the software Multi Quant (Molecular Dynamics, Sunnyvale, California).

Results

Effects of Transient Ca^{2+} Removal with EGTA

Confluent MDCK monolayers growing on polycarbonate filters were transferred to a Ca^{2+}-free medium and treated with 2.5 mM EGTA. TER was measured at 0, 1, 3, 5, and 10 min after the EGTA treatment. After 1 min of EGTA treatment the TER decreased to 43% of the pretreatment value. The TER progressively dropped to 29.2%, 20.8%, and 13.8% after 3, 5 and 10 min of EGTA treatment, respectively. Removal of EGTA after 10 min and reintroduction of Ca^{2+} in the medium restored the TER to 90% of its pretreatment value within 5 hr (Fig. 1*a*).

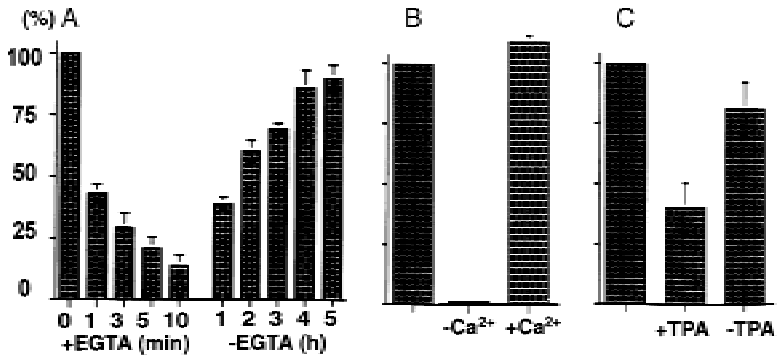

Fig. 1. The effects of EGTA, prolonged Ca^{2+} removal, and TPA on the TER of MDCK cells. (*A*) MDCK cells were grown to confluency on TER filters and pretreatment TER (pre TER) was measured and expressed as 100%. Then the cells were incubated in Ca^{2+} free medium containing 2.5 mM EGTA. The TER was measured after 1, 3, 5, and 10 min of incubation. Following 10 min incubation the cells were allowed to recover in normal culture medium. During recovery, the TER was measured every hour for five hours. Values are expressed as % change in the TER from the pre TER levels. (*B*) To see the effects of prolonged Ca^{2+} removal on TER, MDCK cells were grown for 20–22 hr in a Ca^{2+}-free medium and TER was measured to be zero. Then the cells were allowed to recover in normal Ca^{2+} medium for 20 hr and the TER was measured again. (*C*) To study the effects of 2 μM TPA, cells were grown on filters and the pre TER was measured. Then, the cells were incubated with DMSO (control) or with 2 μM TPA for 90 min and the TER was measured again. For recovery experiments, the filters were gently washed in TPA free medium and allowed to recover for 24 hr. Following recovery, the TER was measured and the cells were fixed for immunocytochemistry. The values are expressed as means ± SD of at least 3 coverslips.

Since both the occludin antibodies gave similar immunofluorescent staining patterns and on Western blots recognized a 62–65 kD doublet as the major occludin band in MDCK cell extracts (*data not shown*), we used the custom-made antibody for all immunofluorescence experiments. Our immunofluorescence results showed that within minutes of Ca^{2+} removal the tight junctions open and the cells begin separating. However, at these early times we could not detect any changes in the distribution and localization of occludin and ZO-1 (Fig. 2). After 30 min of Ca^{2+} removal, when a large number of cells have been separated from the monolayer, the occludin/ZO-1 labeling changes from a continuous to a punctated pattern (Fig. 2). Restoration of Ca^{2+} by removal of EGTA from the extracellular medium and incubation of the cells in normal Ca^{2+} medium (1.8 mM) for 5 hr induced the monolayers to recover, with the cells acquiring normal morphology and with the formation of the characteristic continuous honeycomb localization of ZO-1 and occludin (Fig. 2, bottom panel).

Effects of Prolonged Ca^{2+} Removal and Ca^{2+} Switch

TER was measured to be zero after 20–22 hr incubation of confluent monolayers in a Ca^{2+}-free medium. Cells were then allowed to recover in a normal Ca^{2+} medium for 20–22 hr. As a result, the TER returned to control levels (Fig. 1*b*).

Immunocytochemistry showed that after 3–4 hr of Ca^{2+} switch ZO-1 and occludin began to appear at the cell periphery (Fig. 3). Although TJs were not completely formed in all the cells within 3–4 hr of the Ca^{2+} switch, this time was enough to show that TJ-associated proteins have started to migrate to the cell periphery. It also appears that the arrival of ZO-1 to the periphery precedes that of occludin (*see* arrows in Fig. 3). After 20–22 hr the TER was reestablished (Fig. 1*b*) and the ZO-1 and occludin form the characteristic continuous honeycomb pattern of the TJ (Fig. 3 bottom left and right).

Effect of TPA

Incubation of the MDCK monolayers with TPA for 60–90 min caused a significant decrease of the TER (Fig. 1*c*). The TER decrease was accompanied by a change in the pattern of localization of ZO-1 and occludin (Fig. 4). The labeling, while still forming the honeycomb pattern outlining the cell-cell contacts of the monolayers, appeared distinctively discontinuous. Freeze-fracture replicas show, that the network of strands changed from a continuous belt, made of a network of 3–5 mostly parallel strands, into discontinuous patches of meandering strands (Fig. 4). It was frequently seen in the TPA-treated monolayers regions of cell-cell contact sealed only with a single TJ strand or not sealed at all (Fig. 4). The size and the distribution of the patches of meandering TJ strands correlated with the pattern of the distribution of ZO-1 and occludin immunofluorescence, suggesting that a redistribution of these two proteins accompanies the rearrangement of the TJ strands. The TPA induced rearrangements of the TJ strands and of the distribution of ZO-1 and occludin were not reversed to their normal continuous appearance even 24 hr after removal of the TPA from the medium (Fig. 4) and the TER never recovered to 100%.

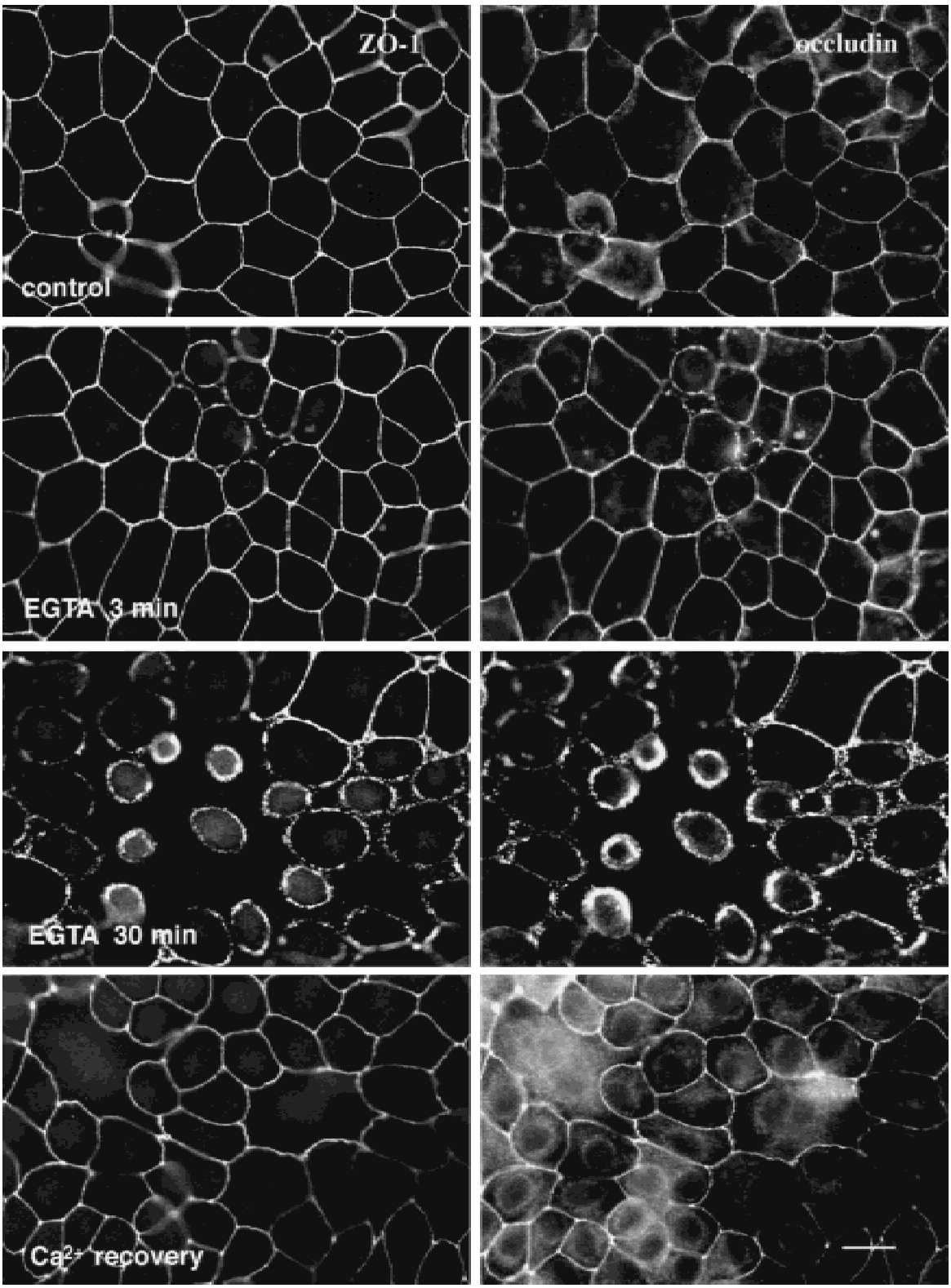

Fig. 2. Immunofluorescent localization of ZO-1 and occludin in MDCK cells after treatment with 2.5 mM EGTA. MDCK cells were grown on glass coverslips, rinsed in Ca^{2+} free medium and incubated with 2.5 mM EGTA. The cells were fixed as described in Materials and Methods at 0 (control), 3, and 30 min intervals and double labeled with affinity purified ZO-1 (left) and occludin (right) antibody. The EGTA treatment induces the MDCK cells to separate from each other, but the belt of ZO-1/occludin in each cell is maintained. After 30 min EGTA treatment cells round up and ZO-1/occludin show punctate patterns along the regions of cell-to-cell contacts. When the monolayer is returned to the normal Ca^{2+} medium, the TJs reseal and the ZO-1 and occludin labeling returns to normal (bottom left and right). Bar, 10 μm.

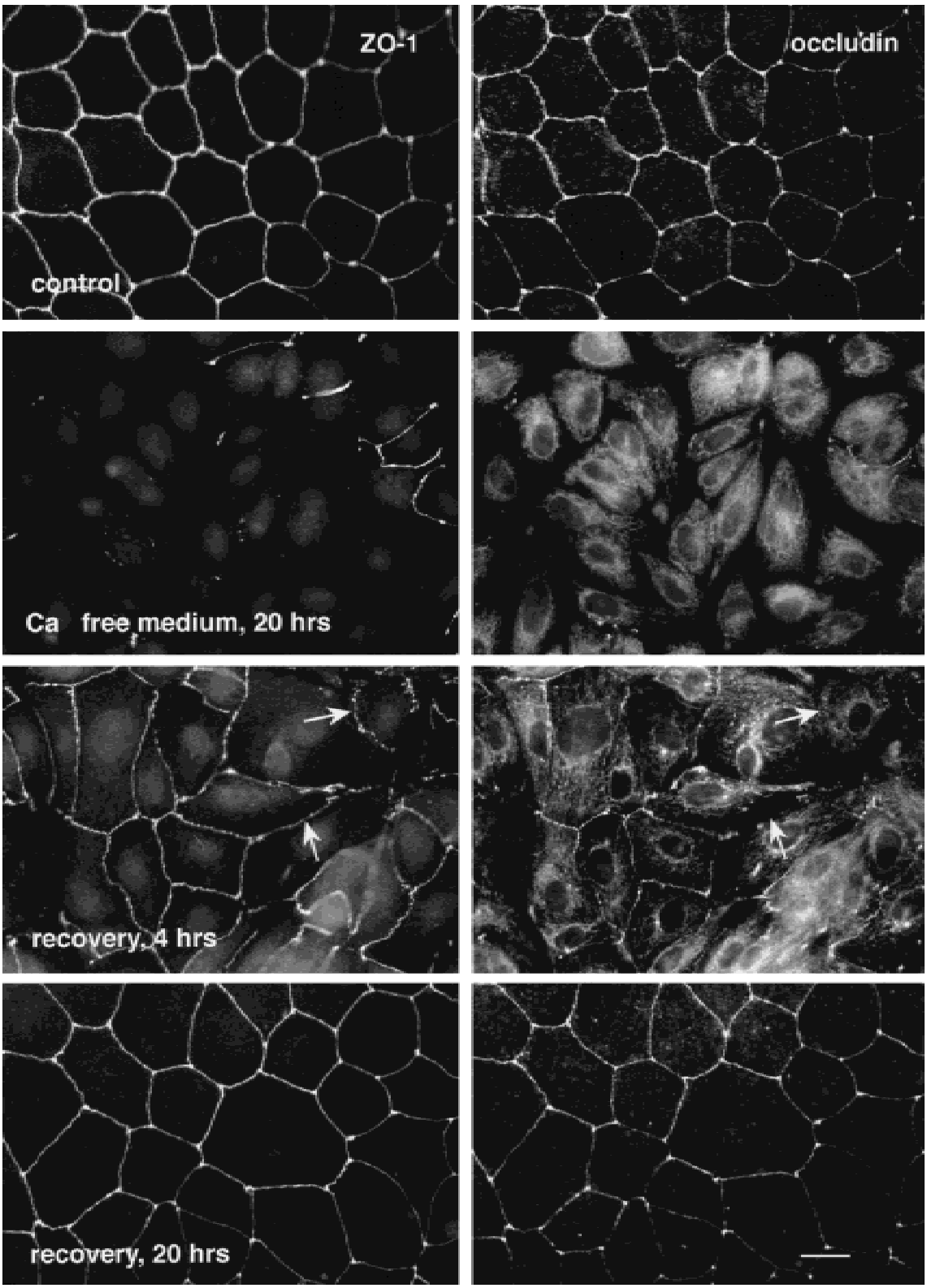
ZO-1
occludin
control
Ca free medium, 20 hrs
recovery, 4 hrs
recovery, 20 hrs

Fig. 3.

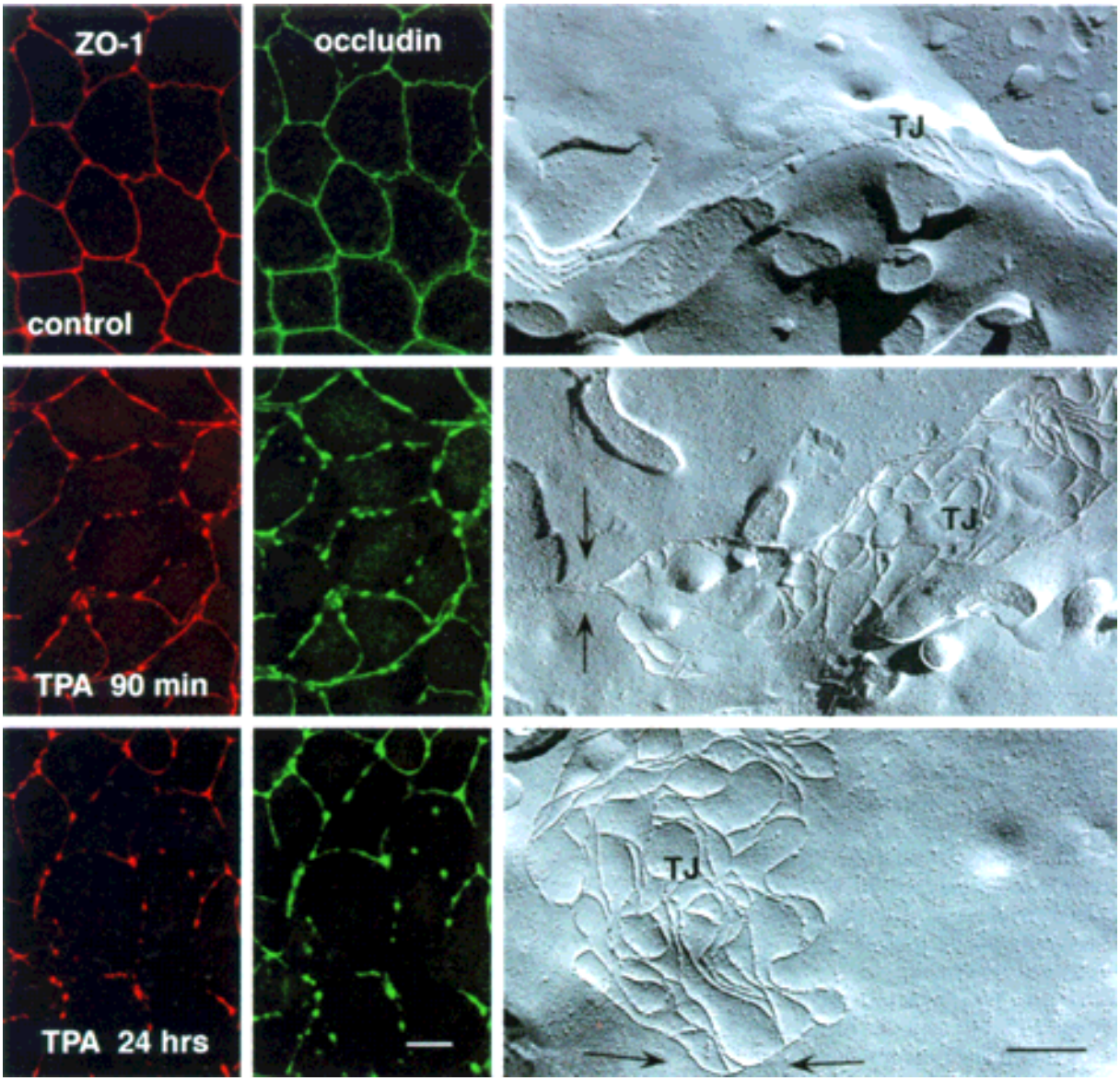

Fig. 4. Immunofluorescent micrographs and freeze-fracture views of MDCK cells to show the effects of TPA on the TJ associated proteins, ZO-1 and occludin. MDCK cells were grown to confluency on glass coverslips and treated with 2 μM TPA for 0 min (controls) and 90 min, or treated for 90 min and allowed to recover for 24 hr, before fixation. Fixed cells were double labeled with affinity purified ZO-1 (left) or occludin (center) antibody. Bar, 10 μm. Figures to the right represent freeze fracture views of TJs of confluent MDCK cells. The size and the distribution of the patches of meandering TJ strands correlated with the pattern of distribution of ZO-1 and occludin immunofluorescence, suggesting that a redistribution of these two proteins accompanies the rearrangement of the TJ strands. Bar, 0.2 μm.

Fig. 3. Immunofluorescent localization of ZO-1 and occludin in MDCK cells after 20 hr of Ca^{2+} starvation, followed by recovery in Ca^{2+} containing medium. The MDCK cells were plated on glass coverslips in culture medium. After 90 min incubation in the normal medium the cells were gently washed (3×) in Ca^{2+}-free medium and incubated for 20 hr in this medium. Following the incubation the cells were allowed to recover in normal Ca^{2+} medium (Ca^{2+} switch) for 4 hr or 20 hr, fixed, and double labeled with affinity purified ZO-1 (left) and occludin (right) antibody. A 20 hr Ca^{2+} starvation causes a nearly complete removal of occludin and ZO-1 from the cell periphery, except for few cell-to-cell contact points, where ZO-1 is still intact. Four hours after returning to normal Ca^{2+} medium, occludin and ZO-1 start to reappear at the TJs. Our results show that there are many points where occludin and ZO-1 do not colocalize (arrows). It appears that ZO-1 arrives at the cell periphery faster than occludin. After 4 hr of Ca^{2+} switch, most of the occludin is still in the cytoplasm, while ZO-1 appears to have reached the cell periphery. However, after 20 hr of recovery, occludin and ZO-1 colocalize at the cell periphery. Bar, 10 μm.

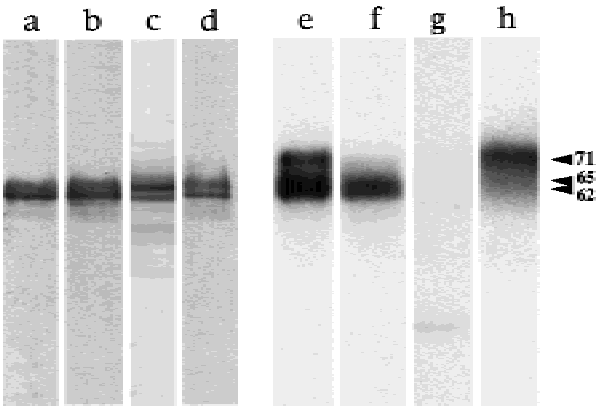

Fig. 5. Immunoblot analysis of cell extracts (*a–d*) and in vivo phosphorylation (*e–h*) after treatments with EGTA, TPA and prolonged Ca^{2+} starvation. (*a*) Immunoblots of cell extracts of MDCK control cells incubated with DMSO alone; (*b*) treated with 2.5 mM EGTA; (*c*) no Ca^{2+} for 20 hr; (*d*) 2 μM TPA. Panels *e–h* represent autoradiographs of immunoprecipitated occludin from phosphorylated (*e*) DMSO treated MDCK control cells; (*f*) treated with EGTA; (*g*) no Ca^{2+} for 20 hr; (*h*) 2 μM TPA for 90 min.

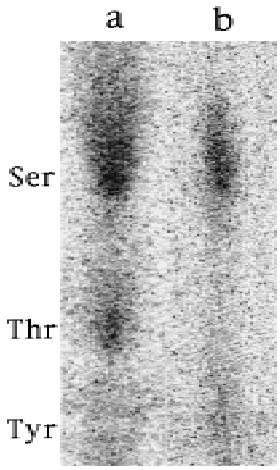

Fig. 6. Phosphoaminoacid analysis of occludin immunoprecipitated from metabolically labeled MDCK cells. MDCK cells were metabolically labeled, occludin immunoprecipitated, electrophoresed and transferred to the immobilon membrane. Phosphoaminoacid analysis was performed on (*a*) the occludin doublet or (*b*) the 71 kD band by the procedure described in Materials and Methods.

Western Blot Analysis and Phosphorylation of Occludin

Western blot analysis of MDCK cell extracts using affinity-purified occludin antibodies showed that the 62–65 kD doublet band was present after either the short or the prolonged Ca^{2+} removal from the culture medium (Fig. 5) and during or after the TPA treatment. Since total cell extracts were used for Western blot analysis we could not distinguish between the pool of occludin that is associated with the TJ from the pool of occludin in the cytoplasmic compartment.

In control monolayers metabolically labeled with ^{32}P-orthophosphoric acid the 62–65 kD doublet bands showed substantial ^{32}P incorporation (Fig. 5*e–f*). The affinity purified antibodies from cell extracts of these monolayers consistently immunoprecipitated a phosphorylated 71 kD band in addition to the 62–65 kD doublet band (Fig. 5*a–d*). The 71 kD band was not observed in the EGTA-treated (Fig. 5*f*) and 20 hr Ca^{2+}-starved (Fig. 5*g*) monolayers, nor did we detect the phosphorylated 62–65 kD bands in the 20 hr Ca^{2+}-starved monolayers (Fig. 5*g*). Also, from these Ca^{2+}-starved metabolically labeled extracts, our occludin antibody consistently immunoprecipitated a 25 kD phosphorylated polypeptide (Fig. 5*g*). Since in all our experiments the cells were pelleted and immediately sonicated in a boiling Tris-SDS buffer, it is unlikely that this band was a result of proteolytic fragmentation.

TPA treatment caused extensive dephosphorylation of the 62–65 kD occludin doublet band, leaving only traces of the phosphorylation signal, while the 71 kD band remained phosphorylated (Fig. 5*h*).

Phosphoamino-acid analysis of the immunoprecipitated occludin showed that occludin was phosphorylated predominantly on serine residues, less on threonine residues (Fig. 6*a*). Phosphoamino-acid analysis of the 71 kD band showed phosphorylation exclusively on serine residues (Fig. 6*b*).

Discussion

We studied the expression, distribution, and phosphorylation of occludin in MDCK cell monolayers following three different procedures for disruption and re-assembly of TJs. When Ca^{2+} was removed from the culture medium in the presence of EGTA, the TJs opened as indicated by the rapid drop of the TER. Immunofluorescence showed that the cells rounded up but retained the belt of occludin around their apical membrane for up to 10–30 min, after the cells had completely separated from the monolayer. When Ca^{2+} was reintroduced, the TJs rapidly resealed. Earlier studies have established the role of Ca^{2+} in maintaining the normal TJs (Gonzalez-Mariscal et al., 1985, 1990). In fact, if Ca^{2+} is restored within minutes of removal, the TER can be fully restored within minutes (Lacaz-Vieira et al., 1999). When monolayers were exposed to prolonged Ca^{2+} starvation, the entire TJ complex is disassembled. Similar results have been reported previously (Meldolesi et al., 1978; Pitelka, Taggart & Hamamoto, 1983). We observed that after the prolonged Ca^{2+} starvation, the occludin and ZO-1 are no longer expressed at the cell surface and most of the occludin is found in the cytoplasm. Three to four hours after reintroduction of Ca^{2+}, occludin reappeared at the

cell surface and after 20 hr the TJs were formed and the TER was fully reestablished. During this *de novo* formation of TJs it appears that ZO-1 arrival at the cell surface precedes that of occludin (Fig. 3, arrows). Even 4 hr after the Ca^{2+}-switch most of the occludin is still seen in the cytoplasm, while ZO-1 is already localized along the cell-cell contact lines.

Our Western blots show that the 62–65 kD doublet remained present during opening or closing of tight junctions. We do not know if these two bands represent two isoforms of occludin, or if one of the bands represents a post-translationally modified occludin. Previous studies have shown that occludin is a phosphorylatable protein (Cordenonsi et al., 1997; Farshori & Kachar, 1997; Sakakibara et al., 1997; Wong, 1997). Sakakibara et al. (1997) also showed that phosphorylation causes an upward shift in the migration of occludin in the Western blots and that little or nonphosphorylated occludin is distributed on the basolateral membranes or in the cytoplasm, while the phosphorylated form of occludin is concentrated at the TJ. In our in vivo phosphorylation study we confirmed that occludin is a phosphorylatable protein and that the level of phosphorylation changes with the TJ permeability.

In addition to the doublet, both occludin antibodies immunoprecipitated a 71 kDa polypeptide. However, this phosphorylated 71 kD band is not visible in the EGTA-treated and Ca^{2+}-starved monolayer cells. These results suggest that the 71 kD band either becomes dephosphorylated by the Ca^{2+} starvation or that it no longer co-immunoprecipitates with the occludin complex, when the TJs are disrupted and the occludin is no longer part of a sealed TJ.

It is intriguing that we did not detect the phosphorylated 62–65 kD doublet in cell monolayers which were Ca^{2+} starved for 20–22 hr (Fig. 5*g*) while the Western blot showed clearly that the occludin doublet is present and the immunofluorescence showed localization of occludin in a cytoplasmic compartment. While we cannot rule out the possibility that the absence of the phosphorylated doublet in our experiments is due to an insufficient amount of immunoprecipitated occludin it is likely that the occludin in the Ca^{2+}-starved cells is present in a nonphosphorylated form (Wong, 1997) in a cytoplasmic compartment.

Phosphoaminoacid analysis shows that the 62–65 kD occludin doublet is predominantly phosphorylated on serine, with some incorporation on threonine in agreement with the studies of Sakakibara et al. (1997), which suggests that occludin function is regulated by serine/threonine kinases. Phosphoamino-acid analysis of the 71 kD band shows that it is exclusively phosphorylated on serine residues. This further suggests that this protein may be distinct from occludin.

Previous studies have shown that treating epithelial cells with TPA increases the permeability of TJs (Ojakian, 1981; Mullin et al., 1992, 1996, 1997). It has been reported that TPA prevents TJ resealing even when added in the last 15 min of the Ca^{2+} switch (Balda, 1991). In our experiments, TPA treatment of MDCK cells opened the TJ by disrupting the organization of the TJ strands. While occludin was significantly dephosphorylated it still colocalized with ZO-1 at the cell-cell contact areas (Fig. 4).

In the TPA treated monolayers the phosphorylated 71 kD band co-immunoprecipitates with the occludin complex, suggesting that this protein is another phosphoprotein that makes up the TJ macromolecular complex.

In conclusion, our results provide further support for the view that occludin and other phosphorylatable occludin-associated proteins are involved in the structure and regulation of TJ permeability in MDCK cells.

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