

Vinculin but not α -actinin is a target of PKC phosphorylation during junctional assembly induced by calcium

M. Perez-Moreno, A. Avila, S. Islas, S. Sanchez and L. González-Mariscal*

Center for Research and Advanced Studies (CINVESTAV), Department of Physiology, Biophysics and Neurosciences, Ap. Postal 14-740, Mexico D.F. 07000, Mexico

*Author for correspondence

Accepted 20 September; published on WWW 12 November 1998

SUMMARY

The establishment of the junctional complex in epithelial cells requires the presence of extracellular calcium, and is controlled by a network of reactions involving G-proteins, phospholipase C and protein kinase C. Since potential candidates for phosphorylation are the tight junction associated proteins ZO1, ZO2 and ZO3, in a previous work we specifically explored these molecules but found no alteration in their phosphorylation pattern. To continue the search for the target of protein kinase C, in the present work we have studied the subcellular distribution and phosphorylation of vinculin and α -actinin, two actin binding proteins of the adherent junctions. We found that during the junctional sealing induced by Ca^{2+} , both proteins move towards the cell periphery and, while there is a significant increase in the phosphorylation of vinculin, α -actinin remains unchanged. The increased

phosphorylation of vinculin is due to changes in phosphoserine and phosphothreonine content and seems to be regulated by protein kinase C, since: (1) DiC8 (a kinase C stimulator) added to monolayers cultured without calcium significantly increases the vinculin phosphorylation level; (2) H7 and calphostin C (both protein kinase C inhibitors) completely abolish this increase during a calcium switch; (3) inhibition of phosphorylation during a calcium switch blocks the subcellular redistribution of vinculin and α -actinin. These results therefore suggest that vinculin phosphorylation by protein kinase C is a crucial step in the correct assembly of the epithelial junctional complex.

Key words: Vinculin, α -Actinin, Phosphorylation, PKC, Calcium-switch, MDCK, Junctional complex

INTRODUCTION

The tight junction (TJ) is the outermost component of the junctional complex. It is responsible for controlling the passage of ions and molecules through the paracellular pathway, and maintaining a polarized distribution of lipids and proteins between the apical and basolateral plasma membranes (Cereijido, 1992; Cereijido et al., 1989, 1993; Mandel et al., 1993).

A useful tool to study junction formation is the so called 'Ca-switch' (CS): newly plated monolayers incubated in a medium without Ca^{2+} develop no junctional complex, yet the addition of this cation triggers the assembly and sealing of the TJ (Gonzalez-Mariscal et al., 1990; Contreras et al., 1992a,b) through a cascade of transducers and enzymes, such as G proteins, phospholipase C, protein kinase C (PKC) and calmodulin (Balda et al., 1991; Denker et al., 1996). The role played by PKC has been difficult to understand, since PKC inhibitors not only block junction assembly during the CS procedure (Balda et al., 1991; Denisenko et al., 1994; Nigam et al., 1991; Stuart and Nigam, 1995), but also prevent the disassembly induced by removal of Ca^{2+} from the medium (Citi, 1992). Consistent with the view that Ca^{2+} triggers a cascade of intercellular reactions, the addition of DiC8, a PKC

agonist, in the absence of extracellular Ca^{2+} , induces the relocation of the tight junction protein ZO1 and F-actin at the cell borders, and decreases the paracellular permeability (Balda et al., 1993). In spite of this effect, DiC8 does not induce redistribution of cadherin, suggesting that PKC may act downstream from this molecule.

When stimulators and inhibitors of PKC are used, the specific phosphorylation of the tight junction phosphoproteins ZO1, ZO2, ZO3 and cingulin is not significantly modified, in spite of the important changes in the assembly and maintenance of the tight junctions elicited by those agents (Balda et al., 1993; Citi and Denisenko, 1995). This opens up the possibility that PKC would not act directly on the tight junction proteins themselves, but may affect instead the actin cytoskeleton. This would be consistent with the fact that H7, a PKC inhibitor, is known to alter the contractility of microfilaments (Citi et al., 1994; Volberg et al., 1994). It will also agree with the observation that actin-disturbing drugs, such as cytochalasin, disrupt the assembly and maintenance of the TJ, and open up the paracellular pathway, (Gonzalez-Mariscal et al., 1985; Madara et al., 1988; Meza et al., 1982; Stevenson and Begg, 1994). The relationship between the cadherin-catenin system, which is an actin dependent cell-cell contact, and the tight junctions is very strong, since these contacts are a prerequisite for TJ formation, as exemplified by

the inhibitory action that antibodies against E-cadherin exert on the appearance of TJ components at the cell membrane (Gumbiner et al., 1988).

Therefore, to pursue our systematic search for the target of PKC during junctional assembly, in the present work we have explored the level of specific phosphorylation of vinculin and α -actinin. The reasons for choosing these molecules are: (1) α -actinin and vinculin are located at the attachment points of actin filaments to the plasma membrane and both molecules are involved in the organization of actin filament bundles (Craig and Pardo, 1979; Geiger, 1982; Geiger et al., 1980; Lazarides and Burridge, 1975); (2) at the adherens junctions, α -actinin is a linkage between the cadherin-catenin complex and the cytoskeleton, since it binds α -catenin (Knudsen et al., 1995; Nieset et al., 1997), vinculin (Kroemker et al., 1994; McGregor et al., 1994; Wachsstock et al., 1987), and actin (Burridge and Feramisco, 1981; Duhaiman and Bamburg, 1984; Kuhlmann et al., 1992); (3) α -actinin and vinculin are substrates of PKC phosphorylation in vitro and in vivo (Kawamoto and Hidaka, 1984; Schwienbacher et al., 1996; Werth et al., 1983; Werth and Pastan, 1984). Furthermore, since phosphorylated vinculin is enriched in the insoluble fraction of detergent treated cells (Geiger, 1982), phosphorylation might be important in vinculin function; and (4) vinculin is a crucial molecule in the maintenance of the epithelial phenotype (Coll et al., 1995; Goldman et al., 1995; Rodriguez-Fernandez et al., 1992; Samuels et al., 1993).

To investigate the phosphorylation level of vinculin and α -actinin during junctional assembly, we use the CS procedure in the presence of PKC stimulators and inhibitors. We found that the subcellular distribution of both α -actinin and vinculin changes during the CS and is sensitive to PKC inhibitors. α -Actinin and vinculin are both phosphorylated in serine, threonine and tyrosine residues in confluent epithelial monolayers, yet during the CS there is a significant increase in vinculin serine and threonine phosphorylation that is sensitive to PKC inhibitors. These results indicate that PKC regulates the assembly of the epithelial junctional complex, acting through the cytoskeleton and, more specifically, on vinculin.

MATERIALS AND METHODS

Cell culture

Starter Madin Darby canine kidney (MDCK) cultures were obtained from the American Type Culture Collection (MDCK, CCL 34). Cells between the 60th and 80th passages were grown at 36.5°C in disposable plastic bottles (Costar 3151, Cambridge, MA) with a 95% air-5% CO₂ atmosphere (SteriCult 200, Forma Scientific, Ohio) and 20 ml of Dulbecco's modified Eagle's basal medium (DMEM; D1152, Sigma Chemical Co. St Louis, MO) with penicillin (100 i.u./ml), streptomycin (100 µg/ml; In Vitro, México), insulin (0.08 U/ml; Eli Lilly, Mexico), and 10% iron supplemented certified calf serum (Gibco BRL, 10371-029, Grand Island, NY). Cells were harvested with trypsin EDTA (In Vitro, México) and plated at confluence. After 1 hour was allowed for cell attachment, medium was removed and replaced with a fresh medium without cells.

Calcium switch (CS)

In the CS experiments, cells were plated as previously described (Gonzalez-Mariscal et al., 1985). Briefly: trypsinized cells were plated at confluence in DMEM without phosphate or without phosphate and Ca²⁺ (Microlab, México DF) to which trypsin inhibitor was added

7,000 units of 0.5% trypsin inhibitor/ml of medium (Gibco 840-7074, Grand Island, NY). Twenty hours later the monolayers incubated in DMEM without phosphate and Ca²⁺, were transferred to DMEM without phosphate and with a normal Ca²⁺ concentration (NC, normal calcium), a process here referred to as CS, or to minimal essential medium without phosphates and Ca²⁺ (LC, low calcium) (Gibco 410-1400, Grand Island, NY3).

Immunofluorescence

Monolayers grown on glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7.4, and permeabilized for 30 minutes with 0.2% Triton X-100 in PBS (PBS-TX). Cells were washed with PBS-TX, blocked with 1% BSA in PBS-TX for 30 minutes, and incubated overnight at 4°C with specific antibodies against vinculin (N9131, Sigma Chemical Co., St Louis MO) or α -actinin (A5044, Sigma Chemical Co. St Louis MO) diluted in 1% BSA-PBS-TX. After washing, incubation of the primary antibody was followed by the secondary antibody (1:25, 55516: FITC sheep anti-mouse; Cappel, West Chester, PA) for 1 hour at room temperature. After washing three times, coverslips were mounted with a (1:19) mixture of *p*-phenyldiamine (Sigma Chemical Co., St Louis MO)-Gelvatol (Montsanto, Indian Orchard, Mass). Unless otherwise stated, images represent an optical section taken with a Bio-Rad confocal microscope MRC-600.

Metabolic labeling

20 hours after plating the cells in NC or LC media without phosphate (Microlab, México DF), monolayers were labeled in either LC or NC medium supplemented with 200 µCi/ml of [³²P]orthophosphate (acid free, 10 mCi/ml, PBS-13, Amersham Corp., Little Chalfont, Buckinghamshire, UK) for 4 hours at 37°C in a CO₂ incubator. Drugs were added to the phosphate-free labeling medium, 30 minutes before [³²P]orthophosphate labeling.

Immunoprecipitation

After labeling, cells were washed twice with PBS and lysed with RIPA buffer (150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 1 mM PMSF, 15 µg/ml leupeptin, 25 µg/ml pepstatin, 40 U/ml aprotinin, 0.2 mM sodium orthovanadate in 40 mM Tris-HCl, pH 7.6) for 30 minutes at 4°C with gentle rotation. The extracts were collected and homogenized vigorously with a syringe, and centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatants were collected and cleared with *Staphylococcus aureus* cells (Pansorbin cells 10%, 507851, Calbiochem, La Jolla, CA) for 1 hour at 4°C. After centrifugation (10 minutes at 14,000 rpm) supernatants were incubated for 3 hours at 4°C with anti-vinculin or anti- α -actinin specific antibodies under continuous rotation; *Staphylococcus* cells conjugated with IgG goat antibodies to IgG mouse antibodies (Tachisorb cells 10%, 575541, Calbiochem, La Jolla, CA) were added and incubated 1 hour at 4°C. Immunoprecipitates were washed twice with RIPA buffer followed by three washes with 50 mM Tris-HCl, pH 7.4. The pellets were boiled 10 minutes in sample buffer (Laemmli, 1970) and electrophoresed onto 8% polyacrylamide-SDS gels.

Protein blotting and analysis

Proteins in polyacrylamide-SDS gels were either stained for 1 hour with 0.25% Coomassie Blue, or transferred to nitrocellulose papers (Hybond-C, RPN 303C, Amersham, Little Chalfont, Buckinghamshire), according to the method of Towbin et al. (1979). In order to identify the proteins, blotting was performed with mouse monoclonal antibodies against vinculin (N9131, Sigma Chemical Co., St Louis MO) or α -actinin (A5044, Sigma Chemical Co., St Louis MO). Peroxidase-conjugated goat IgG to mouse IgG (Sigma Chemical Co., A5278, St Louis MO) was used as a secondary antibody, followed by a chemiluminescence detection system (ECL, RPN 2105, Amersham, Little Chalfont, Buckinghamshire, England).

Determination of specific phosphorylation

Total [^{32}P]phosphate labeling was detected by autoradiography, and total protein was determined in the same gel by immunoblotting using specific antibodies. Specific amino acid phosphate incorporation was detected by performing immunoblots in parallel samples with specific antibodies against phosphoserine (P-ser) (P3430, Sigma Chemical Co., St Louis MO), phosphothreonine (P-threo) (P3555, Sigma Chemical Co., St Louis MO), phosphotyrosine (P-tyr) (P3300, Sigma Chemical Co., St Louis MO), vinculin and α -actinin. A peroxidase-conjugated secondary antibody followed by a chemiluminescence detection system (ECL) was used as described above. Autoradiographs were quantified with an Image Densitometer (Molecular Analysis, Bio-Rad GS-670). The ratios of [^{32}P]protein/total protein and Pser-Pthreo-Ptyr/total protein represent the total specific phosphorylation and the residue specific phosphorylation, respectively.

Phosphoaminoacid analysis

Immunoprecipitates resolved by polyacrylamide-SDS gel electrophoresis were transferred to PVDF membranes (Hybond-P, RPN-2020F, Amersham, Little Chalfont, Buckinghamshire), phosphoaminoacids were analyzed based on the method of Boyle et al. (1991) with minor modifications. ^{32}P -labeled phosphorylated vinculin bands were excised from membranes and hydrolyzed with 6 N HCl at 110°C for 2 hours. The hydrolysates were lyophilized using a Speed-Vac concentrator (VR-1, Heto Lab Equipment, Gydevang, Denmark) and resuspended in 10 μl of pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid) containing 2 μg of each cold phosphoaminoacid standard. The samples were spotted onto thin-layer-cellulose-plates (5716 Merck, KGart Darmstad, Germany) and amino acid separation was performed by thin layer electrophoresis on a HTLE-7000 apparatus (CBS Scientific Co., Del Mar, CA) running at 1,500 V for 45 minutes in the first dimension with the pH 1.9 buffer. The second dimension thin layer chromatography was carried out in a mixture of *n*-butanol:acetic acid:H₂O (100:22:50). The individual amino acids were identified by comparison with the internal standards detected by ninhydrin staining. The position of ^{32}P -labeled phosphoaminoacids was determined by autoradiography.

Specific activity of PKC

The specific activity of PKC in monolayers of MDCK cells was determined with a PKC assay system (13161-013 Gibco-BRL, Grand Island, NY). Briefly, a Triton X-100 extract of MDCK monolayers is prepared, purified in a DEAE-52 column, and the incorporation of ^{32}P from [^{32}P]ATP to myelin basic protein under PKC stimulation with PMA is measured.

PKC fluorescence detection

PKC was detected using RIM-1 (Chii-Shiarng and Poenie, 1993), a rhodaminated specific inhibitor of PKC (1:100, 0081: Teflabs, Austin, Texas). Monolayers grown on glass coverslips were fixed and permeabilized with -20°C methanol. Cells were washed with PBS, blocked with 3% FBS in PBS for 30 minutes, and incubated for 1 hour with RIM-1. After washing three times, coverslips were mounted with a (1:19) mixture of *p*-phenylendiamine (Sigma Chemical Co., St Louis MO)-Gelvatol (Montsanto Indian Orchard, Mass). Images represent a projection of 20, 0.5 μm optical sections taken with a Bio-Rad confocal microscope MRC-600.

Drugs

H7, PMSF, EGD, DiC8, calphostin C and orthovanadate, were all obtained from Sigma Chemical Co. (St Louis, MO). Stocks of DiC8 (5 mg/ml), calphostin C (1 mg/ml) and GF 109203X (20 μM) were prepared in DMSO attaining a final concentration of DMSO in DMEM <0.1%. Stocks of H7 (25 mg/ml), EGD (100 mg/ml), and orthovanadate (10 mM) were prepared in water. All stocks were kept at -20°C. A stock of PMSF (57 $\mu\text{g}/\text{ml}$) was prepared fresh in

isopropyl alcohol. The following concentrations of drugs were used: H7, 50 μM ; EGD, 100 μM ; DiC8, 100 $\mu\text{g}/\text{ml}$, calphostin C, 500 nM and GF 109203X, 5 μM .

Results are expressed as means \pm s.e.m., where *n* is the number of observations.

RESULTS

The subcellular distribution of vinculin and α -actinin changes during the CS

The distribution of vinculin as well as α -actinin during the formation of tight junctions initiated by the CS, was examined by immunofluorescence microscopy using confluent MDCK cells. In the LC medium, vinculin (Fig. 1a) and α -actinin (Fig.

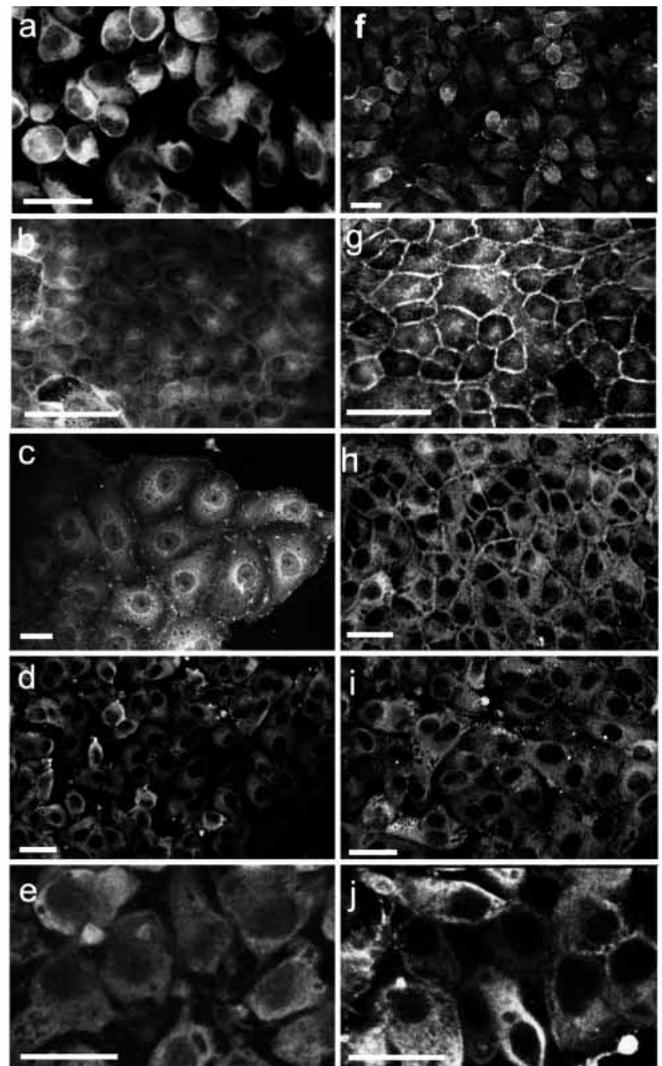
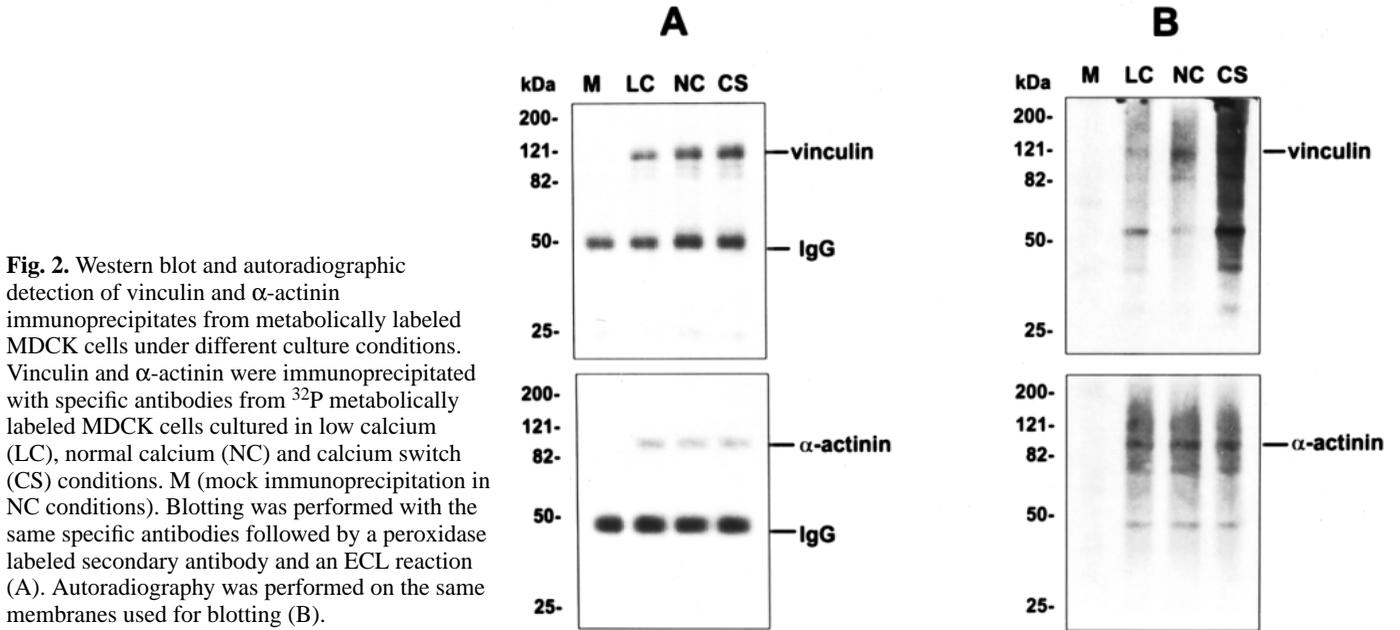


Fig. 1. Subcellular distribution of vinculin and α -actinin in the CS and under the effect of H7 and calphostin C. Immunofluorescence microscopy of vinculin (a-e) and α -actinin (f-j) in MDCK cells after 20 hours in LC (a,f) or in NC (b,g); 4 hours after the CS (c,h); and 4 hours after the CS in the presence of 500 nM calphostin C (d,i) or 50 μM H7 (e,j). The CS induced the translocation of vinculin and α -actinin from the cytoplasm to the sites of cell-cell contact. Note that both calphostin C and H7 prevent the localization of vinculin and α -actinin along the cell borders during the CS. Bar, 20 μm .

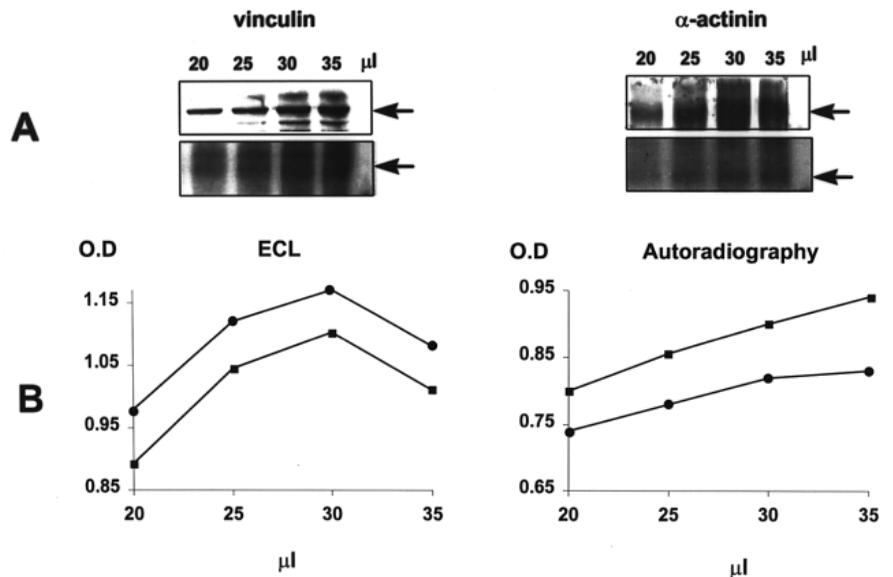


If signals were cytoplasmic and diffuse. When cells were stained 4 hours after the CS, the patterns for vinculin (Fig. 1c) and α -actinin (Fig. 1h) were almost identical to that observed in confluent monolayers that were maintained at NC (Fig. 1b and g, respectively). Since the establishment of the junctional complex is sensitive to PKC activity (Balda et al., 1991, 1993; Denisenko et al., 1994; Nigam et al., 1991; Stuart and Nigam, 1995), and vinculin as well as α -actinin are phosphorylated proteins (Egerton et al., 1996; Ito et al., 1983; Kawamoto and Hidaka, 1984; Schwienbacher et al., 1996; Sefton et al., 1981; Werth and Pastan, 1984; Werth et al., 1983), we explored whether their distribution was sensitive to the activity of PKC. Inhibition of PKC with calphostin C (Fig. 1d,i) or H7 (Fig. 1e,j) during a CS induced distribution patterns that resembled those found in cells maintained in LC. In summary, both vinculin and α -actinin are proteins whose subcellular distribution changes during the establishment of the junctional complex.

Vinculin but not α -actinin becomes phosphorylated during the CS

We next analyzed whether the level of phosphorylation of vinculin and α -actinin changes during the CS. ^{32}P -labeled monolayers cultured in LC, NC and CS were immunoprecipitated with specific antibodies to both proteins (Fig. 2A) and the phosphate incorporated was detected by autoradiography (Fig. 2B) in the same membrane. Since phosphorylated vinculin and α -actinin are found in LC, NC and CS conditions (Fig. 2B), a quantitative determination became necessary to ascertain if an increase in phosphorylation occurs during a CS.

Western blots (Fig. 3A above) and autoradiographs (Fig. 3A below) of different amounts of vinculin (left) and α -actinin (right) immunoprecipitates were quantified by densitometry. Fig. 3B shows that ECL determinations of total vinculin and α -actinin content were on the linear range whenever the OD



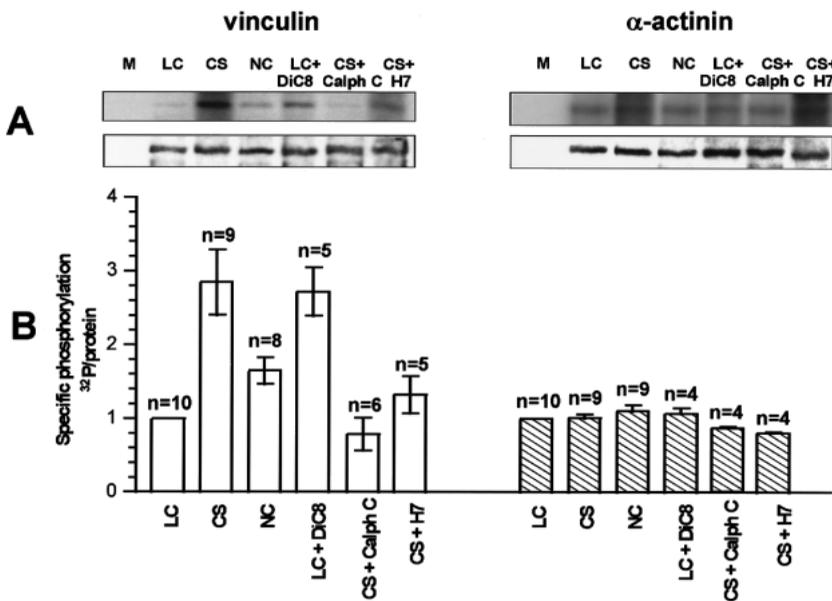


Fig. 4. Specific phosphorylation of vinculin and α -actinin. Vinculin and α -actinin were immunoprecipitated from metabolically ^{32}P -labeled MDCK cells cultured in LC without and with DiC8, NC and CS condition with and without H7 (50 μM) and calphostin C (500 nM). (A) An example of phosphorylated vinculin and α -actinin is shown in the autoradiographs (upper) together with their corresponding ECL western blot determinations (lower). (B) Autoradiographs and ECL immunoblots were quantified with an image densitometer and specific phosphorylation was determined as the ratio of phosphorylated protein to total protein content, as described in Materials and Methods. Phosphorylation at 20 hours in LC was taken as 1 to normalize the results of different experiments. The standard error is shown, and n indicates the number of experiments performed for each experimental condition. The changes in vinculin phosphorylation were significant as determined by a t -test: LC Vs CS $P < 0.0001$; LC Vs NC $P < 0.001$; LC Vs LC + DiC8 $P < 0.0001$; CS Vs CS + H7 $P < 0.05$ and CS Vs CS + calphostin C $P < 0.005$. The changes in α -actinin phosphorylation were not statistically significant ($P > 0.05$) with the exception of CS Vs CS + H7 ($P = 0.039$).

values obtained were below 1. For the phosphate incorporation studies, densitometric analysis of autoradiographs showed a linear increase when OD measurements were below 0.85.

Data on the linear range of Fig. 3B were used to obtain the specific phosphorylation (ratio of protein phosphorylation/total protein content) of each protein during the CS. Fig. 4 shows a significant increase ($P < 0.0001$) in vinculin phosphorylation during the CS. The increase is greater than the value obtained in monolayers permanently cultured in NC. When the CS is done in the presence of PKC inhibitors, a significant blockade in phosphorylation is found, being more significant for calphostin C ($P < 0.005$) than for H7 ($P < 0.05$). For α -actinin no significant changes in phosphorylation were found, with the exception of a CS performed in the presence of H7, where the blockade in phosphorylation by the PKC inhibitor gave values

below those obtained in monolayers cultured in LC ($P < 0.0395$).

During the CS vinculin becomes phosphorylated in serine and threonine residues in a process that suggests the participation of PKC

To determine the type of phosphorylation that takes place during the CS we studied the specific phosphate incorporation per amino acid residue, using western blot analysis (Fig. 5A). Quantifications of vinculin in Fig. 5B show that while no phosphorylation changes are found in P-tyr, a very significant ($P < 0.0001$) increase in P-ser and P-threo phosphorylation occurs during the CS. These changes can be suppressed with the PKC inhibitor H7. As expected from the results shown in Fig. 4, no changes in α -actinin specific phosphate incorporation per amino acid residue were found (Fig. 5B).

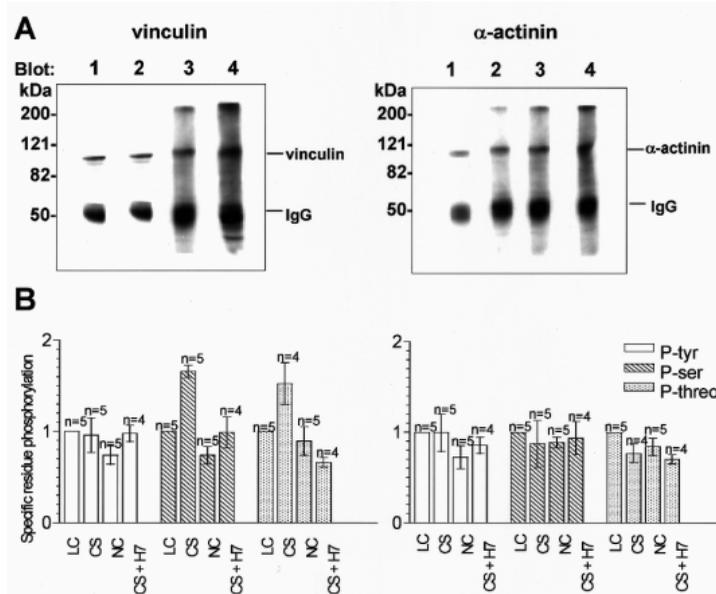


Fig. 5. Specific phosphate incorporation per amino acid residue of vinculin and α -actinin. (A) Immunoprecipitated vinculin and α -actinin were analyzed by western blot using antibodies against the immunoprecipitated protein (lanes 1); anti-P-tyr (lanes 2), anti-P-ser (lanes 3) and anti-P-threo (lanes 4). (B) Specific quantification of P-tyr, P-ser and P-threo to vinculin and α -actinin in cells cultured in LC, NC, CS and CS + H7. Specific residue phosphorylation was quantified as the ratio of P-tyr, P-ser and P-threo determined with specific antibodies to total protein content, as described in Materials and Methods. Determinations at 20 hours in LC were taken as 1 to normalize the results of different experiments. The standard error is shown, and n indicates the number of experiments performed for each experimental condition. The changes in vinculin phosphorylation for P-tyr were not statistically significant. Changes in vinculin P-ser were significant as determined by a t -test: LC Vs CS, $P < 0.0001$; LC Vs NC $P < 0.05$; and CS Vs CS + H7, $P < 0.005$. With the exception of LC Vs NC all the changes in P-threo were significant according to a t -test: LC Vs CS, $P < 0.0001$; and CS Vs CS + H7, $P < 0.05$. The changes in α -actinin P-tyr, P-ser and P-threo were not statistically significant ($P > 0.05$) with the exception of LC Vs CS ($P = 0.0395$) in P-threo.

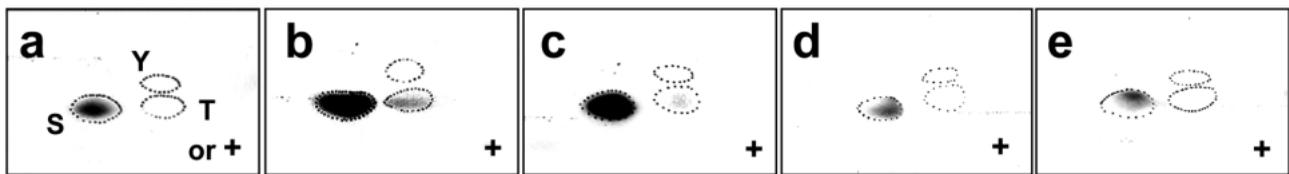


Fig. 6. Phosphoaminoacid analysis of vinculin. Total hydrolysates of phosphorylated vinculin from cells cultured in LC, CS, LC + DiC8, CS + calphostin C and CS + GF 109203X, were spotted onto thin layer cellulose plates at the origin (or). Separation was performed in the first dimension by thin layer electrophoresis and in the second dimension by thin layer chromatography. Dotted ovals indicate the migration of standard phosphoaminoacids, identified by ninhydrin staining: phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y). The TLC plates were simultaneously autoradiographed for 24 hours.

Table 1. PKC activity

Condition	Activity \pm s.e.m.	<i>n</i>
Normal calcium	1	11
Low calcium	1.91 \pm 0.33	10
Calcium switch	1.76 \pm 0.37	7

Determinations at NC were taken as 1 to normalize the results of different experiments. The standard error is shown, and *n* indicated the number of experiments performed for each experimental condition.

The identity of the phosphorylated amino acids was corroborated by partial hydrolysis and thin layer chromatography of the ^{32}P -labeled vinculin (Fig. 6). In cells cultured in LC medium, vinculin was found to contain P-ser (Fig. 6A). Ca-switched cells had increased amounts of both P-ser and P-threo (Fig. 6B). While cells cultured in LC with DiC8 showed an increased P-ser and P-threo signal similar to that found in Ca-transferred cells (Fig. 6C), those switched in the presence of the PKC inhibitors calphostin C (Fig. 6D) or GF 109203X (Fig. 6E) gave patterns resembling those that remained in LC medium.

The cellular distribution and content of PKC is Ca^{2+} dependent

To study whether PKC becomes activated during the CS, we analyzed its subcellular distribution before and after this process. Fig. 7 shows that the distribution of the enzyme in the cell is strongly dependent on the concentration of Ca^{2+} in

the bathing medium. Thus, in the absence of this ion (Fig. 7B), PKC is retained mainly in the cytoplasm, and upon switching the monolayers to Ca^{2+} containing medium (Fig. 7C), a fraction of PKC is transferred to the cell border. Ca^{2+} not only affects the position of PKC in the cell, but the content of this enzyme in the cell as well. Thus, using an assay that measures the amount of PKC that can be activated by the phorbol ester PMA, we found that this amount is higher in cells incubated in the absence than in the presence of Ca^{2+} (Table 1). Four hours after switching the cell from LC to NC does not suffice to reduce the amount of PKC to the levels it achieves in NC. However, it cannot be ruled out that part of the PKC that is cell junction associated in the presence of Ca^{2+} , becomes Triton insoluble and thus undetectable by the phorbol ester assay.

DISCUSSION

We have studied the role of Ca^{2+} in the subcellular distribution and phosphorylation of vinculin and α -actinin in epithelial monolayers. We found that: (1) while in LC, both molecules present a perinuclear distribution, the addition of Ca^{2+} makes them spread towards the cell membrane; (2) under both conditions vinculin and α -actinin are phosphorylated in serine, threonine and tyrosine residues; (3) Ca^{2+} addition increases phosphorylation in serine and threonine residues of vinculin; (4) vinculin phosphorylation is closely related to activation of PKC; and (5) experimental conditions that prevent vinculin

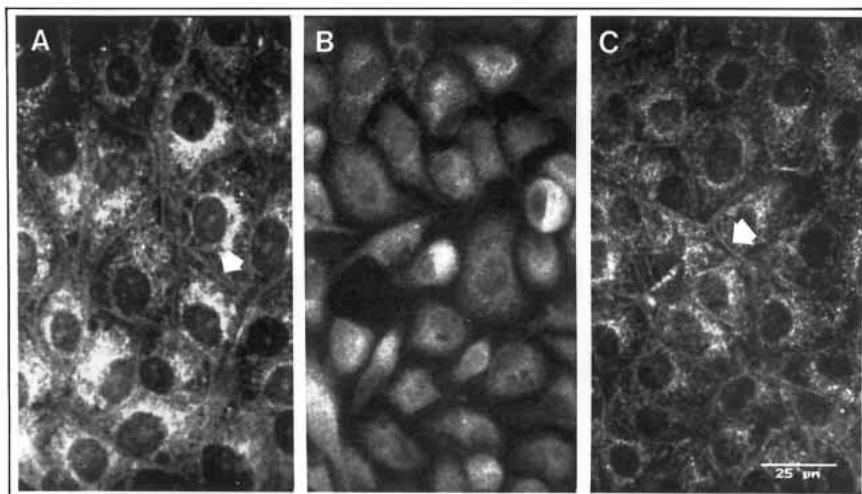


Fig. 7. The distribution of PKC in MDCK cells changes during the CS. Monolayers of MDCK cells were fixed and permeabilized with methanol -20°C , and stained with Rim-1, a rhodaminated specific PKC inhibitor. (A) Monolayers incubated for 20 hours in NC. The enzyme distributes around the nucleus, and shows a clear accumulation at the cell borders (arrow). (B) Monolayers incubated for 20 hours in LC show no accumulation of PKC at the cell border. (C) In monolayers incubated for 20 hours in LC, and switched to NC for 4 hours, PKC moves from the cytoplasmic region to the cell borders (arrow). Images represent projections of 20 optical sections of $0.5\ \mu\text{m}$ each.

phosphorylation, also block the subcellular redistribution of vinculin and α -actinin. Taken together, these results indicate that vinculin phosphorylation by PKC plays a central role in the assembly of the epithelial junctional complex.

Our observations of vinculin and α -actinin relocation are in accordance with studies of reorganization of these two proteins following ATP depletion and replenishment (Muhs et al., 1997; Wang, 1986) and Ca^{2+} induced assembly of adherens junctions (O'Keefe et al., 1987). In all these studies, while dispersion of vinculin is accompanied by a loss of cell adhesion, concentration of this protein at cell borders results in the formation of stable intercellular junctions.

The phosphorylation of vinculin in serine, threonine and tyrosine residues in NC and LC conditions, is consistent with previous reports that have shown that vinculin is a substrate of tyrosine kinase Src (Sefton et al., 1981) and PKC (Kawamoto and Hidaka, 1984; Schwienbacher et al., 1996; Werth et al., 1983; Werth and Pastan, 1984). Ca^{2+} caused a pronounced increase in vinculin phosphorylation as indicated by [^{32}P]orthophosphate incorporation. As shown by western blot and phosphoaminoacid analysis this increase is due to phosphorylated serine and threonine residues. The fact that calphostin C, GF 109203X and H7 strongly inhibit this phosphorylation suggests that it is operated by PKC. When we measured the total activity of PKC during a CS, we did not find any significant change. Presumably, the changes of activity of a particular PKC isoform that may have occurred remain undetectable in the total measurement. This is consistent with our finding that only a portion of PKC detected with the rhodaminated PKC inhibitor peptide, RIM, is translocated from the cytosol to the cell borders during the calcium switch. PKC is not just a single molecular species, but a family of serine/threonine kinases comprising at least 12 known isoforms. According to their molecular analysis, two groups of isoenzymes have been identified: the first group includes α , βI , βII and γ , which are active in the presence of calcium and phospholipids, while the second group comprises δ , ϵ , η , θ , λ and ζ , which do not require calcium for their activity (reviewed by Buchner, 1995; Dekker and Parker, 1994). While MDCK cells express PKC isoforms ζ , ϵ , β , α , δ , γ , ι (Dodane and Kachar, 1996), only ζ and α isoforms have been reported to concentrate at cell junctions in epithelial cells (Dodane and Kachar, 1996; Dong et al., 1993). In fibroblasts PKC α , has been found to codistribute with vinculin in focal contacts (Jaken et al., 1989).

Balda et al. (1991) have shown that stimulation and inhibition of PKC increases and blocks, respectively, the development of transepithelial electrical resistance (TER) during a CS, in confluent epithelial monolayers. However, the target of PKC phosphorylation remained obscure, due to the fact that ZO1, ZO2, ZO3 and cingulin do not change their phosphorylation status during the CS with and without PKC stimulators and inhibitors. Here we show that during this process, vinculin but not α -actinin becomes a target of PKC phosphorylation. We therefore propose that transfer from LC to NC medium moves vinculin from the cytosol to adherent junctions located at the cell borders. Vinculin is comprised of a large globular head domain and a smaller, rod-like tail region (Milam, 1985), and once it comes into close contact with phosphatidylinositol 4,5 biphosphate at the plasma membrane, the molecule changes its conformation from a closed form governed by head-tail

interactions, to an opened one (Tempel et al., 1995). This contact, in turn exposes, the vinculin interaction site for F-actin (Bubeck et al., 1997; Huttelmaier et al., 1997; Westmeyer et al., 1990) and a phosphorylation site for PKC, located at the tail region of the molecule (Johnson and Craig, 1995; Weekes et al., 1996). Therefore the activation of the tail would allow vinculin to crosslink and bundle actin, and to recruit by oligomerization more active vinculin molecules to cell-cell adhesion sites. We hence suggest that phosphorylation of vinculin at the membrane is a crucial process for the assembly of the junctional complex. This process might be finely regulated; accordingly it has been shown that binding of vinculin to F-actin decreases serine-threonine phosphorylation, presumably by overlapping binding sites (Kawamoto and Hidaka, 1984). This might explain the fact that serine/threonine phosphorylation of vinculin is somewhat decreased in NC cells when compared to calcium switched monolayers. This process could also be regulated by growth factors, since it has been shown that platelet derived growth factor (PDGF) decreases the amount of phosphatidylinositol 4,5 biphosphate bound to vinculin (Fukami et al., 1994), and nerve growth factor (NGF) treatment increases phosphorylation and redistribution of vinculin (Halegoua, 1987).

Several studies have recently shown that phosphorylation of vinculin may also play a role in cell adhesion disassembly (Bussolino et al., 1994; Dwyer-Nield et al., 1996; Halegoua, 1987; Jaken et al., 1989; Muhs et al., 1997). Therefore vinculin phosphorylation produces the apparently contradictory effects of increasing paracellular permeability in certain systems and yet in others such as MDCK cells, it seems to be a crucial step in the reassembly of the junctional complex after calcium replacement. This might not be so unexpected after all, since in MDCK cells inhibitors of PKC have both been shown to prevent junction disassembly induced by calcium chelation (Citi, 1992) or after treatment with cytochalasin D (Citi et al., 1994), and to block junctional reassembly during a calcium switch (Balda et al., 1991; Nigam et al., 1991).

The results of this study have shown that during CS procedure, vinculin becomes a target of PKC phosphorylation at serine and threonine residues and accumulates at cell-cell borders, thus suggesting that phosphorylation of vinculin plays a key role in cell adhesion. Further research both on the type of PKC participating in this process and on the identification of the specific serine and threonine residues that become phosphorylated may provide new insights into the understanding of why vinculin phosphorylation in different systems may give rise to opposite cell-cell adhesion outcomes.

We thank Dr Maria Teresa Hernandez (Center for Scientific Research of Yucatan, CICY, Mexico) and Dr Amparo Cano (Madrid Autonomous University, Spain) for their advice and Dr Marcelino Cerejido (CINVESTAV, Mexico) for critical reading of the manuscript. We also thank Dr Gerardo Contreras and biologist Jose Luna (CINVESTAV, Mexico) for their help with the confocal microscope. This work was supported by the National Research Council of Mexico (CONACYT). M. Perez-Moreno and A. Avila are both recipients of CONACYT fellowships.

REFERENCES

- Balda, M. S., Gonzalez-Mariscal, L., Contreras, R. G., Macias-Silva, M. E., Torres-Marquez, M., Garcia-Sainz, J. A. and Cerejido, 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.
- Balda, M. S., Gonzalez-Mariscal, L., Matter, K., Cerejido, M. and Anderson, J. M. (1993). Assembly of the tight junction: the role of diacylglycerol. *J. Cell Biol.* **123**, 293-302.
- Boyle, W. J., van der Geer, P. and Hunter, T. (1991). Phosphopeptide mapping and phosphoaminoacid analysis by two dimensional separation on thin layer cellulose plates. *Meth. Enzymol.* **201**, 110-148.
- Bubeck, P., Pistor, S., Wehland, J. and Jockusch, B. M. (1997). Ligand recruitment by vinculin domains in transfected cells. *J. Cell Sci.* **110**, 1361-1371.
- Buchner, K. (1995). Protein kinase C in the transduction of signals toward and within the cell nucleus. *Eur. J. Biochem.* **228**, 211-221.
- Burridge, K. and Feramisco, J. R. (1981). Non-muscle α -actinins are calcium-sensitive actin binding proteins. *Nature* **294**, 565-567.
- Bussolino, F., Silvagno, F., Garbarino, G., Costamagna, C., Sanavio, F., Arese, M., Soldi, R., Aglietta, M., Pescarmona, G., Camussi, G. and Bosia, A. (1994). Human endothelial cells are targets for platelet-activating factor (PAF). *J. Biol. Chem.* **269**, 2877-2886.
- Cerejido, M., Ponce, A. and Gonzalez-Mariscal, L. (1989). Tight junctions and apical/basolateral polarity. *J. Membr. Biol.* **110**, 1-9.
- Cerejido, M. (1992). Evolution of ideas on the tight junction. In *Tight Junctions* (ed. M. Cerejido), pp. 1-3. Boca Raton, FL: CRC.
- Cerejido, M., Gonzalez-Mariscal, L., Contreras, R. G., Gallardo, J. M., Garcia-Villegas, R. and Valdes, J. (1993). The making of a tight junction. *J. Cell Sci.* **17**, 127-132.
- Chii-Shiarn, C. and Poenie, M. (1993). New fluorescent probes for protein kinase C. Synthesis, characterization, and application. *J. Biol. Chem.* **268**, 15812-15822.
- Citi, S. (1992). Protein kinase inhibitors prevent junction dissociation induced by low extracellular calcium in MDCK epithelial cells. *J. Cell Biol.* **117**, 169-178.
- Citi, S., Volberg, T., Bershadsky, A. D., Denisenko, N. and Geiger, B. (1994). Cytoskeletal involvement in the modulation of cell-cell junctions by the protein kinase inhibitor H-7. *J. Cell Sci.* **107**, 683-692.
- Citi, S. and Denisenko, N. (1995). Phosphorylation of the tight junction protein cingulin and the effects of protein kinase inhibitors and activators in MDCK epithelial cells. *J. Cell Sci.* **108**, 2917-2926.
- Coll, J. L., Ben-Ze'ev, A., Ezzell, R. M., Rodriguez-Fernandez, J. L., Baribault, H., Oshima, R. G. and Adamson, E. D. (1995). Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion, and locomotion. *Proc. Nat. Acad. Sci. USA* **92**, 9161-9165.
- Contreras, R. G., Gonzalez-Mariscal, L., Balda, M. S., Garcia Villegas, M. R. and Cerejido, M. (1992a). The role of calcium in the making of a transporting epithelium. *News Physiol. Sci.* **7**, 105-108.
- Contreras, R. G., Miller, J. H., Zamora, M., Gonzalez-Mariscal, L. and Cerejido, M. (1992b). Interaction of calcium with the plasma membrane of epithelial (MDCK) cells during junction formation. *Am. J. Physiol.* **263**, C313-C318.
- Craig, S. W. and Pardo, J. V. (1979). Alpha-actinin localization in the junctional complex of intestinal epithelial cells. *J. Cell Biol.* **80**, 203-210.
- Dekker, L. and Parker, P. J. (1994). Protein kinase C-a question of specificity. *Trends Biochem. Sci.* **19**, 73-77.
- 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.
- Denker, B. M., Sahar, Ch., Khawaja, S. and Nigam, S. K. (1996). Involvement of a heterotrimeric G protein α subunit in tight junction biogenesis. *J. Biol. Chem.* **271**, 25750-25753.
- 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.
- Dong, L., Stevens, J. L. and Jaken, S. (1993). Transformation-sensitive localization of α -protein kinase C at cell-cell contacts in rat renal proximal tubule epithelial cells. *Cell Growth Differ.* **4**, 793-798.
- Duhaiman, A. S. and Bambang, J. R. (1984). Isolation of Brain α -Actinin. Its characterization and a comparison of its properties with those of muscle α -actinins. *Biochemistry* **23**, 1600-1608.
- Dwyer-Nield, L. D., Miller, A. C., Neighbors, B. W., Dinsdale, D. and Malkinson, A. M. (1996). Cytoskeletal architecture in mouse lung epithelial cells is regulated by protein kinase C- α and calpain II. *Am. J. Physiol.* **270**, L526-L534.
- Egerton, M., Moritz, R. L., Druker, B., Kelso, A. and Simpson, R. J. (1996). Identification of the 70 kD heat shock cognate protein (Hsc70) an α -actinin-1 as novel phosphotyrosine-containing proteins in T lymphocytes. *Biochem. Biophys. Res. Commun.* **224**, 666-674.
- Fukami, K., Endos T., Imamura, M. and Takenawa, T. (1994). α -Actinin and vinculin are PIP₂-binding proteins involved in signaling by tyrosine kinase. *J. Biol. Chem.* **269**, 1518-1522.
- Geiger, B., Tokoyasu, K. T., Dutton, A. H. and Singer, S. J. (1980). Vinculin, an intracellular protein localized at specialized sites where microfilaments bundles terminate at cell membranes. *Proc. Nat. Acad. Sci. USA* **77**, 4127-4131.
- Geiger, B. (1982). Microheterogeneity of avian and mammalian vinculin: distinctive subcellular distribution of different isoformins. *J. Mol. Biol.* **159**, 685-701.
- Goldman, W. H., Schindl, M., Cardozo, T. J. and Ezzell, R. M. (1995). Motility of vinculin-deficient F9 embryonic carcinoma cells analyzed by video, laser confocal and reflection interference contrast microscopy. *Exp. Cell Res.* **221**, 311-319.
- Gonzalez-Mariscal, L., Chavez de Ramirez, B. and Cerejido, M. (1985). Tight junction formation in cultured epithelial cells (MDCK). *J. Membr. Biol.* **86**, 113-125.
- Gonzalez-Mariscal, L., Contreras, R. G., Bolivar J. J., Ponce, A., Chavez de Ramirez, B. and Cerejido, M. (1990). Role of calcium in tight junction formation between epithelial cells. *Am. J. Physiol.* **259**, C978-C986.
- Gumbiner B., Stevenson B. and Grimaldi A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* **107**, 1575-1588.
- Halegoua, S. (1987). Changes in the phosphorylation and distribution of vinculin during nerve growth factor induced neurite outgrowth. *Dev. Biol.* **121**, 97-104.
- Huttelmaier, S., Bubeck, P., Rudiger, M. and Jockusch, B. M. (1997). Characterization of two F-actin-binding and oligomerization sites in the cell-contact protein vinculin. *Eur. J. Biochem.* **247**, 1136-1142.
- Ito, S., Werth, D. K., Richert, N. D. and Pastan, I. (1983). Vinculin phosphorylation by the *src* kinase. *J. Biol. Chem.* **258**, 14626-14631.
- Jaken, S., Leach, K. and Klauck, T. (1989). Association of type three protein kinase C with focal contacts in rat embryo fibroblasts. *J. Cell Biol.* **109**, 697-704.
- Johnson, R. P. and Craig, S. W. (1995). F-actin binding site masked by the intramolecular association of vinculin head and tail domains. *Nature* **373**, 261-264.
- Kawamoto, S. and Hidaka, H. (1984). Ca²⁺-activated phospholipid-dependent protein kinase catalyzes the phosphorylation of actin-binding proteins. *Biochem. Biophys. Res. Commun.* **118**, 736-742.
- Knudsen, K. A., Peralta-Soler, A., Johnson, K. R. and Wheelock, M. J. (1995). Interaction of α -actinin with the cadherin/catenin cell-cell adhesion complex via α -catenin. *J. Cell Biol.* **130**, 67-77.
- Kroemker, M., Rudiger, A. H., Jockusch, B. M. and Rudiger, M. (1994). Intramolecular interactions in vinculin control α -actinin binding to the vinculin head. *FEBS Lett.* **355**, 259-262.
- Kuhlman, P. A., Hemmings, L. and Critchley, D. R. (1992). The identification and characterization of an actin binding site in α -actinin by mutagenesis. *FEBS Lett.* **304**, 201-206.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lazarides, E. and Burridge, K. (1975). α -actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell* **6**, 289-298.
- Madara, J. L., Stafford, J., Barenberg, D. and Carlson, S. (1988). Functional coupling of tight junctions and microfilaments in T84 monolayers. *Am. J. Physiol.* **254**, G416-G423.
- Mandel, L. J., Bacallao, R. and Zampighi, G. (1993). Uncoupling of the molecular fence and paracellular gate functions in epithelial tight junctions. *Nature* **361**, 552-555.
- McGregor, A., Blanchard, A. D., Rowe, A. J. and Critchley, D. R. (1994). Identification of the vinculin-binding site in the cytoskeletal protein α -actinin. *Biochem. J.* **301**, 225-233.
- Meza, I., Sabanero, M., Stefani, E. and Cerejido, M. (1982). Occluding junctions in MDCK cells: modulation of transepithelial permeability by the cytoskeleton. *J. Cell Biol.* **87**, 746-754.
- Milam, L. M. (1985). Electron microscopy of rotary shadowed vinculin and vinculin complexes. *J. Mol. Biol.* **184**, 543-545.
- Muhs, A., Noll, T. and Piper, H. M. (1997). Vinculin phosphorylation and

- barrier failure of coronary endothelial monolayers under energy depletion. *Am. J. Physiol.* **273**, H608-H617.
- Nieset, J. E., Redfield, A. R., Jin, F., Knudsen, K. A., Johnson, K. R. and Wheelock, M. J.** (1997). Characterization of the interaction of α -catenin with α -actinin and β -catenin/plakoglobin. *J. Cell Sci.* **110**, 1013-1022.
- Nigam, S. K., Denisenko, N., Rodriguez-Boulan, 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.
- O'Keefe, E. J., Briggaman, R. A. and Herman, B.** (1987). Calcium-induced assembly of adherens junctions in keratinocytes. *J. Cell Biol.* **105**, 807-817.
- Rodriguez-Fernandez, J. L., Geiger, B., Salomon, D., Sabanay, I., Zoller, M. and Ben-Ze'ev A.** (1992). Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA. *J. Cell Biol.* **119**, 427-438.
- Samuels, M., Ezzell, R., Cardozo, T. J., Critchley, D. R., Coll, J. and Adamson, E. D.** 1993. Expression of chicken vinculin complements the adhesion defective phenotype of a mutant mouse F9 embryonal carcinoma cell. *J. Cell Biol.* **121**, 909-921.
- Schwiabacher, C., Jockusch, B. M. and Rudiger, M.** (1996). Intramolecular interactions regulate serine/threonine phosphorylation of vinculin. *FEBS Lett.* **384**, 71-74.
- Sefton, B. M., Hunter, T., Ball, E. H. and Singer, S. J.** (1981). Vinculin: a cytoskeletal target of the transforming protein of the Rous Sarcoma virus. *Cell* **24**, 165-174.
- Stevenson, B. M. and Begg, D. A.** (1994). Concentration-dependent effects of cytochalasin D on tight junctions and actin filaments in MDCK epithelial cells. *J. Cell Sci.* **107**, 367-375.
- Stuart, R. O. and Nigam, S. K.** (1995). Regulated assembly of tight junctions by protein kinase C. *Proc. Nat. Acad. Sci. USA* **92**, 6072-6076.
- Tempel, M., Goldmann, W. H., Isenberg, G. and Sackmann, E.** (1995). Interaction of the 47-kDa talin fragment and the 32-kDa vinculin fragment with acidic phospholipids: a computer analysis. *Biophys. J.* **69**, 228-241.
- Towbin, H., Staehelin, T. and Gordon, J.** (1979). Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.
- Volberg, T., Geiger, B., Citi, S. and Bershadski, A. D.** (1994). Effect of protein kinase inhibitor H-7 on the contractility, integrity and membrane anchorage of the microfilament system. *Cell Motil. Cytoskel.* **29**, 321-338.
- Wachsstock, D. H., Wilkins, J. A. and Lin, S.** (1987). Specific interaction of vinculin with α -actinin. *Biochem. Biophys. Res. Commun.* **146**, 554-560.
- Wang, Y.** (1986). Reorganization of α -actinin and vinculin in living cells following ATP depletion and replenishment. *Exp. Cell Res.* **167**, 16-28.
- Weekes, J., Barry, T. and Critchley, D.** (1996). Acidic phospholipids inhibit the intramolecular association between the N- and C-terminal regions of vinculin, exposing actin-binding and protein kinase C phosphorylation sites. *Biochem. J.* **314**, 827-832.
- Werth, D. K., Niedel, J. E. and Pastan, I.** (1983). Vinculin, a cytoskeletal substrate of protein kinase C. *J. Biol. Chem.* **258**, 11423-11426.
- Werth, D. K. and Pastan, I.** (1984). Vinculin phosphorylation in response to calcium and phorbol esters in intact cells. *J. Biol. Chem.* **259**, 5264-5270.
- Westmeyer, A., Ruhnau, K., Wegner, A. and Jockusch, B.** (1990). Antibody mapping of functional domains in vinculin. *EMBO J.* **9**, 2071-2078.