Tight-junction protein zonula occludens 2 is a target of phosphorylation by protein kinase C

Antonia AVILA-FLORES*, Erika RENDÓN-HUERTA†, Jacqueline MORENO*, Socorro ISLAS*, Abigail BETANZOS*, Martha ROBLES-FLORES† and Lorenza GONZÁLEZ-MARISCAL*¹

*Department of Physiology, Biophysics and Neuroscience, Center for Research and Advanced Studies (CINVESTAV), A. Postal 14-740, México D.F. 07000, and

†Department of Biochemistry, Faculty of Medicine, Autonomous National University of México (UNAM), Ap 70-159, México D.F. 04510

Zonula occludens 2 (ZO-2) protein is a tight-junction phosphorylated protein that belongs to the membrane-associated guanylate kinase ('MAGUK') family. Here we study the interaction between ZO-2 and protein kinase C (PKC). We have constructed two ZO-2 fusion proteins of the middle (3PSG) and C-terminal (AP) regions of the molecule and demonstrate that they are phosphorylated by PKC isoenzymes β , ϵ , λ and ζ . To understand the physiological significance of the interaction between ZO-2 and PKC, we analysed the phosphorylation state of ZO-2 immunoprecipitated from monolayers with mature tight

junctions or from cells that either lack them or have them disassembled through Ca^{2+} chelation. We found that in the latter condition the phosphorylation level of ZO-2 is significantly higher and is due to the action of both PKC and cAMP-dependent protein kinase. These results therefore suggest that the phosphorylated state of ZO-2 restrains its capacity to operate at the junctional complex.

Key words: membrane-associated guanylate kinase, MDCK cells, protein kinase A, protein phosphorylation.

INTRODUCTION

The intercellular junctional complex of epithelia and endothelia separates different compartments. At the uppermost portion of this complex, between the apical and the lateral membrane, is the tight junction (TJ). TJs regulate the passage of ions and molecules through the paracellular pathway and maintain the polarized distribution of lipids and proteins at the plasma membrane [1]. TJs display an architectural organization of two domains: (1) an extracellular region, in which contacts are established homotypically by the transmembrane proteins occludin, claudins and junctional adhesion molecule ('JAM'), and (2) a cytoplasmic undercoat, where a scaffold of proteins links the transmembrane molecules to actin-cytoskeletal components. The membrane-associated guanylate kinase (MAGUK) proteins zonula occludens 1 (ZO-1), ZO-2 and ZO-3 are found in this domain. As with all MAGUKs, they consist of modular units that comprise three PDZ domains, an SH3 (Src homology 3) region and a guanylate kinase (GK) domain [2].

In addition, TJ MAGUK proteins have a unique C-terminal domain that in ZO-1 and ZO-2 contains an acidic section and a proline-rich section, in which several alternatively spliced regions have been detected [2].

ZO-2 is a 160 kDa phosphoprotein [3–5] present at the TJs of epithelia and endothelia and at adherent junctions of non-TJ-containing cells [6]. ZO-2 and ZO-1 co-immunoprecipitate as heterodimers through PDZ-2/PDZ-2 interactions [7,8]. Soluble ZO-1, ZO-2 and ZO-3 are found as independent ZO-1–ZO-2 and ZO-1–ZO-3 complexes [9]. Whereas the N-terminal domain of ZO-2 directly binds to claudins [10], occludin and α -catenin [11], the C-terminal domain of the protein co-localizes with actin filaments and interacts with the actin-binding protein 4.1 [12].

During the assembly and sealing of the TJ, a cascade of transducers and enzymes, such as G-proteins, phospholipase C, protein kinase C (PKC) and calmodulin, is involved [13]. The role

of PKC has been difficult to understand because PKC inhibition blocks not only the junction assembly induced by Ca^{2+} [13–16] but also the disassembly triggered by the removal of Ca^{2+} [17]. Furthermore, purified PKC phosphorylates co-immunoprecipitated ZO-1, ZO-2 and ZO-3 [17]. Although Madin–Darby canine kidney (MDCK) cells express classical (α , β and γ), new (δ and ϵ) and atypical (λ and ζ) PKC isoforms, only the last of these isoenzymes have been reported to concentrate at the TJ [18,19].

The identification of PKC targets during junctional assembly and disassembly is crucial for understanding TJ regulation. Previously we showed that the phosphorylation of vinculin by PKC modulates the assembly of the junctional complex [20]. Here we explore the action of PKC on the TJ protein ZO-2. Employing phosphorylation assays *in vitro* we demonstrate that the middle and C-terminal portions of ZO-2 are targets of PKC phosphorylation. Moreover, native ZO-2 interacts *in vitro* and co-immunoprecipitates with several PKC isoforms. When TJs are either absent or are being disassembled owing to Ca^{2+} removal, native ZO-2 is significantly more phosphorylated than in monolayers with well-established TJs. This increased phosphorylation is due to the action of both PKC and cAMP-dependent protein kinase (PKA).

EXPERIMENTAL

Expression and purification of ZO-2 polypeptides as glutathione S-transferase (GST) fusion proteins

RNA isolated from MDCK cells was purified by filtration through poly(U)–Sephadex. ZO-2 cDNA was obtained by reverse transcription with primer 1, which was complementary to nt 4691–4674 of the canine sequence [7]. By means of PCR with *Pfu* DNA polymerase (catalogue no. 600135; Stratagene, La Jolla, CA, U.S.A.) we generated the following fragments: 3PSG, which encompassed the last PDZ domain, the SH3 region and the GK

Abbreviations used: GK, guanylate kinase; GST, glutathione S-transferase; LC, low Ca^{2+} (1–5 μM); MAGUK, membrane-associated guanylate kinase; MDCK, Madin–Darby canine kidney; NC, normal Ca^{2+} (1.8 mM); PKA, cAMP-dependent protein kinase; PKC, protein kinase C; TER, transepithelial electrical resistance; TJ, tight junction; ZO-2, zonula occludens 2 protein.

¹ To whom correspondence should be addressed (e-mail lorenza@fisio.cinvestav.mx).

Figure 1 Western blot identification of 3PSG protein and its location within ZO-2

Upper panel: schematic representation of ZO-2 cDNA. This diagram shows the regions that were amplified to obtain the 3PSG and AP fusion proteins. Asterisks and arrowheads indicate the putative PKC and PKA phosphorylation sites respectively. The numbers in parentheses identify the nucleotides employed for the PCR primers. Lower panel: identification of 3PSG in a Western blot with an anti-(ZO-2) antibody. The number at the left is the molecular mass in kDa.

domain [primers 2 (nt 1595–1621) and 3 (nt 3019–2989)], and AP, which contained the acidic and the proline-rich regions of ZO-2 [primers 4 (nt 3029–3058) and 5 (nt 3933–3905)]. Primers 3 and 5 contained a 5' *EcoRI* site. The PCR products were cloned into *SmaI-EcoRI*-cut pGEX-3X (catalogue no. 27480301; Amersham Pharmacia Biotech, Uppsala, Sweden). The identities of the constructs pGEX-3PSG and pGEX-AP were confirmed by double-strand sequencing with primers 5' and 3' to the PGEX cloning site (catalogue nos. 27141001 and 27141101; Amersham Pharmacia Biotech). Competent *Escherichia coli* (strain JM109) cells were transformed with the constructs and the expression of GST-3PSG and GST-AP was induced for 4 h with 5 mM isopropyl β -D-thiogalactoside. Fusion proteins were purified by standard methods. The identity of the 3PSG fusion protein was confirmed by Western blotting of the molecule with the anti-(ZO-2) antibody (see Figure 1, lower panel). This procedure was not performed for the AP protein because the anti-(ZO-2) antibody does not recognize the AP region.

Protein blotting and analysis

Proteins were separated by SDS/PAGE and either stained with Coomassie Blue or transferred to nitrocellulose membranes. Proteins were identified with mouse monoclonal antibodies against GST (GST 3-4C, catalogue no. 136700; Zymed Laboratories, San Francisco, CA, U.S.A.) and rabbit polyclonal antibodies against PKC ζ , β II, ϵ and λ (catalogue no. sc-216, sc-210, sc-214 and sc-1091; Santa Cruz Biotechnologies, Santa Cruz, CA, U.S.A.), the catalytic subunit of PKA α (catalogue no. sc-903; Santa Cruz Biotechnologies) and phosphatase PP1 (catalogue no. sc-443; Santa Cruz Biotechnologies). Peroxidase-conjugated goat IgG against either mouse or rabbit IgG (catalogue nos 62–6520 and 62–6120 respectively; Zymed Laboratories) were used as secondary antibodies, followed by a chemi-

luminescence detection system (ECL + Plus, catalogue no. RPN 2132; Amersham Life Science, Little Chalfont, Bucks., U.K.). Alternatively, secondary goat anti-rabbit or anti-mouse antibodies coupled to alkaline phosphatase (catalogue nos. 170–6518 and 170–6520 respectively; Bio-Rad Laboratories) were used and a 5-bromo-4-chloroindol-3-yl phosphate/Nitro Blue Tetrazolium colorimetric detection kit (catalogue no. 170-6432; Bio-Rad) was subsequently employed.

Cell culture

MDCK cells were cultured in normal- Ca^{2+} medium (NC; 1.8 mM Ca^{2+}) and low- Ca^{2+} medium (LC; 5 μM Ca^{2+}) as described previously [20].

Phosphorylation assays *in vitro* with total cellular extracts

Fusion protein (10 mg) preadsorbed in 50 ml of glutathione-agarose beads [1:2 (w/v) slurry] were used for each precipitation with 200 ml of MDCK cell extract containing 800 mg of total soluble proteins. The fusion proteins with the cell extract were incubated overnight at 4 °C on a rotator. In mock reactions the contact with MDCK cell extracts was omitted. Phosphorylation reactions were performed in a kinase buffer [50 mM Hepes (pH 7.4)/10 mM MgCl_2 /2 mM MnCl_2] containing [γ - ^{32}P]ATP. Reactions were performed at 30 °C for 30 min. The fusion proteins were eluted with 5 mM glutathione and subjected to SDS/PAGE [12% (w/v) gel]. The gel was dried under vacuum and the phosphorylated bands were detected by autoradiography. To test the specificity of the kinase interaction, GST and the fusion proteins were incubated with the cell extracts; after extensive washing, 10 μg of the fusion proteins was added before the *in vitro* kinase assay.

Phosphoamino acid analysis

Fusion proteins phosphorylated *in vitro* and resolved by SDS/PAGE [10% (w/v) gel] were transferred to PVDF membranes, and the phosphoamino acids were analysed by the method of Boyle et al. [21] with minor modifications previously described [20].

Metabolic labelling and immunoprecipitation of ZO-2

Cells that had previously been plated and incubated for 20 h in either NC or LC without phosphate were labelled in medium supplemented with 200 μ Ci/ml [32 P] P_i (acid-free, 10 mCi/ml, catalogue no. PBS-13; Amersham, Little Chalfont, Bucks., U.K.) as previously described [20].

Determination of specific phosphorylation

Total [32 P]phosphate labelling was detected by autoradiography, and total protein was determined in the same gel by either staining with Coomassie Blue (for 3PSG and AP proteins) or immunoblotting as described previously [20]. The ratios of [32 P]protein to total protein were designated the total specific phosphorylation.

Partial purification of PKC

PKC was purified from hepatocytes as described previously [22].

Phosphorylation of ZO-2 fusion proteins by PKC immune complexes

Aliquots of partly purified and concentrated PKCs (1 mg/ml) were immunoprecipitated with antibodies against PKC isoenzymes β II, ϵ , λ or ζ . The phosphorylation assays were performed as described previously [23]. The non-specific PKC immunoprecipitation was performed with an antibody against glial fibrillar acidic protein, generously provided by Dr Jose Segovia from CINVESTAV, México.

PKC overlay assay

PKC binding was performed as previously described [23].

Electrical measurements

The degree of sealing of the TJ was evaluated by measuring the electrical resistance across the monolayer as previously described [24].

Immunofluorescence

Monolayers grown on glass coverslips were fixed, permeabilized and processed for immunofluorescence with a polyclonal antibody against ZO-2 [Zymed 71-1400, diluted 1:100 in 1% (w/v) IgG-free BSA] as described previously [20].

Drugs

The following concentrations of drugs were used: 6 μ M GF 109203X (catalogue no. 203290; Calbiochem) and 1 μ M H-89 (catalogue no. 371963; Calbiochem). These drugs were prepared as stocks in DMSO attaining a final concentration of DMSO in Dulbecco's modified Eagle's medium of less than 0.1%.

RESULTS

ZO-2 fusion proteins specifically interact with kinases and become phosphorylated on serine and threonine residues

Because TJ sealing [3,13] and disassembly [17] are regulated by kinases, we investigated whether ZO-2 was one of their TJ targets. We constructed two ZO-2 GST fusion proteins: the first (3PSG) included the third PDZ domain, the SH3 region and the guanylate kinase module (residues 400–874 of canine cDNA [7]); the second (AP) covered the acidic and proline-rich domains of

Figure 2 ZO-2 fusion proteins specifically interact with a kinase and become phosphorylated

Upper panel: GST and fusion proteins bound to glutathione–agarose beads were incubated with or without an MDCK cell extract. Associated proteins were tested for kinase activity *in vitro*. Asterisks in the autoradiograph denote the phosphorylated 3PSG and AP fusion proteins. Lower panel: the specificity of the kinase interaction was assessed by incubating GST or fusion proteins with the cell extract and, after extensive washing, 3PSG or AP was added before the *in vitro* kinase assay. Note that only the fusion protein in contact with the cell extract became phosphorylated (asterisks). The positions of molecular mass markers are indicated (in kDa) at the left in the upper panel; the molecular masses of the major bands are indicated at the right in both panels.

Figure 3 Two-dimensional phosphoamino acid analysis of ZO-2 fusion proteins

Total hydrolysates of ZO-2 fusion proteins phosphorylated by cellular extracts were spotted on cellulose TLC plates at the origin (Or). Separation was performed in the first dimension by thin-layer electrophoresis and in the second by thin-layer chromatography. Dotted circles indicate the migration of standard phosphoamino acids, identified by ninhydrin staining: phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y).

strong phosphorylation of both was detected (Figure 2, upper panel). Mock incubation of the fusion proteins and of GST revealed no phosphorylation. To test that the kinases associated to the ZO-2 region of protein and not to the GST portion, we incubated GST with cell extracts and, after being washed extensively, the fusion proteins were added before the kinase assay. Figure 2 (lower panel) shows that the phosphorylation of ZO-2 fusion proteins was specific, because no phosphorylation was obtained when GST and not the fusion protein was in contact with the MDCK cell extract. To analyse whether a kinase bound to one fusion protein was capable of phosphorylating both fusion proteins, we incubated one hybrid

Figure 5 PKC isoforms β , ϵ , λ and ζ bind ZO-2 in an overlay assay

Samples of immunoprecipitated (IP) ZO-2, histone and a ZO-2 precipitation with a preimmune serum were subjected to SDS/PAGE [10% (w/v) gel], transferred to nitrocellulose and incubated with or without a partly purified PKC extract. PKC binding to nitrocellulose-immobilized ZO-2 and histone was detected with antibodies against PKC isoenzymes. PKC_{mix} refers to the mixture of β , ϵ , λ and ζ antibodies. To confirm that the 160 and 220 kDa bands stained with the anti-PKC antibodies corresponded to ZO-2 and ZO-1 respectively, parallel lanes were blotted with anti-(ZO-1) and anti-(ZO-2) antibodies.

Figure 6 PKC isoenzymes and PKA are present in the ZO-2 immunoprecipitate

The presence of PKCs, PKA and phosphatases in confluent cells (upper panel) and a ZO-2 immunoprecipitate (Ip) derived from confluent and subconfluent cultures was detected in a Western blot (lower panel); — indicates blots not treated with the first specific antibody. Filled and open arrowheads show the positions of the blotted proteins and the location of the IgG used for the immunoprecipitation respectively. The asterisks mark a non-specific band revealed when the blot was overexposed. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 7 Inhibition of PKC phosphorylation delays the TJ disassembly induced by Ca^{2+} chelation

Upper panels: subcellular localization of ZO-2 in monolayers cultured in LC and NC and treated with 4 mM EGTA with or without a PKC inhibitor. In LC, ZO-2 stains the cytoplasm diffusely, whereas in NC the cell-cell contacts are clearly marked. At 30 min after treatment with 4 mM EGTA, ZO-2 is still found at some cellular borders but at 60 min of treatment ZO-2 is found scattered in the cytoplasm. However, if PKC phosphorylation is inhibited with 6 μM GF 109203X, ZO-2 remains at the cellular boundaries. Scale bar, 20 μm . Lower panel: TER of monolayers cultured in LC, NC and treated with 4 mM EGTA with or without a PKC inhibitor. Monolayers cultured in NC ($\triangle$) display a TER that LC ($\blacktriangle$) cultured monolayers lack. The addition of EGTA provokes a collapse of TER ($\bullet$), which is deterred by the addition of GF 109203X ($\circ$).

protein with the cellular extract, washed it extensively and then added the second fusion protein. We then performed the kinase assay and tested whether the latter protein became phosphorylated. Figure 2 (lower panel) shows that only the protein in contact with the cellular extract became phosphorylated, suggesting either that the two proteins were phosphorylated by different kinases or that the kinase bound to the first protein did not have access to the second one.

Kinases can be broadly classified on the basis of the amino acid that they phosphorylate. We therefore performed a phospho-amino acid analysis of the fusion proteins phosphorylated by kinases present in the cellular extract. Figure 3 shows that both fusion proteins were strongly phosphorylated on serine residues, whereas only a slight threonine phosphorylation was detected in AP. Thus the enzymes that specifically phosphorylated the fusion proteins were serine/threonine kinases.

Figure 8 Phosphorylation of native ZO-2 in monolayers cultured in LC and NC

ZO-2 was immunoprecipitated from ^{32}P -labelled MDCK cells cultured in NC and LC. An example of phosphorylated native ZO-2 is shown in the autoradiograph (upper panel), together with its corresponding ZO-2 ECL determination (lower panel).

Table 1 Specific phosphorylation of native ZO-2 in monolayers cultured in LC and NC

ZO-2 was immunoprecipitated from ^{32}P -labelled MDCK cells cultured in NC and LC. Autoradiographs and immunoblots were quantified and the specific phosphorylation was determined as the ratio of phosphorylated protein to total protein content. Phosphorylation in LC was taken as 1 to normalize the results of different experiments. Results are means $\pm$ S.E.M. for n experiments performed for each condition. The changes in ZO-2 phosphorylation were significant ($P < 0.0001$) as determined by a t test.

Condition	n	Specific phosphorylation
NC	8	0.69 ± 0.12
LC	8	1.00

3PSG and AP regions of ZO-2 are targets of PKC phosphorylation

Because we and others had previously demonstrated the participation of PKC in the regulation of TJ formation and disassembly [3,13,15–17] and our ZO-2 fusion proteins possessed various PKC consensus sites (in 3PSG these were Ser-425, Ser-434, Thr-478, Ser-595, Ser-677, Ser-686, Thr-744, Thr-762, Ser-819 and Ser-820; in AP they were Ser-962, Thr-984, Ser-1028, Ser-1032 and Ser-1131) (see Figure 1, upper panel), we investigated whether PKC isoenzymes could phosphorylate the ZO-2 fusion proteins.

We focused on PKC isoforms expressed by MDCK cells, either at the TJ region (λ and ζ) or the nucleus (β and ϵ) [18,19]. The latter were explored because we have observed that in subconfluent cultures ZO-2 is present at the nucleus [25].

Immunoprecipitated PKC isoenzymes β , ϵ , λ and ζ were incubated with the fusion proteins, and kinase assays were performed. A representative example is shown (Figure 4, upper panel). The four isoforms phosphorylated the PKC substrate histone H1-IIIS, which was added where indicated (0.2 mg/ml) (Figure 4, upper panel, arrows). Faint bands of the same molecular mass as histone were observed in some autoradiographic lanes. We suspect that they corresponded to trace amounts of histone present in the PKC immunoprecipitate, as shown previously [26]. However, a histone Western blot of the PKC immunoprecipitate did not reveal the expected bands above background level (results not shown), possibly owing to their low abundance. We therefore cannot confirm that such bands corresponded to co-precipitated histone. Two bands of 45 and 47 kDa (Figure 4, upper panel, filled arrowheads), phosphorylated by all the PKC isoenzymes tested, did not appear in the immunoprecipitate assay with the non-specific antibody. We therefore

consider them to have been contaminants in the PKC immunoprecipitate.

No phosphorylation of 3PSG and AP was detected in the non-specific kinase immunoprecipitate. Instead, the results of three independent assays indicated that the specific phosphorylation of 3PSG and AP by the PKC isoenzymes was superior to (AP by λ), similar to (3PSG by ϵ ; AP by β and ζ) or lower than (3PSG by β and AP by ϵ) that of histone (Figure 4, lower-left panel). 3PSG was not a substrate of isoforms λ and ζ because its specific phosphorylation was equal to that of a non-specific band present in the negative control lanes (Figure 4, upper panel).

When the specific inhibitor GF 109203X was added at a concentration reported to repress all PKC isoforms (6 μM) [27], a decrease in the phosphorylation of 3PSG and AP was detected (Figure 4, upper panel), thus confirming the participation of PKC on 3PSG and AP phosphorylation.

The identities of the PKC isoforms in the immunoprecipitate were determined in a Western blot (Figure 4, lower-right panel).

PKC isoforms β , ϵ , λ and ζ bind *in vitro* to endogenous ZO-2

Several proteins that bind PKC have been identified by overlay assays, an immunoblotting modification that uses PKC rather than antibodies to probe protein bands [28]. To investigate whether PKC could bind endogenous ZO-2, we immunoprecipitated ZO-2 from MDCK lysates, subjected it to SDS/PAGE, transferred it to nitrocellulose membranes and overlaid it with a partly purified PKC extract. We then used antibodies against PKC isoforms β , ϵ , λ and ζ that could stain the ZO-2 band only if a PKC had specifically associated to the ZO-2 molecule present in the nitrocellulose during the overlay procedure. Figure 5 shows that, whereas antibodies against isoforms β , ϵ , λ and ζ did not interact with the precipitation of ZO-2 by preimmune serum, they bound to histone and ZO-2 and co-immunoprecipitated ZO-1 immobilized in the membrane. The identities of ZO-1 and ZO-2 were confirmed by blotting parallel lanes with specific antibodies. The present result prompted us to study whether the interaction between ZO-2 and PKC could occur *in vivo*.

PKC isoforms ϵ , λ and ζ and PKA are associated with ZO-2 *in vivo*

Because the location of kinases and phosphatases near their substrates seems to enhance the modulation of protein phosphorylation, we studied whether PKC and certain serine/threonine phosphatases are associated with ZO-2 *in vivo*. Because ZO-2 contains putative PKA phosphorylation sites (Figure 1), we also investigated the presence of PKA. To maintain natural protein–protein interactions, we immunoprecipitated ZO-2 under conditions of low stringency and analysed the immunoprecipitate by Western blotting. Figure 6 (upper panel) shows the presence of PKA, phosphatase PP1 and PKC isoenzymes ϵ , λ and ζ in the cellular extract. Although isoform β is present in the nuclei of MDCK cells [18] and is immunoprecipitated from a PKC concentrate, it remained undetectable in the total cellular extract, probably owing to its low abundance. ZO-2 immunoprecipitate obtained from confluent cells contained PKA and PKCs λ and ζ but lacked the ϵ isoform (Figure 6, lower panel). The nuclei of MDCK cells contained PKC ϵ and those derived from subconfluent cultures also possessed ZO-2. We therefore analysed a ZO-2 immunoprecipitate obtained from subconfluent cells and detected the presence of PKC ϵ . The results obtained indicate that although there were robust signals from PKC ϵ , λ and ζ in the cell (Figure 6, upper panel) a barely distinguishable quantity of them was associated with ZO-2 (Figure 6, lower panel).

Figure 9 Phosphorylation of native ZO-2 during Ca^{2+} chelation in the presence or absence of PKC and PKA inhibitors

ZO-2 was immunoprecipitated from ^{32}P -labelled MDCK cells cultured in NC and treated for 30 min with 2.5 mM EGTA, with or without 6 μM GF 109203X (GF) and/or 1 μM H-89. An example of phosphorylated native ZO-2 is shown in the autoradiograph (upper panel), together with its corresponding blot determination (lower panel).

Table 2 Specific phosphorylation of native ZO-2 during Ca^{2+} chelation in the presence or absence of PKC and PKA inhibitors

ZO-2 was immunoprecipitated from ^{32}P -labelled MDCK cells cultured in NC and treated for 30 min with 2.5 mM EGTA, with or without 6 mM GF 109203X (GF) and/or 1 mM H-89. The specific phosphorylation was determined as specified in Table 1. Phosphorylation in NC was taken as 1 to normalize the results. Significances as determined by a *t* test: EGTA versus NC, $P < 0.0003$; EGTA versus EGTA + GF-109203X, $P < 0.0001$; EGTA versus EGTA + H-89, $P < 0.0001$; EGTA versus EGTA + GF 109203X + H-89, $P < 0.002$; EGTA + GF 109203X versus EGTA + GF 109203X + H-89, $P = 0.2590$.

Condition	<i>n</i>	Specific phosphorylation
NC	16	1.00 ± 0.12
NC + EGTA	16	1.50 ± 0.03
NC + EGTA + GF	6	1.07 ± 0.07
NC + EGTA + H-89	6	1.15 ± 0.09
NC + EGTA + GF + H-89	5	1.21 ± 0.11

In our search for the presence of major serine/threonine phosphatases in the ZO-2 immunoprecipitate, we employed an antibody against PP1 that was capable of cross-reacting with phosphatases PP2A, PP2B and PPX. Even though a strong signal for PP1 phosphatase was found in the total cellular extract, no signal was detectable in the ZO-2 immunoprecipitate. To explore the point further, we employed an antibody specific for PP2B and found no reactivity in both the cellular extract and

the immunoprecipitate (results not shown). Therefore no major serine/threonine phosphatase seemed to be strongly associated with the kinase-ZO-2 complex.

At low Ca^{2+} concentrations PKA and PKC phosphorylate ZO-2

Next we studied the relation between the phosphorylation condition of ZO-2 and the physiological status of TJs. Previously we had observed that on plating MDCK cells at confluence in NC medium, they synthesized, assembled and sealed their TJs in a process that could be gauged through the development of transepithelial electrical resistance (TER) [24]. However, if 1 h after being plated the monolayers are transferred to LC, they do not establish their TJs [24,29]. If Ca^{2+} is chelated in confluent and mature monolayers cultured in NC, TJs disassemble and TER decreases [30]. Therefore we studied here whether this process was modulated by phosphorylation by PKC. Figure 7 shows that cells cultured in LC displayed no TER (lower panel, $\blacktriangle$) and ZO-2 stained their cytoplasm diffusely. In contrast, in cells cultured in NC ZO-2 was located at the cell borders and the monolayer displayed a TER of $250 \Omega \cdot \text{cm}^2$ ($\triangle$). In monolayers treated with 4 mM EGTA, TER immediately decreased ($\bullet$) and ZO-2 staining moved from the cell borders to the cytoplasm. When the PKC inhibitor GF 109203X was added together with EGTA ($\circ$), ZO-2 remained at the cell boundaries and a significant delay in TER collapse was detected.

These results prompted us to explore the phosphorylation state of ZO-2 obtained from monolayers that either possessed (NC medium) or lacked (LC medium) TJs, as well as in those whose TJs were being disassembled as a result of Ca^{2+} chelation.

^{32}P -labelled monolayers cultured in LC, and in NC with or without 4 mM EGTA, were immunoprecipitated with antibodies against ZO-2, and the phosphate incorporated was detected by autoradiography in the same membrane in which Western blots against ZO-2 were performed later.

Figure 8 and Table 1 demonstrate a significantly higher ($P < 0.0001$) degree of ZO-2 phosphorylation in cells cultured in LC compared with those grown in NC. Moreover, we observed an increase in the apparent molecular mass of ZO-2 in LC (Figure 8, lower panel). This mobility shift might have been due to the enhanced phosphorylation found in this state.

To determine whether the higher phosphorylation of ZO-2 in the LC condition was triggered by the absence of Ca^{2+} , we evaluated the phosphorylation state of ZO-2 in monolayers of NC-cultured cells treated for 30 min with 4 mM EGTA. Figure 9 and Table 2 show that in effect the lack of Ca^{2+} induced a significant increase in the specific phosphorylation of ZO-2

Figure 10 ZO-2 two-dimensional phosphoamino acid analysis

ZO-2 was immunoprecipitated from cells cultured in NC or with 4 mM EGTA in the absence and in the presence of 6 μM GF 109203X or 1 μM H-89. Dotted ovals indicate the migration of standard phosphoamino acids from the origin (Or), identified by ninhydrin staining: phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y).

($P < 0.0003$). PKC seemed to be responsible for this phosphorylation because treatment with 6 μ M GF 109203X abolished the EGTA-induced hyperphosphorylation of ZO-2 ($P < 0.0001$). This concentration of inhibitor corresponded to the IC_{50} for the compound for the atypical PKCs (λ and ζ) [27] and surpassed those of new and classical PKCs.

Because we had found that PKA co-immunoprecipitated with ZO-2, we also explored the possible participation of this kinase in ZO-2 phosphorylation during Ca^{2+} removal. Figure 9 and Table 2 show that 1 μ M of the PKA-specific inhibitor H-89 effectively blocked ZO-2 hyperphosphorylation ($P < 0.0001$) and that the addition of both PKA and PKC inhibitors together with EGTA did not decrease ZO-2 phosphorylation below the level found in NC-cultured cells. The search for putative PKA phosphorylation motifs in canine ZO-2 revealed two sites: one located between the first and second PDZ repeats (Ser-140) and the other at the acidic region (Ser-950) (see Figure 1). Although the number of PKA consensus sites contrasted with the abundance of PKC motifs ([31]; see Figure 1) found in ZO-2, the phosphorylation of the former might have been crucial, because treatment with H-89 apparently restrained PKC-mediated phosphorylation. This could be speculatively explained if PKA phosphorylation were to induce a conformational change in ZO-2 that was necessary for exposing PKC phosphorylation sites to the enzyme.

To determine the nature of the phosphorylated residues in ZO-2, we performed a two-dimensional phosphoamino acid analysis of immunoprecipitated ZO-2. Figure 10 shows that ZO-2 was predominantly serine phosphorylated. This phosphorylation increased significantly during TJ disassembly triggered by Ca^{2+} chelation and could be blocked with GF 109203X or H-89, suggesting the participation of PKC and PKA.

DISCUSSION

In the present study we have generated two GST fusion proteins of the middle (3PSG) and C-terminal (AP) domains of ZO-2 that are phosphorylated by kinases present in a cellular extract. The two-dimensional phosphoamino acid analyses demonstrated intense phosphorylation of serine residues in 3PSG and AP proteins and a faint threonine spot in the latter. Tyrosine phosphorylation of endogenous ZO-2 has been reported previously [4,5], and sequence analysis reveals the presence of six putative tyrosine phosphorylation sites: four located in the basic region between PDZ-1 and PDZ-2 (Tyr-222, Tyr-226, Tyr-230 and Tyr-234), one in the SH3 region (Tyr-622) and another in the GK domain (Tyr-787). However, the two-dimensional phosphoamino acid analysis of native ZO-2 does not show phosphorylated tyrosine residues in NC monolayers or in those subjected to Ca^{2+} chelation (Figure 9 and Table 1). However, this does not exclude tyrosine phosphorylation under other conditions, as has been found in v-Src-transfected cells [4] and in cultures treated with a tyrosine phosphatase inhibitor [32].

Both 3PSG and AP proteins seem to be excellent PKC substrates, although the degree of their phosphorylation varies with the isoform assayed. The use of GF 109203X, an inhibitor capable of acting on classical, new and atypical PKCs, confirmed the point.

ZAK, an uncharacterized kinase different from PKC, is responsible for the serine phosphorylation of ZO-1 at the C-terminus of SH3 [31]. This domain contains only two putative serine phosphorylation sites, which are located immediately adjacent to each other (Ser-597 and Ser-598) and are conserved in other members of the MAGUK family: ZO-2 (Ser-677, canine), ZO-3 (Ser-557, Ser-558) and Tamou (Ser-592). Therefore

the serine PKC phosphorylation found in the 3PSG fusion protein might not take place at the SH3 domain and might instead occur at any of the PKC consensus sites found between PDZ-2 and PDZ-3 (Ser-425 and Ser-434) and/or at the GK domain (Ser-819 and Ser-820).

When immunoprecipitated endogenous ZO-2 was overlaid with an enriched PKC fraction, β , ϵ , λ and ζ isoforms became associated. Because under these conditions ZO-2 is exposed in a denatured form, its binding to PKCs reflects the strong nature of the interaction. This interaction seems to be present *in vivo*, because isoforms λ and ζ co-immunoprecipitate with native ZO-2. It is noteworthy that PKC ϵ can be found associated to ZO-2 only when derived from subconfluent cells, a condition in which ZO-2 concentrates at the nucleus [25]. The absence of isoform β from the ZO-2 immunoprecipitate was expected because it remained almost undetectable in the total cellular extract. The observation that different PKC isoforms located at the TJ and the nucleus are complexed to ZO-2 suggests diverse functions for this protein that could be regulated by phosphorylation by different isoenzymes and maybe even on distinct residues.

The hyperphosphorylation of ZO-2 in cells that either lack TJs owing to being cultured in LC or disassemble them as a result of Ca^{2+} chelation might suggest that the phosphorylated state of this molecule restrains its capacity to operate at the junctional complex. Citi [17] observed that the addition of a PKC inhibitor during Ca^{2+} chelation prevents the removal of ZO-1 and cingulin from the cell borders. We have obtained the same result for ZO-2 with GF 109203X.

We showed previously that a PKC sensitive to 1,2-dioctanoyl-glycerol (DiC8) participated in the assembly of the TJ induced by Ca^{2+} [3,13]. We then demonstrated that the phosphorylation of vinculin by this type of PKC is crucial for the correct assembly of the epithelial junctional complex [20]. Therefore classical and new PKCs, both sensitive to DiC8, seem to participate in the process of TJ formation, whereas atypical and therefore non-DiC8-responsive kinases might regulate the disassembly process through their action on ZO-2.

To co-ordinate multiple components of signal transduction pathways, cells have resorted to the use of scaffolding proteins that hold selected kinases and phosphatases near the precise proteins that they are supposed to regulate. To organize their function, these proteins harbour multiple protein-protein binding regions, a condition met in the MAGUK proteins by their PDZ, SH3 and GK domains [2]. One well-studied scaffolding MAGUK protein is PSD-95, a synaptic molecule that links an *N*-methyl-D-aspartate glutamate receptor, an inward rectifier K^+ channel and a tyrosine kinase [2]. The presence of both PKA and PKC in ZO-2 immunoprecipitates favours the concept of ZO-2 as a scaffolding TJ protein. Although we were unable to detect PP1 and PP2A in the ZO-2 immunoprecipitate, we expect that this complex contains another serine/threonine phosphatase. It has been shown recently that the polarity determinant protein Par-6 forms a stable complex with Rac1 or Cdc42 and atypical PKCs. This association results in the stimulation of the activity of atypical PKCs and cell transformation [33]. Par-6 interacts through PDZ domains with Par-3/ASIP/Bazooka [34], a TJ-located protein [19] that is in turn directly associated to atypical PKCs [35]. Therefore a close link between TJs and atypical PKCs is starting to emerge.

In summary, we have demonstrated that, during TJ disassembly, ZO-2 becomes serine phosphorylated by atypical PKCs located at the TJ and complexed with ZO-2. PKA and the new PKC ϵ , present in the nuclei of MDCK cells, also phosphorylate this molecule.

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REFERENCES

- Cereijido, M. (1992) Evolution of ideas on the tight junction. In *Tight Junctions* (Cereijido, M., ed.), pp. 1–3. CRC Press, Boca Raton, FL.
- González-Mariscal, L., Betanzos, A. and Avila Flores, A. (2000) MAGUK proteins: structure and role in the tight junction. *Semin. Cell. Dev. Biol.* **11**, 315–324.
- Balda, M. S., Gonzalez-Mariscal, L., Matter, K., Cereijido, M. and Anderson, J. M. (1993) Assembly of the tight junction: the role of diacylglycerol. *J. Cell Biol.* **123**, 293–302.
- Takeda, H. and Tsukita, S. (1995) Effects of tyrosine phosphorylation on tight junctions in temperature-sensitive v-src-transfected MDCK cells. *Cell Struct. Funct.* **20**, 387–393.
- Gopalakrishnan, S., Raman, N., Atkinson, S. J. and Marrs, J. A. (1998) Rho GTPase signaling regulates tight junction assembly and protects tight junctions during ATP depletion. *Am. J. Physiol.* **275**, C798–C809.
- Itoh, M., Morita, K. and Tsukita, S. (1999) Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. *J. Biol. Chem.* **274**, 5981–5986.
- Beatch, M., Jesaitis, L. A., Gallin, W. J., Goodenough, D. A. and Stevenson, B. R. (1996) The tight junction protein ZO-2 contains three PDZ (PSD-95/Discs-Large/ZO-1) domains and an alternatively spliced region. *J. Biol. Chem.* **271**, 25723–25726.
- Fanning, A. S., Jameson, B. J., Jesaitis, L. A. and Anderson, J. M. (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J. Biol. Chem.* **273**, 29745–29753.
- Wittchen, E. S., Haskins, J. and Stevenson, B. R. (1999) Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J. Biol. Chem.* **274**, 35179–35185.
- Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M. and Tsukita, S. (1999) Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J. Cell Biol.* **147**, 1351–1363.
- Itoh, M., Morita, K. and Tsukita, S. (1999) Characterization of ZO-2 as a MAGUK family member associated with tight as well as adherens junctions with a binding affinity to occludin and alpha catenin. *J. Biol. Chem.* **274**, 5981–5986.
- Mattagajasingh, S. N., Huang, S. C., Hartenstein, J. S. and Benz, Jr., E. J. (2000) Characterization of the interaction between protein 4.1R and ZO-2. A possible link between the tight junction and the actin cytoskeleton. *J. Biol. Chem.* **275**, 30573–30585.
- Balda, M. S., Gonzalez-Mariscal, L., Contreras, R. G., Macias-Silva, M., Torres-Marquez, M. E., Garcia-Sainz, J. A. and Cereijido, M. (1991) Assembly and sealing of tight junctions: possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. *J. Membr. Biol.* **122**, 193–202.
- Denisenko, N., Burighel, P. and Citi, S. (1994) Different effects of protein kinase inhibitors on the localization of junctional proteins at cell–cell contact sites. *J. Cell Sci.* **107**, 969–981.
- Nigam, S. K., Denisenko, N., Rodriguez-Boulant, E. and Citi, S. (1991) The role of phosphorylation in development of tight junctions in cultured renal epithelial (MDCK) cells. *Biochem. Biophys. Res. Commun.* **181**, 548–553.
- Stuart, R. O. and Nigam, S. K. (1995) Regulated assembly of tight junctions by protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6072–6076.
- Citi, S. (1992) Protein kinase inhibitors prevent junction dissociation induced by low extracellular calcium in MDCK epithelial cells. *J. Cell Biol.* **117**, 169–178.
- Dodane, V. and Kachar, B. (1996) Identification of isoforms of G proteins and PKC that colocalize with tight junctions. *J. Membr. Biol.* **149**, 199–209.
- Izumi, Y., Hirose, T., Tamai, Y., Hirai, S., Nagashima, Y., Fujimoto, T., Tabuse, Y., Kempfues, K. J. and Ohno, S. (1998) An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of *Caenorhabditis elegans* polarity protein PAR-3. *J. Cell Biol.* **143**, 95–106.
- Perez-Moreno, M., Avila, A., Islas, S., Sanchez, S. and Gonzalez-Mariscal, L. (1998) Vinculin but not alpha-actinin is a target of PKC phosphorylation during junctional assembly induced by calcium. *J. Cell Sci.* **111**, 3563–3571.
- Boyle, W. J., van der Geer, P. and Hunter, T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* **201**, 110–149.
- Robles-Flores, M. and Garcia-Sainz, J. A. (1993) Activated protein kinase C binds to intracellular receptors in rat hepatocytes. *Biochem. J.* **296**, 467–472.
- Robles-Flores, M., Alcantara-Hernandez, R. and Garcia-Sainz, J. A. (1991) Differences in phorbol ester-induced decrease of the activity of protein kinase C isozymes in rat hepatocytes. *Biochim. Biophys. Acta* **1094**, 77–84.
- Gonzalez-Mariscal, L., Chavez de Ramirez, B. and Cereijido, M. (1985) Tight junction formation in cultured epithelial cells (MDCK). *J. Membr. Biol.* **86**, 113–125.
- Gonzalez-Mariscal, L., Islas, S., Gonzalez-Robles, A. and Ponce, L. (1999) Expression of the tight junction associated protein ZO-2 at the nuclei of subconfluent epithelial cells. *Mol. Biol. Cell* **10**, 407a, abstract number 2358.
- Rendon-Huerta, E., Mendoza-Hernandez, G. and Robles-Flores, M. (1999) Characterization of calreticulin as a protein interacting with protein kinase C. *Biochem. J.* **344**, 469–475.
- Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem.* **268**, 9194–9197.
- Wolf, M. and Sahyoun, N. (1986) Protein kinase C and phosphatidylserine bind to *M*₁ 110,000/115,000 polypeptides enriched in cytoskeletal and postsynaptic density preparations. *J. Biol. Chem.* **261**, 13327–13332.
- Gonzalez-Mariscal, L., Contreras, R. G., Bolivar, J. J., Ponce, A., Chavez de Ramirez, B. and Cereijido, M. (1990) Role of calcium in tight junction formation between epithelial cells. *Am. J. Physiol.* **259**, C978–C986.
- Cereijido, M., Robbins, E. S., Dolan, W. J., Rotunno, C. A. and Sabatini, D. D. (1978) Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J. Cell Biol.* **77**, 853–880.
- Balda, M. S., Anderson, J. M. and Matter, K. (1996) The SH3 domain of the tight junction protein ZO-1 binds to a serine protein kinase that phosphorylates a region C-terminal to this domain. *FEBS Lett.* **399**, 326–332.
- Staddon, J. M., Herrenknecht, K., Smales, C. and Rubin, L. L. (1995) Evidence that tyrosine phosphorylation may increase tight junction permeability. *J. Cell Sci.* **108**, 609–619.
- Qiu, R. G., Abo, A. and Steven, M. G. (2000) A human homolog of the *C. elegans* polarity determinant Par-6 links Rac and Cdc42 to PKC ζ signaling and cell transformation. *Curr. Biol.* **10**, 697–707.
- Joberty, G., Petersen, C., Gao, L. and Macara, I. G. (2000) The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat. Cell Biol.* **2**, 531–539.
- Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D. and Pawson, T. (2000) A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and α PKC signalling and cell polarity. *Nat. Cell Biol.* **2**, 540–547.

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