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Regulated assembly of tight junctions by protein kinase C

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ABSTRACT **We have previously shown that protein phosphorylation plays an important role in the sorting and assembly of tight junctions. We have now examined in detail the role of protein kinases in intercellular junction biogenesis by using a combination of highly specific and broad-spectrum inhibitors that act by independent mechanisms. Our data indicate that protein kinase C (PKC) is required for the proper assembly of tight junctions. Low concentrations of the specific inhibitor of PKC, calphostin C, markedly inhibited development of transepithelial electrical resistance, a functional measure of tight-junction biogenesis. The effect of PKC inhibitors on the development of tight junctions, as measured by resistance, was paralleled by a delay in the sorting of the tight-junction protein, zona occludens 1 (ZO-1), to the tight junction. The assembly of desmosomes and the adherens junction were not detectably affected, as determined by immunocytochemical analysis. In addition, ZO-1 was phosphorylated subsequent to the initiation of cell–cell contact, and treatment with calphostin C prevented ≈85% of the phosphorylation increase. Furthermore, *in vitro* measurements indicate that ZO-1 may be a direct target of PKC. Moreover, membrane-associated PKC activity more than doubled during junction assembly, and immunocytochemical analysis revealed a pool of PKC ζ that appeared to colocalize with ZO-1 at the tight junction. A preformed complex containing ZO-1, ZO-2, p130, as well as 330- and 65-kDa phosphoproteins was detected by coimmunoprecipitation in both the presence and absence of cell–cell contact. Identity of the 330- and 65-kDa phosphoproteins remains to be determined, but the 65-kDa protein may be occludin. The mass of this complex and the incorporation of ZO-1 into the Triton X-100-insoluble cytoskeleton were not PKC dependent.**

The regulation of the assembly of intercellular junctions is poorly understood but appears to be initiated by cadherin-mediated cell–cell contact, followed by formation of the adherens junction and the assembly of desmosomes. Completion of tight-junction assembly then occurs along with establishment of apical–basolateral polarity (1). In epithelial cell culture models, this entire process occurs, with impressive synchrony, over the relatively short span of a few hours (2–4); however, during the compaction stage of epithelial tissue development, these events occur over several days with considerable spatiotemporal heterogeneity.

MDCK cells maintained in low calcium (LC) medium lack cell–cell contact, intercellular junctions, and apical–basolateral polarization of lipids and protein (2–4). Upon switching to normal calcium (NC) medium, the cells rapidly develop characteristics of a polarized, tight, transporting epithelium. In many respects, the MDCK cell calcium switch recapitulates *in vitro* key events in epithelial morphogenesis (1, 5). Tight-junction formation can be followed by measuring the development of transepithelial electrical resistance (TER), and the formation of desmosomes, adherens junctions, as well as tight junctions, can be monitored immunocytochemically and biochemically.

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We have previously observed localized changes in intracellular calcium at sites of cell–cell contact as MDCK cells establish intercellular junctions and hypothesized a role for this ion in signaling relevant to intercellular-junction formation (4, 6). In addition, previous studies have suggested a role for phosphorylation in the assembly of tight junctions and sorting of tight-junction proteins, zona occludens 1 (ZO-1) and cingulin, to the tight junction (3, 7). Using a combination of highly specific, as well as broad-spectrum, inhibitors of protein kinases, we now detect apparent selective regulation of the biogenesis of tight junctions by protein kinase C (PKC) without immunocytochemical evidence of an effect on desmosome and adherens junction biogenesis. Moreover, membrane-associated PKC activity increases during tight-junction biogenesis, and the tight-junction protein ZO-1 appears to be a specific target of PKC as tight junctions assemble.

METHODS

Materials. All reagents were purchased from Sigma with the following exceptions. Calphostin C was from Calbiochem, H7 [2-methyl-1-(5-isoquinolinesulfonyl)-piperazine] was from Seikagaku America (Rockville, MD), and iso-H7, the 3-methyl isomer of H7, was from Sigma. Purified PKC was purchased from Upstate Biotechnology (Lake Placid, NY). R40.76 ZO-1 hybridoma was provided by D. Goodenough (Harvard University) (8). Anti-E-cadherin rr1 hybridoma was from B. Gumbiner (Memorial Sloan–Kettering) (9) and anti-desmoplakin NW6 polyclonal antibody was from K. Green (Northwestern University) (10).

TER and Immunocytochemistry. Single-cell suspensions of MDCK cells were plated at ≈2 × 10^5 cells per cm^2 on polycarbonate filters (Transwells; Costar) and allowed to establish confluent monolayers over 24 hr before incubation for 16 hr in LC medium, containing 1.6 μM Ca^{2+}, as described (4). At the start of each experiment, cells were switched to NC medium, containing 1.8 mM Ca^{2+}, with or without inhibitor. TER was measured as described (4), and the results were expressed in Ω·cm^2. Immunocytochemical studies were conducted on identical monolayers on glass coverslips using standard procedures as described (4).

Metabolic Labeling and Immunoprecipitation. MDCK cell monolayers were grown in 6-well tissue culture plates and prepared as for TER experiments. After phosphate starvation by two incubations of 30 min in LC medium prepared without phosphate, monolayers were switched to either fresh phosphate-free LC medium, NC medium, or NC medium with inhibitors, each containing [^{32}P]orthophosphate at 20 μCi/ml (1 Ci = 37 GBq). At various times after the calcium switch, monolayers were rinsed twice in phosphate-buffered saline and solubilized in immunoprecipitation buffer (100 mM NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.2% SDS/2 mM EDTA/10 mM Hepes, pH 7.5/1 mM sodium orthovanadate) and a protease inhibitor cocktail [1 mM phenylmethylsulfonyl fluoride/1 mM iodoacetamide/1 mM ben-

Abbreviations: LC, low calcium; NC, normal calcium; PKC, protein kinase C; TER, transepithelial electrical resistance; ZO-1 ZO-2, zonula occludens 1 and 2, respectively.

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zamidine/aprotinin, leupeptin, pepstatin A, and antipain (each at 20 μg/ml)]. DNA was sheared by passing the cell extract five times through a 23-gauge needle. Samples were cleared of insoluble material by centrifugation (10,000 × *g* for 10 min); the small pellet that was discarded was shown by immunoblot to contain no ZO-1 (data not shown). The supernatants were transferred to fresh tubes and placed on a rotator overnight at 4°C after addition of 400 μl of ZO-1 hybridoma supernatant and 30 μl of goat anti-rat Sepharose beads (Cappel). The procedure was shown to quantitatively immunoprecipitate all immunoreactive ZO-1 from the supernatant (data not shown). Beads were subsequently washed four times in 1 ml of immunoprecipitation buffer and boiled in 30 μl of 2× sample buffer, followed by SDS/PAGE and autoradiography of the dried gel or transfer to nitrocellulose for immunodetection of ZO-1 followed by autoradiography. Single bands at 225 kDa corresponding to radiolabeled ZO-1 were excised and quantified by scintillation counting.

PKC Activity Analysis and *in Vitro* Phosphorylation of ZO-1. PKC activity was measured in cytosolic and membrane fractions of MDCK cells using the Biotrak PKC enzyme assay kit (Amersham). At various times after initiation of cell–cell contact, monolayers of MDCK cells were scraped into a buffer (50 mM Tris·HCl, pH 7.5/10 mM EDTA/5 mM EGTA/ protease inhibitors as for immunoprecipitations) and triturated five times through a 23-gauge needle. Particulate and cytosolic fractions were separated by centrifugation at 10,000 × *g* for 1 hr. The particulate fraction was solubilized in lysis buffer containing 0.5% Triton X-100, and the cytosolic fraction was made 0.5% in Triton X-100. Aliquots of each fraction were analyzed according to the manufacturer's directions.

Immunopurified ZO-1 prepared as described above was incubated for 30 min at 25°C with 1 ng of purified PKC (Upstate Biotechnology) in 50 mM Tris·HCl, pH 7.5/1 mM calcium acetate/50 μM magnesium ATP/15 mM magnesium acetate/0.67 mol % α-phosphatidyl-L-serine/0.1% Triton X-100, phorbol 12-myristate 13-acetate at 2.7 μg/ml/2.5 mM dithiothreitol/0.1 μCi of [γ-^{32}P]ATP in a total reaction volume of 60 μl. The reaction was terminated by addition of 60 μl of 2× sample buffer and boiling. Control samples using histone (Sigma) as a substrate for PKC or ZO-1 in the absence of PKC and in the absence of phosphatidylserine and calcium were prepared as indicated in the figure legends. Samples were separated by SDS/PAGE, and autoradiograms were obtained from the dried gels.

Extraction of MDCK Cells and Rate-Zonal Centrifugation. Six hours after the induction of cell–cell contact, monolayers were rinsed twice with 10 mM Tris·HCl, pH 7.5/150 mM NaCl/1 mM phenylmethylsulfonyl fluoride. Cells were extracted for 20 min at 4°C on a gently rotating platform after overlaying the cells with 1 ml of CSK buffer (0.5% Triton X-100/100 mM NaCl/10 mM Tris·HCl, pH 7.5/300 mM sucrose), and the same protease inhibitor mixture was used as for immunoprecipitations. The resulting extract was completely aspirated, and the residue was dissolved in sample buffer. Aliquots of soluble and insoluble fractions were analyzed by SDS/PAGE and immunoblotting. For rate-zonal centrifugation analysis MDCK cell extracts were layered on top of 5–20% linear sucrose gradients prepared in the lysis buffer without detergents. The samples were centrifuged for 24 hr at 32,000 rpm in a Beckman SW-40 rotor. Gradients were fractionated from top to bottom into 16 fractions plus residue. Samples were separated on SDS/5–15% PAGE gels, transferred to nitrocellulose, and probed with anti-ZO-1 hybridoma supernatant.

RESULTS

Regulation of Tight-Junction Biogenesis by PKC. Using potent and highly specific protein kinase inhibitors, as well as a combination of less specific inhibitors, we sought to determine precisely which kinase(s) is critical for tight-junction biogenesis. In contrast to H7, which is broadly active against many kinases due to competitive inhibition with ATP (11), calphostin C is a highly specific and irreversible inhibitor of PKC; the inhibition constant (50 nM) is three orders of magnitude smaller for PKC than other kinases for which data are available (12–14). Its specificity for PKC coupled with a lack of cellular toxicity by the MTT (Sigma; 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test (15) and trypan blue exclusion (data not shown) suggested that it was a valuable tool with which to investigate the role of PKC in junction formation.

We observed that inhibitors with activity against PKC (such as calphostin C and H7) profoundly inhibited TER development (Fig. 1) in a dose-dependent manner (data not shown), whereas agents specific for a variety of other kinases such as cAMP-dependent protein kinase (Rp-cAMPS), Ca^{2+} calmodulin-dependent kinase II (KN-62), or myosin light chain kinase (ML-7) had no effect on TER (Fig. 1 *Inset*). In addition, we exposed confluent monolayers in NC medium to calphostin C and H7 and found no change in TER or staining pattern of ZO-1, indicating that fully assembled tight junctions were unaffected (data not shown). Taken together, the effects of calphostin C and H7 on TER development indicated an essential role for PKC in the tight-junction assembly process.

Given the profound effect of PKC inhibitors on tight-junction formation, we sought evidence of PKC activation. Enzymatic assay revealed that total cellular PKC activity increased 33% during the calcium switch (Fig. 2*A*). In addition, membrane-associated PKC activity more than doubled by 1 hr after the calcium switch (Fig. 2*B*). Immunocytochemical analysis of the ζ isoform of PKC revealed a clear translocation to the lateral surface of the plasma membrane (Fig. 2*C*). Moreover, by standard immunofluorescence microscopy PKC ζ appeared to colocalize with ZO-1 at sites of junction formation (Fig. 2*D*).

Treatment with both H7 and calphostin C retarded ZO-1 movement from its intracellular location to the lateral plasma membrane during tight-junction assembly. In untreated monolayers, by 2 hr ZO-1 had appropriately sorted to the lateral surface of the plasma membrane (Fig. 3*b*). Treatment of monolayers with H7 (Fig. 3*d*) and, to a slightly lesser extent, with calphostin C (Fig. 3*c*) during the induction of cell–cell contact consistently and markedly retarded sorting (six of six experi-

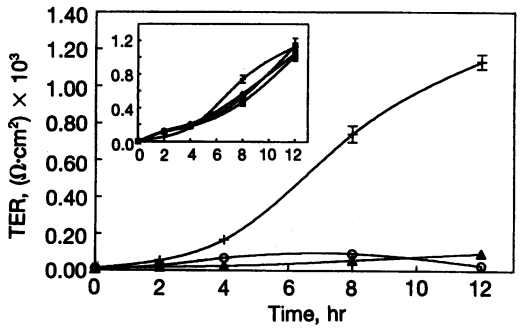

FIG. 1. Effect of calphostin C on TER during the calcium switch. MDCK cell monolayers were prepared on polycarbonate filters and incubated in LC medium for 16 hr. Cells were then switched to NC medium (+) or NC medium with 500 nM calphostin C (○) or 25 μM H7 (△), and transepithelial electrical resistance was measured at the indicated time points. (*Inset*) Results for other kinase inhibitors: Ca^{2+}/calmodulin-dependent kinase II (KN-62; 10 μM) (■), myosin light chain kinase (ML-7; 10 μM) (□), and cAMP-dependent protein kinase (Rp-cAMPS; 10 μM) (◆) along with controls (+) at the indicated time points. Data shown are means ± SEMs for $n = 7$ experiments.

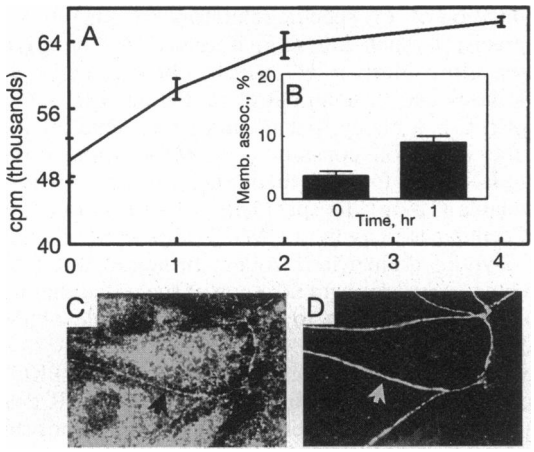

FIG. 2. (*A*) PKC activity during the MDCK calcium switch. At indicated times after initiation of junction formation, MDCK monolayers were lysed, and aliquots were assayed for PKC activity by using the Amersham Biotrak PKC enzyme assay kit according to the manufacturer's directions. Total regulatable PKC enzyme activity was calculated after subtraction of blank and control samples lacking calcium or phospholipid. (*B*) Inset shows increase in membrane-associated (memb. assoc.) (particulate fraction) PKC activity by 1 hr after initiation of cell–cell contact from 3.7% to 9.0% of total PKC activity. (*C* and *D*) Double-label immunofluorescence staining for PKC ζ (*C*) and ZO-1 (*D*) 1 hr after initiation of cell contact. Note that a distinct pool of PKC ζ redistributes to the plasma membrane and colocalizes with ZO-1 (arrow).

ments), such that even by 2 hr most cells did not display complete rings of ZO-1 staining.

In contrast to the defect in sorting of the tight-junction protein ZO-1 resulting from PKC inhibition, we observed no effect on the accumulation at the adherens junction of the adhesion molecule E-cadherin (Fig. 3*e*). In addition, we observed that the desmosomal protein desmoplakin (Fig. 3*f*) sorted appropriately in the presence of the calphostin C concentrations used. Although reorganization of F-actin into the cortical actin ring appeared to proceed normally despite PKC inhibition by calphostin C, H7-treated cells displayed subtle alterations in their actin staining pattern. Light microscopic examination of thick sections of Transwell filters showed that treatment with calphostin C did not affect the establishment of cell–cell contact (data not shown). These data, together with the effect of the inhibitors on TER, indicate that PKC inhibition causes a defect in tight-junction sorting and function and suggests some relatively selective role for PKC in tight-junction assembly.

Inhibition of PKC Inhibits Phosphorus Incorporation into ZO-1 During Formation of Tight Junctions. Two hours after induction of cell–cell contact, a clear and highly reproducible (nine of nine experiments) increase in the signal from radioactive phosphorus incorporated into ZO-1 was seen (Fig. 4*A*, compare first and second lanes). However, ZO-1 from cells treated with calphostin C (lane 3) and 25 μM H7 (lane 4) incorporated significantly less phosphate than did control cells, whereas ZO-1 from cells treated with 25 μM iso-H7 appeared similar to control cells. Quantification of the excised bands by scintillation counting revealed that cell treatment with calphostin C prevented 85% of the increase in ZO-1 phosphorus content seen after induction of cell–cell contact, whereas treatment with H7 prevented 69.8% of the increase, and equal concentrations of iso-H7 (a relatively impotent isomer of H7) (16) prevented only 12.5% of the increase in ZO-1 phosphate incorporation. Thus, the decreased ZO-1 phosphorylation seen with inhibitors paralleled the results from immunocytochemical and TER measurements.

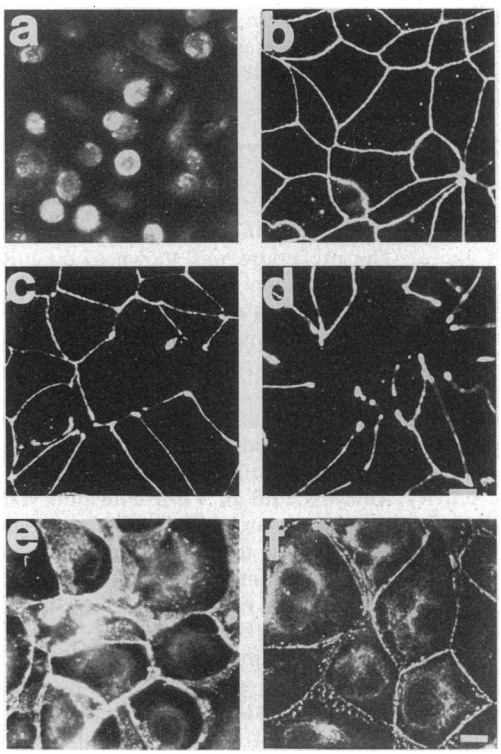

FIG. 3. Effect of kinase inhibitors on redistribution of the tight-junction protein ZO-1 and representative intercellular junction proteins. Data for ZO-1 shown before (*a*) and 2 hr after (*b*) the induction of cell–cell contact in untreated monolayers, as well as monolayers treated at the time of the switch with 500 nM calphostin C (*c*) and 25 μM H7 (*d*). There was no apparent effect of calphostin C treatment on the redistribution of either E-cadherin (*e*) or desmoplakin (*f*). Essentially the same results were obtained in five separate experiments. (Bar = 10 μm.)

Longer-exposure autoradiograms of the SDS/PAGE-resolved immunoprecipitates demonstrated four additional bands of molecular mass different from ZO-1 (Fig. 4*C*) that were not apparent on immunoblot for ZO-1. The phosphoproteins at 160 kDa (ZO-2) and 130 kDa apparently represent previously identified proteins (7, 17, 18). The band at 160 kDa specifically reacted with anti-ZO-2 antiserum, and double labeling for ZO-1 and ZO-2 showed that the two proteins colocalize in areas of punctate staining in the absence of

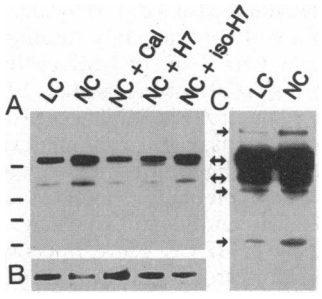

FIG. 4. (*A*) Autoradiogram of ZO-1 immunoprecipitates from MDCK cells labeled with [^{32}P]orthophosphate during the calcium switch. Double arrows at left indicate ZO-1 at 225 kDa and ZO-2 at 160 kDa. The data were gathered 2 hr after initiation of cell–cell contact. Molecular mass markers at left represent 205, 117, 80, and 50 kDa. Cal, calphostin C. (*B*) Immunoblot of the identical filter probed with anti-ZO-1 antibody. (*C*) Longer-exposure autoradiogram reveals the presence of coimmunoprecipitated phosphoproteins. In addition to bands corresponding to ZO-1 at 225 kDa and ZO-2 at 160 kDa (double arrows), upon longer exposure of the autoradiograms, bands at 130, 330, and 65 kDa become apparent (single arrows).

cell–cell contact (data not shown). The 330-kDa band, which is comparable in intensity to p130, and the 65-kDa band are possibly distinctive; however, p65 may represent the mammalian form of occludin (19). It is noteworthy that the same multimolecular complex appeared to be present before and after junction assembly.

To investigate the possibility that ZO-1 could be a direct target of PKC in a regulatable fashion, we tested the ability of purified PKC to phosphorylate immunopurified ZO-1. Immunoprecipitates of ZO-1 incubated with purified PKC in the presence of [γ-^{32}P]ATP revealed specific bands at 225 kDa (Fig. 5, lane 1). In addition, phosphorylated bands were seen at 160 and 130 kDa and likely corresponded to ZO-2 and p130. It was notable that the coimmunoprecipitated proteins at 65 and 330 kDa did not appear to be phosphorylated *in vitro* by PKC. The band at 81 kDa represents auto-phosphorylation of the added PKC (20). ZO-1 immunoprecipitates incubated in the same buffer without PKC showed that no significant kinase activity coimmunoprecipitated with ZO-1 (Fig. 5, lane 3). In addition, the phosphatidylserine dependence of the enzyme preparation was demonstrated (Fig. 5, lanes 2 and 5), indicating the *in vitro* phosphorylation by PKC is regulatable. These data, taken together with the demonstration that PKC inhibition *in vivo* retards ZO-1 phosphorylation, were consistent with a direct action of PKC on ZO-1.

PKC Inhibition Does Not Alter Association of ZO-1 with Triton X-100-Insoluble Fraction or Apparent Size of the Complex Containing ZO-1 During Tight-Junction Formation. We speculated that the association of junctional proteins with the cytoskeleton, for which morphologic and physiologic data exist (21, 22), might be a distal step in the assembly of tight junctions mediated by PKC. We therefore analyzed the stabilization of ZO-1 into a Triton X-100-insoluble pool, a measure of cytoskeletal association (4) during formation of tight junctions.

Both ZO-1 and desmoplakin underwent significant stabilization during the calcium switch, being relatively extractable before initiation of cell–cell contact and relatively resistant to extraction by 6 hr after initiation of cell–cell contact (Fig. 6*A*). Inhibition of PKC during formation of junctions did not affect incorporation of either desmoplakin I or ZO-1 into the Triton X-100-insoluble "cytoskeleton."

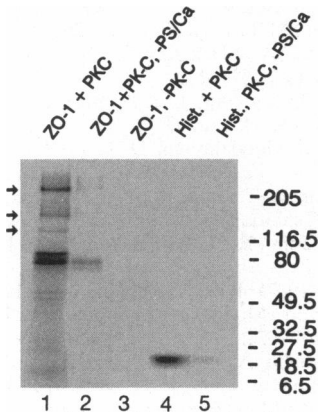

FIG. 5. *In vitro* phosphorylation of ZO-1 by purified PKC. Immunopurified ZO-1 was incubated with purified PKC in a buffer containing [γ-^{32}P]ATP followed by SDS/PAGE and autoradiography. The band at 81 kDa represents phosphorylation of the added PKC (lane 1). Phosphorylation of ZO-1 by purified PKC occurs in a regulatable fashion as indicated by its sensitivity to withdrawal of phosphatidylserine (PS) and calcium (lanes 2 and 5). No kinase activity was coimmunoprecipitated, as indicated by lack of detectable bands when PKC was omitted (lane 3). The ability of the PKC preparation to phosphorylate histone (Hist.) (lane 4) serves as a positive control; the inability to phosphorylate IgG heavy or light chains (lanes 1 and 2) serves as negative controls. Arrows on left, 225 kDa, 160 kDa, and 130 kDa.

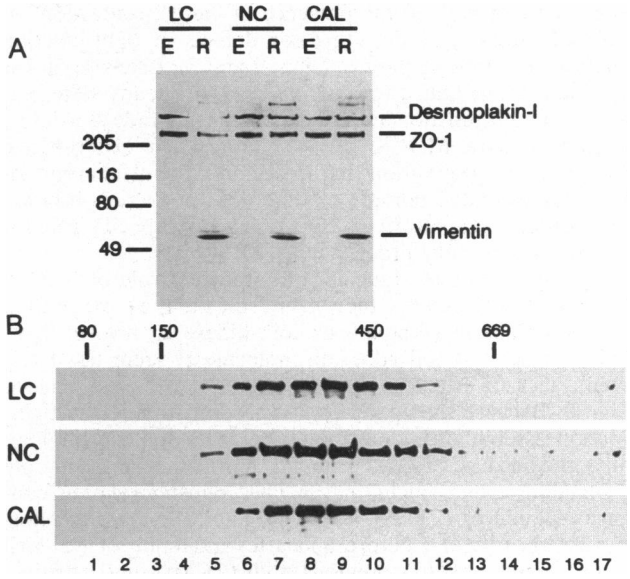

FIG. 6. (*A*) Triton X-100 extractability of ZO-1. Monolayers of MDCK cells in 6-well tissue culture plates were extracted in a Triton X-100-containing buffer 6 hr after induction of cell–cell contact or after change to fresh LC medium. Aliquots of extract (E) and residue (R) were separated by SDS/PAGE, transferred to nitrocellulose, and probed with indicated antisera. Treatment with calphostin (CAL) had no clear effect on association of ZO-1 (or desmoplakin I) with Triton-insoluble structures during establishment of intercellular junctions. (*B*) Rate-zonal centrifugation analysis of ZO-1-containing complex. Three hours after induction of cell–cell contact, monolayers of MDCK cells were extracted with the same buffer used in immunoprecipitations, and the extract was layered on top of 5–20% sucrose gradients. The apparent molecular mass of ZO-1 ranged from its native ≈225 kDa through ≈700 kDa in both LC and NC media; inhibition of PKC by calphostin C had no effect. The wide range of apparent molecular mass of ZO-1 is consistent with the participation of a fraction of, but not all, cellular ZO-1 in the complex identified by coimmunoprecipitation.

Although immunoprecipitation did not reveal any change in the number of coimmunoprecipitated phosphoproteins (Fig. 4), we speculated about the possibility of a molecular mass increase of the soluble complex during the assembly of tight junctions. Data shown for 3 hr after initiation of cell–cell contact (a time point characterized by large differences in both the sorting and Triton solubility between cells in LC medium, NC medium, or calphostin C treated medium) (4) showed no significant change in the apparent size of the ZO-1-containing complex. Both before cell contact and 3 hr after the induction of cell–cell contact, the apparent mass of ZO-1 ranged from its native ≈225 kDa to ≈700 kDa. Inhibition of PKC with calphostin C did not significantly change the mass of the ZO-1-containing complex.

DISCUSSION

Very little is known about regulatory pathways involved in assembly of the junctional complex. Our results suggest that sorting and/or the assembly of various components of the junctional complex (tight junctions, adherens junctions, desmosomes) may be differentially regulated. The development of TER is a very late event in the assembly of the junctional complex; the presence of significant TER implies a completely assembled junctional complex. A defect in the development of TER could, in principle, be secondary to a defect in cell–cell contact or formation of the adherens junction, as opposed to an effect on tight junction assembly *per se*. The immunocytochemical data in the present study, while not quantitative, suggest that cell–cell contact and adherens junction formation

proceeded normally in the presence of the concentrations of PKC inhibitors used despite clear defects in tight-junction function and staining pattern (Figs. 2 and 3). Likewise, it was possible *a priori* that a role for PKC in the steady-state regulation of tight-junction integrity could masquerade as a defect in biogenesis in MDCK cell calcium switch experiments. However, our observation that treatment of confluent monolayers for extended periods of time does not alter observed TER (or staining pattern of ZO-1) suggests a specific role for PKC in the assembly process.

Although the data argue for an important role of PKC in tight-junction biogenesis, they do not rule out the participation of another kinase, although protein kinase A, myosin light-chain kinase, and cell adhesion molecule II seem less likely given a lack of effect on TER by inhibitors of these kinases (Fig. 1). Because the action of PKC seemed limited to tight-junction assembly (as determined by TER and morphologically) and because of ZO-1 sequence motifs that suggest a role in signaling events, including 34 PKC phosphorylation consensus sequences (23–25), we investigated the state of phosphorylation of ZO-1 before and after induction of cell–cell contact. In contrast to a previous study (7), we found a distinct increase in ZO-1 phosphorylation (Fig. 4*A*). However, another recent study did reveal a greater steady-state incorporation of phosphate into ZO-1 from MDCK cells grown in NC medium as opposed to LC medium (26). In the present work, we also found that treatment with calphostin C nearly abolished the incorporation of phosphorus into ZO-1 after the initiation of cell–cell contact. This finding, while suggestive, did not prove that ZO-1 was an actual target of PKC. That ZO-1 may be directly phosphorylated by PKC *in vivo* during assembly of tight junctions is supported by the fact that ZO-1 is easily phosphorylated *in vitro* with purified enzyme in a regulatable fashion (Fig. 5).

Furthermore, total cellular PKC activity and membrane-associated PKC activity increased during the calcium switch. Moreover, the ζ isoform of PKC colocalized with ZO-1 at the lateral surface of the plasma membrane (Fig. 2). While intriguing, these results do not necessarily demonstrate a functional role for PKC ζ in tight-junction assembly. However, the colocalization results do show that, at least in principle, an isoform of PKC is in intimate proximity to ZO-1 and could be the enzyme responsible for its phosphorylation.

In addition to the apparent involvement of PKC in the phosphorylation of ZO-1, we observed the coimmunoprecipitation of phosphoproteins at 160 kDa and 130 kDa, as has been previously reported, as well as potentially distinctive phosphoproteins at 65 kDa and ≈330 kDa (Fig. 4*C*). The 65-kDa protein could be the mammalian form of occludin (19), while the 330-kDa protein may represent a member of the spectrin–fodrin family (27). The apparent degree of phosphorylation of the 160-kDa phosphoprotein (likely ZO-2) paralleled the changes seen in ZO-1 phosphate content. That ZO-1 and ZO-2 (or at least that fraction of ZO-2 associated with ZO-1) should become phosphorylated during the calcium switch in a coordinate manner is not surprising, given their large degree of homology, which includes numerous PKC consensus sequences (34 sites on ZO-1 and 15 sites on ZO-2) (25).

The Triton solubility data taken together with the results of rate-zonal centrifugation help to elucidate the behavior of the ZO-1-containing complex during the biogenesis of the tight junction. The stability of the heteromeric complex (in the face of a stringent buffer containing SDS) suggests the presence of relatively high-affinity associations between these proteins. Nevertheless, the wide range of apparent molecular mass was consistent with the participation of a fraction of, but not all, cellular ZO-1 in the complex identified by coimmunoprecipitation (Fig. 6*B*). Uncomplexed ZO-1 and ZO-1-containing complex are initially localized to the cytoplasm and subsequently translocate to the lateral surface of the plasma membrane by 2 hr after initiation of cell contact, as indicated by ZO-1 immunofluorescence. Subsequent to initiation of cell–cell contact, the ZO-1-containing complex associates with cytoskeletal elements and becomes relatively inextractable in Triton X-100-containing buffers. However, although association of the ZO-1-containing complex with the Triton-insoluble cytoskeleton has been shown to depend on intracellular calcium (4), this cytoskeletal link is apparently not dependent on PKC (Fig. 6*A*), suggesting at least partial dissociation of the effects of Ca^{2+} and PKC on tight-junction biogenesis.

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