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Phosphorylation of Claudin-4 By PKC ϵ Regulates Tight Junction Barrier Function in Ovarian Cancer Cells

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Abstract

Claudin proteins belong to a large family of transmembrane proteins essential to the formation and maintenance of tight junctions (TJs). In ovarian cancer, TJ protein claudin-4 is frequently overexpressed and may have roles in survival and invasion, but the molecular mechanisms underlying its regulation are poorly understood. In this report, we show that claudin-4 can be phosphorylated by protein kinase C (PKC) at Thr189 and Ser194 in ovarian cancer cells and overexpression of a claudin-4 mutant protein mimicking the phosphorylated state results in the disruption of the barrier function. Furthermore, upon phorbol ester-mediated PKC activation of OVCA433 cells, TJ strength is decreased and claudin-4 localization is altered. Analyses using PKC inhibitors and siRNA suggest that PKC ϵ , an isoform typically expressed in ovarian cancer cells, may be important in the TPA-mediated claudin-4 phosphorylation and weakening of the TJs. Furthermore, immunofluorescence studies showed that claudin-4 and PKC ϵ are co-localized at the TJs in these cells. The modulation of claudin-4 activity by PKC ϵ may not only provide a mechanism for disrupting TJ function in ovarian cancer, but may also be important in the regulation of TJ function in normal epithelial cells.

Introduction

In epithelial and endothelial cells, tight junctions (TJs) provide a dynamic barrier to paracellular transport of solutes and ions. TJs also behave as a fence at the apical-most part of the intercellular junctional complex, establishing cellular polarity [1–4]. In addition, TJ signaling complexes are involved in a wide array of physiological and pathological processes, including proliferation and differentiation pathways. TJs are composed of several proteins including the claudin proteins, a family of related transmembrane proteins that form the backbone of TJs. Recently, a number of claudin proteins have been implicated in human cancer [5,6]. Consistent with the observation that TJs are dismantled in tumors, certain claudin family members have been found to be down-regulated in cancer. However, paradoxically, claudins have also been shown to be elevated in several cancers [5]. For example, we and others have shown that claudin-3 and claudin-4 are highly increased in ovarian cancer [7–14]. These claudins have also been shown to be elevated in prostate, pancreatic, uterine, and breast cancer [15–17]. So far, claudin-1,3,4,5,7,10,16 have been shown altered in various cancers [5]. The functions of these proteins in tumorigenesis are still being elucidated, but they may have important roles in cell survival, motility, and invasion of cancer cells [18–20]. The mechanisms leading to the

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overexpression of claudins in cancer as well as the mechanisms of post-translational regulation/modification of these proteins in cancer are not well understood.

Numerous agents and cell signaling pathways interact with the TJ protein complex [21,22] and the involvement of kinases in the biogenesis and regulation of the TJ components is well established [23–28]. Interestingly, several studies have demonstrated the involvement of various kinases in the phosphorylation and regulation of claudin proteins [29–37], and we have recently shown that phosphorylation of claudin-3 by PKA can affect TJ properties in ovarian cancer cells [38]. Protein kinase C (PKC) isoforms are present in ovarian cancer and are known to modulate TJ function by phosphorylation of the proteins in the complex [24,34,39–43], but it is unclear whether PKC can directly phosphorylate and regulate claudin proteins. We have previously shown that claudin-4 can be phosphorylated in ovarian cancer cells upon 12-O-Tetradecanoylphorbol-13-acetate (TPA) stimulation [38], but the exact PKC isoforms involved, the phosphorylation sites on claudin-4, and the consequences of this phosphorylation have remained unknown. Here, we show that claudin-4 is phosphorylated by PKC at Thr189 and Ser194 in ovarian cancer cells. The TJ strength was reduced in these cells upon phorbol ester-mediated PKC activation and claudin-4 localization was altered. Knockdown of PKC ϵ in TPA-stimulated OVCA433 cells decreased the phosphorylation levels of claudin-4 and prevented the TPA induced reduction in TER. Finally, analysis of mutant claudin-4 proteins demonstrated that both PKC phosphorylation sites were important in mediating the barrier function in OVCA433 cells. Our results suggest that PKC ϵ , an important kinase involved in the regulation of TJs in several epithelial cells, may contribute to claudin-4 regulation in ovarian cancer cells, as well as in normal cells.

Results

Claudin-4 is phosphorylated by PKC in ovarian cancer cell

In order to investigate whether claudin-4 could be phosphorylated by PKC, we treated ovarian cancer cells with the PKC activator TPA and examined claudin-4 status. Claudin-4 immunoprecipitation from $\gamma^{32}\text{P}$ ATP-labeled OVCA433 cells revealed that TPA treatment induced a significant level of phosphorylation in claudin-4 (Fig. 1A). Similar results were obtained using the non-isotopic ProQ Diamond phosphoprotein/SYPRO Ruby stain system (Fig. 1B). We also observed TPA-mediated phosphorylation of claudin-4 in BG-1 and OVCA420 ovarian cancer cell lines (Fig. 1B). For the experiments shown, cells were treated with TPA for 1 hour, however, similar results were obtained with 30 min TPA treatment (data not shown). Addition of the PKC inhibitors Gö6850 and Ro32-432 at 1 μM prior to TPA treatment inhibited the effects of TPA, confirming that the TPA-induced claudin-4 phosphorylation was indeed mediated by PKC (Fig. 1C). Gö6850 blocks the activity of the classical (α , $\beta 1$, $\beta 2$, γ) and novel (δ , ϵ , θ , η , μ) isoforms. At 1 μM , PKC inhibitor Ro32-432 blocks PKC α , $\beta 1$, $\beta 2$, γ and ϵ . In order to further characterize the PKC isoforms responsible for the TPA-induced phosphorylation of claudin-4, additional isoform-selective PKC inhibitors were utilized. The compound indolocarbazole Gö6976 inhibits the Ca^{2+} dependent isoforms α and $\beta 1$, with no effect on the Ca^{2+} -independent PKC isoforms δ , ϵ and ζ [44]. We also tested the effect of Rottlerin, an inhibitor specific for nPKC δ (IC_{50} = 3–6 μM) and a weak antagonist of cPKC isoforms [45,46]. Neither, Gö6976 (1 μM) or Rottlerin (6 μM) prevented TPA-stimulated phosphorylation of claudin-4 (Fig. 1C). Therefore, these results suggest the involvement of PKC ϵ in the phosphorylation of claudin-4 in these cells.

Expression of PKC isoforms and claudin-4 in ovarian cancer cell lines

Since the expression pattern of PKC isoforms in ovarian cancer is not completely clear, we used immunoblotting to examine the expression of various PKC isoforms in a panel of ovarian cancer cell lines (Fig. 2). An immortalized culture of normal ovarian surface epithelial cells

(HOSE-B) was also included in the analysis. Interestingly, PKC ϵ , the isoform which appears important for claudin-4 phosphorylation, was highly expressed in all the ovarian cancer cell lines examined, except in the non-transformed HOSE-B. PKC α was also found expressed in nearly all the cell lines examined, albeit weakly in, BG-1, OVCA432, OVCAR4, and HOSE-B cells. The majority of the cell lines showed appreciable PKC β expression, except for, BG-1, UCI101, OVCA420, and HOSE-B in which PKC β was very low. Most ovarian cancer cell lines expressed both claudin-4 and multiple PKC isoforms.

The PKC ϵ isoform phosphorylates claudin-4 in OVCA433 cells

Our study with PKC inhibitors Gö6976, Ro32-432 and Gö6850 suggested PKC ϵ as a prime candidate for the phosphorylation of claudin-4 (Fig 1C). A titration of the PKC inhibitor Ro32-432 showed that inhibition of claudin-4 phosphorylation became apparent only when the IC₅₀ for PKC ϵ (108 nM) was reached, again suggesting PKC ϵ involvement (Fig. 3A). In order to directly test PKC ϵ involvement in claudin-4 phosphorylation, we specifically knocked down PKC ϵ in these cells using RNAi technology. siRNA treatment was extremely efficient at knocking down PKC ϵ and TPA treatment did not appear to affect its expression levels (Fig. 3B). The immunoprecipitated claudin-4 protein from siRNA-treated cells showed substantial reduction in phosphorylation levels compared to the control cells following TPA treatment (Fig. 3C). This result provides strong evidence for PKC ϵ as the kinase responsible, at least in part, for claudin-4 phosphorylation.

PKC affects transepithelial resistance (TER) in OVCA433 cells

We next assessed whether claudin-4 phosphorylation may have functional consequences on the transport properties of TJs in ovarian cancer cells. We examined OVCA433 cells, which have relatively high TER and low permeability, indicating the presence of a functional TJ barrier. TPA stimulation led to a decrease in TER in OVCA433 cells and both PKC inhibitors Gö6850 and Ro32-0432 at 1 μ M significantly prevented this effect (Fig. 4A). However, similar to what we observed in the claudin-4 phosphorylation experiments (Fig. 1C), Gö6976 and Rottlerin were not effective at preventing the effects of TPA. These results suggest an important role for PKC ϵ in mediating the TPA effects on TJ function, and suggest the involvement of claudin-4 phosphorylation in this phenomenon.

In order to further test the importance of PKC ϵ on the regulation of barrier function, we used siRNA technology to knock down PKC ϵ . For this experiment, TPA was added to siRNA-transfected or control cells after they had established a measurable TER. Immunoblotting shows that PKC ϵ protein could be reduced efficiently in these cells following siRNA treatment (Fig. 4B). TPA treatment of control siRNA-transfected cells significantly reduced TER compared to vehicle-treated cells ($P < 0.05$ n=5) (Fig. 4B). However, cells in which PKC ϵ had been knocked down did not show a significant decrease in TER compared to the vehicle treated cells following TPA treatment. These experiments along with the phosphorylation studies clearly demonstrate that PKC ϵ can influence the TJ properties of OVCA433 cells.

Claudin-4 localization at the membrane is associated with TJ function

We next wished to confirm the location of claudin-4 protein in OVCA433 cells as part of the TJs. Immunofluorescence studies show the presence of claudin-4 and occludin at the intercellular junctions (Fig. 5A). The confocal Z-scan images reveal the colocalization of the two proteins at the junction of the cells (Fig. 5B). Interestingly, areas lacking intercellular junctions did not express claudin-4 on these regions of the membranes (Fig. 5C). In order to examine the role of claudin-4 in the barrier function changes observed upon TPA treatment, we observed its localization. In untreated OVCA433 cells, claudin-4 was found more defined at the intercellular junction (Fig. 5D). After TPA treatment, claudin-4 staining was more diffuse at the intercellular junctions with increasing presence in the cytoplasm. Pre-treatment with

PKC inhibitors Gö6850 and Ro32-0432 restored a claudin-4 staining profile similar to what was observed in untreated cells. On the other hand, PKC inhibitors Gö6976 or Rottlerin were ineffective at blocking the TPA effects (Fig. 5D). These patterns of inhibition are identical to what was observed for claudin-4 phosphorylation (Fig. 1) and barrier function (Fig. 4A) suggesting that claudin-4 phosphorylation by PKC ϵ leads to mislocalization of the protein and altered TJ function.

PKC ϵ isoform co-localizes with claudin-4

Our data strongly suggest an important role for the PKC ϵ isoform in phosphorylating claudin-4 and disrupting the barrier function in OVCA433 cells, and we wondered whether the two proteins co-localized in these cells. Without treatment, PKC ϵ is present predominantly in the nucleus of the cells with uneven staining at the membrane (Fig. 6A). TPA treatment led to a relocalization of PKC ϵ to the cell surface, which could be inhibited by the PKC inhibitor Gö6850. Z-scans of control and TPA-treated OVCA433 cells provided evidence for the co-localization of PKC ϵ and claudin-4 (Fig. 6B). In addition, the pattern of claudin-4 staining at the membranes of TPA-treated cells showed a significant widening, consistent with TJ dissolution and the decreased resistance seen in TER studies (Fig. 4). Both claudin-4 and PKC ϵ were present in the apical and basolateral membranes. Even though the fluorescent emission for PKC ϵ is weaker than that of claudin-4, the staining intensity patterns for PKC ϵ and claudin-4 showed a clear overlap at the membrane (Fig. 6C). To check whether the translocated PKC ϵ was associated with TJs, we also stained the cells with occludin. Both proteins were found to be present at the cell borders (Fig. 6D). These results substantiate our functional data and phosphorylation studies, strengthening the concept of PKC ϵ localizing to the TJs and altering the barrier function through the phosphorylation of claudin-4.

Claudin-4 is phosphorylated at Thr-189 and Ser-194 by PKC

Prediction of the putative PKC phosphorylation sites on claudin-4 was made using the database NetPhos 2.0 Server (<http://www.cbs.dtu.dk/services/NetPhosK/>). Two putative PKC phosphorylation sites were identified, Thr-189 and Ser-194, both of which conformed to the generalized consensus sequence (R/K)₁₋₃-(X)₀₋₂-S/T(X)₀₋₂-(R/K)₁₋₃ where X represents any amino acid [47]. To evaluate whether these two amino acids are phosphorylated in cells, we overexpressed the full-length wild-type claudin-4 and various phosphorylation mutants in HEY cells, an ovarian cancer cell line devoid of claudin-4. Fig. 7A shows that wild-type claudin-4 was phosphorylated upon TPA stimulation in these cells, but the intensity of the phosphorylated band was decreased slightly in immunoprecipitates of cells expressing claudin-4 T189A or S194A mutants. The TPA-induced phosphorylation signal was abolished in the immunoprecipitates of claudin-4 T189A-S194A double mutant indicating that both sites are phosphorylated following TPA stimulation in these cells.

Effects of overexpression of wild-type and mutant claudin-4 proteins on TER

In order to further evaluate the roles of claudin-4 phosphorylation in TJ function, we examined the effect of overexpressing wild-type and mutant claudin-4 proteins on the TJ properties of OVCA433 cells, which already contain endogenous claudin-4 protein. Immunoblotting revealed that expression of claudin-4 wild-type and the various mutants was very efficient in these cells (Fig. 7B). Overexpressed wild-type claudin-4 and the T189A/S194A mutant localized prominently at the intercellular junctions with some staining in the cytoplasm (Fig. 7C). Although, the claudin-4 T189D/S194D mutant protein (mimicking constitutive phosphorylation) was also found at the intercellular junction, the staining pattern appeared disrupted and was not continuous on the cell surface. The localization pattern of this mutant was similar to what was observed in TPA-treated untransfected cells (Fig 5D). The functional effects of wild-type and mutant claudin-4 proteins were evaluated by measuring TER in cells

transfected with the various expression vectors. While overexpression of claudin-4 WT or T189A/S194A mutant did not significantly alter the TER compared to control cells, overexpression of the T189D/S194D mutant led to a significant inhibition of TER by 2.5 fold compared to the vector or claudin-4 WT (Fig. 7D) (n=7, P<0.05). These results suggest that claudin-4 phosphorylation leads to its decrease at the TJ junction as well as a reduction in TJ strength.

Discussion

Several studies have confirmed the overexpression of claudin-3 and claudin-4 in ovarian cancer [7–14], but the roles of these proteins in tumorigenesis or the mechanisms regulating them are unclear. As a first step in elucidating the role of claudin phosphorylation in ovarian cancer, we have recently shown that claudin-3 can be phosphorylated by PKA at Thr192, and that phosphorylation of this residue could lead to the disruption of TJs in ovarian cancer cells [38]. The phosphorylation of claudin proteins is well-established and other claudins have been shown to be phosphorylated by various protein kinases. For example, claudin-1 is phosphorylated at Thr203 probably by MAPK [29], claudin-5 at Thr207 [32], claudin-16 at Ser217 [37], and claudin-4 by EphA2 at Tyr208 [35]. In addition claudin-1 and -4 are substrates for the WNK kinase, although no putative sites have been reported [33]. Some investigations have shown modulation of claudin-4 by aPKC [30] and PKC θ [34]. In Caco-2 cells, constitutively active PKC θ is necessary to maintain barrier function and this process involves the phosphorylation of claudin-1 and -4 [34].

In the current work, we have examined the mechanisms and consequences of claudin-4 phosphorylation by PKC. We show that the inhibition patterns of claudin-4 phosphorylation in the presence of the various PKC inhibitors is consistent with a role for PKC ϵ , an isoform expressed at high levels in all the ovarian cancer cell lines examined, but not in an immortalized ovarian cell line used as control (Fig. 2). In addition, knockdown of PKC ϵ protein using specific siRNA resulted in substantial reduction in the TPA-mediated phosphorylation of claudin-4. Our results thus provide the first evidence for the phosphorylation of endogenous claudin-4 by PKC ϵ in ovarian cancer cells.

We also sought to characterize the effects of PKC ϵ -mediated claudin-4 regulation in ovarian cancer cells. We find that the TPA treatment leads to a breakdown of TJs in OVCA433. The knockdown of PKC ϵ prevented this effect supporting the role of PKC ϵ -mediated regulation of TJ function (Fig. 4). In addition, claudin-4 relocalization from the membrane to the cytoplasm following TPA treatment was associated with its phosphorylation and a decrease in TJ strength. The PKC inhibitors that blocked claudin-4 phosphorylation also inhibited the effects of TPA on TJ barrier, again suggesting claudin-4 as an important target of PKC ϵ for its TJ-related effects. PKC ϵ contribution to cell motility has been linked to β 1 integrin by regulating its trafficking [48]. It is also known to be associated with β 1 integrin through RACK1 and F-actin in mediating cell adhesion and mobility [49,50]. Our results suggest that PKC ϵ 's role in metastasis may be related to its ability to impair the barrier function (and cell adhesion) through phosphorylation of TJ proteins such as claudin-4. These findings are consistent with a number of studies that have previously shown that phosphorylation of various claudin proteins can decrease TJ integrity [32,35,51–54], although phosphorylation of claudin-1 has also been shown to promote barrier function [29,31]. However, it is important to note that TJ disruption likely occurs through multiple mechanisms. For example we found that TPA treatment disturbed actin from the cell borders and led to the appearance of stress fibers (data not shown). We have also observed that occludin staining is altered following TPA treatment (data not shown). Overall, our data show that phosphorylation of claudin-4 represent an important contributing factor in TJ weakening in ovarian cancer cells.

Previous work has suggested that claudin-3 and claudin-4 overexpression in ovarian and other cancers may promote invasion and survival, but overexpression of claudins was not found correlated with barrier function [20]. Our current findings suggest that post-translation modifications of the overexpressed claudins may inhibit their TJ-related function while allowing other pro-oncogenic effects. More specifically, PKA phosphorylation of claudin-3 [38] and PKC phosphorylation of claudin-4 (this work) may both be important in TJ disruption in ovarian cancer.

The PKC ϵ staining showed a prominent presence in the nucleus of the OVCA433 cells with weak presence at the membrane, which was significantly increased following phorbol ester TPA exposure. Significantly, the relocalization of PKC ϵ to the membrane led to a co-localization with claudin-4, a finding consistent with claudin-4 being a target of this kinase. Translocation of activated PKC ϵ to the membrane upon TPA treatment has been shown in other studies [42,55] and alteration of TJs by translocation of PKC ϵ has been shown previously in epithelial cells [42]. Various isoforms of PKC have been implicated in ovarian tumorigenesis and drug resistance [56–62]. Involvement of PKC ϵ in tumor development, invasion, and metastasis have been shown in other tissues [50,63–67]. However, little is known about the roles of PKC ϵ in ovarian cancer and, in light of the results reported here, this PKC isoform certainly deserves further consideration in ovarian tumorigenesis.

After demonstrating that claudin-4 is a substrate for PKC ϵ in OVCA433 cells, we identified the residues phosphorylated by this kinase. Site-directed mutagenesis followed by phosphorylation assays demonstrated that both Thr189 and Ser194 of claudin-4 are phosphorylated upon TPA stimulation. Interestingly, a claudin-4 T189A/S194A mutant which cannot be phosphorylated at these sites still exhibit background phosphorylation (Fig. 7A), suggesting that other kinases are responsible for basal phosphorylation of claudin-4 in these cells. Overexpression of claudin-4 WT or T189A/S194A in OVCA433 did not affect the barrier properties of these cells. However, overexpression of the T189D/S194D mutant, which mimics the phosphorylated state of claudin-4 protein, led to a significant decrease in TER. The immunofluorescence analysis of claudin-4 protein localization demonstrated that the T189D/S194D significantly decreased the amount of claudin-4 observable at the intercellular junction, providing a mechanism for the decreased barrier function. This relocalization was similar to what we observed following treatment of these cells with TPA. Taken together, these results strongly suggest that claudin-4 phosphorylation by PKC ϵ at Thr189 and Ser194 is important in claudin-4 relocalization and TJ loosening, an event that may be important in ovarian cancer progression.

In summary, we have shown that PKC activation can affect TJ function and architecture, and that at least part of this effect is mediated through claudin-4 phosphorylation. We have provided evidence for PKC ϵ as a likely isoform responsible for claudin-4 phosphorylation, an event that leads to a reduction in TJ strength. The specific PKC phosphorylation sites identified will undoubtedly prove to be extremely useful in future studies aimed at identifying the consequences of claudin-4 phosphorylation in ovarian and other tissues. In addition to its TJ functions, claudin-4 overexpression and regulation by PKC may transduce downstream signaling pathways in ovarian cancer. Examination of these pathways may give insights to the mechanisms of ovarian tumorigenesis and provide new directions for the detection and therapy of this disease.

Materials and methods

Cell lines

Ovarian cell lines BG-1, CAOV3, HEY, IGROV-1, UCI101, OVCA420, OVCA429, OVCA432, OVCA433, OVCAR-2, OVCAR-3 and OVCAR-4 were cultured in McCoy's 5A

growth medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) and kept in a humidified incubator at 37 °C with 95% air and 5% CO₂. HOSE-B, an ovarian surface epithelial cell line immortalized with E6 and E7 was maintained in RPMI 1640 supplemented with 10% FCS and 300 µg/ml geneticin (Invitrogen, Carlsbad, CA).

Expression constructs, siRNA, and transfections

Mutant claudin-4 expression constructs were generated by PCR using the pCiNeo-CLDN4 expression plasmid previously described [20]. Point mutations of the serine or threonine residues in the consensus PKC site were introduced using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were confirmed by DNA sequencing. Control non-targeting siRNA and SMARTpool of siRNA PKCε, were obtained from Dharmacon RNA Technologies (Lafayette, CO). OVCA433 cells were transfected with claudin4 WT and mutant constructs using Fugene according to the manufacturer's protocol (Roche). Pooled clones were maintained in McCoy's growth medium containing 500 µg/ml geneticin. OVCA433 cells were transfected with siRNA (100 nM final concentration) using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen). 12-O-Tetradecanoylphorbol-13-acetate (TPA) and PKC inhibitors (Gö6850, Gö6796, Rottlerin, Ro-32-0432) were obtained from Calbiochem (EMD Biosciences, Inc., San Diego, CA).

Antibodies

Mouse monoclonal claudin-4 was purchased from Zymed (South San Francisco, CA) and rabbit claudin-4 antibody from Abcam (Cambridge, UK). Mouse monoclonal PKCα, β, γ, δ, ε, θ were obtained from BD Transduction Laboratories (San Jose, CA). Mouse monoclonal PKCε used in immunofluorescence studies was obtained from Abcam. Rabbit phospho-PKC (pan) antibody was obtained from Cell Signaling (Danvers, MA). Rabbit polyclonal occludin was purchased from Zymed. Mouse monoclonal GAPDH was purchased from Abcam. Mouse monoclonal α-tubulin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-linked donkey anti-rabbit Ig and sheep anti-mouse IgG horseradish antibodies were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Alexafluor 488, 555, 594 conjugated anti-mouse and anti-rabbit antibodies were purchased from Invitrogen (Molecular Probes).

Immunoblotting

Cell lysates, quantitated using Pierce (Rockford, IL) BCA quantitation assay, were resolved by SDS-PAGE (Tris glycine gels; Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat dry milk in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20 (v/v) and incubated overnight with primary antibody (claudin-4, 1:250, PKCα, 1:1000, PKCβ, 1:250, PKCγ, 1:1000, PKCδ, 1:500, PKCε, 1:1000, PKCθ, 250, Phospho-PKC (pan), 1:1000, GAPDH 1:4000, α-tubulin 1: 2000). Following the washes in Tris-buffered saline, membranes were probed with HRP-conjugated antibody (anti-mouse or anti-rabbit 1:10,000). ECL western blotting detection kit (Amersham Pharmacia, Piscataway, NJ) was used for visualization of the positive bands.

In-vivo phosphorylation, immunoprecipitation, and phosphoprotein detection

BG-1, OVCA420, OVCA433, were plated at 80–90% confluence in 60 mm dishes and were serum starved overnight for 16 h. When required, metabolic labeling of OVCA432 cells with [³²P] orthophosphate (500 µCi/ml; MP Biomedicals, Irvine, CA) was carried out as previously described [38]. Cells were stimulated with TPA (100 ng/ml) for 30 mins to 1 hr at 37°C. HEY cells were plated at 70–80% confluence in 100-mm dishes and transfected with a plasmid

encoding either wild-type or mutated human claudin-4 using FuGENE 6 transfection reagents. Transiently transfected cells (18 h post-transfection) were stimulated with TPA (100 ng/ml) for 1 h at 37°C. When necessary, PKC inhibitors were added 60 min prior to the addition of TPA. Immunoprecipitations were performed as previously described [38], and the immunoprecipitates were separated by SDS-PAGE. Phosphoproteins were detected by staining the gels using the fluorescent Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Briefly, the gels were fixed in 50% methanol and 10% acetic acid (30–60 min), washed with deionized water 10–20 min, incubated in the dark in Pro-Q Diamond phosphoprotein gel stain for 1.5–2 h and destained in 20% acetonitrile, 50 mM sodium acetate, pH 4.0 for 1 h, 3 times. After acquiring images, the gels were stained overnight in SYPRO Ruby protein gel stain (Molecular Probes) to visualize total protein. The stained gels were visualized with the Typhoon 9200 Phosphorimager (GE Healthcare, Piscataway, NJ) using appropriate excitation filters for the multiplex gel stains. The bands obtained from the ProQ diamond and SYPRO Ruby staining were quantitated using Scion Image (Frederick, MD). The data shown are normalized to the control in each set of experiments.

Transepithelial Resistance (TER) measurements

OVCA433 cells were seeded at a density of 5×10^5 on 12 mm polycarbonate transwell clear membranes (0.4 μ m-pore size, Costar, Cambridge, MA). TER was measured using a Millicell-ERS V-ohm meter (WPI, New Haven, CT). The values were normalized for the area of the filter and obtained after subtraction of blank values (filter and bath solution). TPA (100 ng/ml) or DMSO vehicle control was added at time zero, with measurements taken every hour thereafter. When appropriate, PKC inhibitors were added 1 h prior to TPA treatment. For the knockdown experiments, OVCA433 cells were seeded at a density of 1×10^5 on 12 mm polycarbonate transwell clear membranes a day prior to siRNA transfections. TPA was administered 72 h after transfection and the TER was followed for 5 h.

Immunofluorescence

OVCA433 cells were grown to 90% confluency on glass slides and were washed with phosphate buffered saline (PBS pH 7.4, with 1 mM CaCl_2 and 1 mM MgCl_2), followed by fixation with cold Methanol for 10 min at -20°C . Cells treated with DMSO (control) or TPA (100 ng/ml) and PKC inhibitors were fixed in a similar fashion. Fixed cells were washed 3 times with PBS without Ca^{2+} and Mg^{2+} , blocked in 3% BSA/0.2% TritonX-100 (in PBS) for 1 h at room temperature or overnight at 4°C , followed by incubation with primary antibodies in blocking buffer (mAb and pAb claudin-4 1:100, mAb PKC ϵ 1:50, pAb occludin 1:50) at room temperature for 1 h. After 3 washes with PBS (with 0.05% Triton X-100), the cells were incubated with the appropriate Alexafluors conjugated secondary antibodies for 1 h at room temperature. After the cells were washed in PBS 5 min, 4 times, they were mounted in Prolong gold anti-fade mounting medium (Invitrogen). Fluorescent signal were examined and imaged using a Zeiss LSM 510 Meta confocal microscope (Thornwood, NY).

Statistical analysis

Data are expressed as means $\pm$ S.E. Statistical analysis was performed using the Students t-test, with $P < 0.05$ considered statistically significant.

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Fig 1. PKC Phosphorylation of claudin-4 in ovarian cancer cells

(A) OVCA433 cells metabolically labeled with [³²P] orthophosphate were treated with TPA (100 ng/ml) or vehicle (DMSO) and then immunoprecipitated with claudin-4 antibody. Phosphorylated claudin-4 protein was confirmed by immunoblot. (B) BG-1, OVCA420, and OVCA433 cells were treated with TPA (100 ng/ml) and then immunoprecipitated with claudin-4 antibody. The gel was stained with Pro-Q Diamond dye (staining phosphorylated proteins) and SYPRO Ruby (staining all proteins). The ratio of ProQ signal to SYPRO Ruby signal gives a measure of phosphorylation levels relative to the total amount of protein. (C) Effects of various PKC inhibitors on TPA-induced claudin-4 phosphorylation. OVCA 433 cells were treated with TPA in the presence of PKC inhibitors and immunoprecipitated with

claudin-4 antibody or with mouse IgG (as control). PKC inhibitors Gö6850 (1 μ M) and Ro32-432 (1 μ M) inhibited the effects of TPA. PKC inhibitors Gö6976 and Rottlerin did not inhibit TPA-mediated claudin-4 phosphorylation. Quantification of phosphorylation levels for each treatment was determined by the ProQ-SYPRO Ruby ratio.

Fig 2. Protein expression of PKC isoforms and claudin-4 in ovarian cancer cell lines
Whole cell lysates of ten ovarian cancer cell lines and one normal ovarian cell line (HOSE-B) were used for immunoblotting with antibodies against different PKC isoforms and claudin-4 to profile endogenous protein expression. PKC ϵ is found in all the ovarian cancer cells lines studied. PKC α is also consistently expressed in all the cell lines examined. In addition, Phosphopan PKC antibody detected the constitutively activated PKC in a majority of the ovarian cancer cells lines (81%).

Fig 3. PKC ϵ isoform phosphorylates claudin-4 in OVCA433 cells

(A) OVCA433 cells were treated with TPA alone and in presence of PKC inhibitor Ro32-0432 at concentrations of 9 nM, 28 nM and 108 nM. Claudin-4 protein was immunoprecipitated, run on a polyacrylamide gel, and the phosphorylated bands were detected with ProQ-Diamond phosphoprotein gel stain. The corresponding protein bands were detected with SYPRO Ruby total protein stain. The inhibitor Ro32-0432 at 108 nM decreases the intensity of phosphorylation band of claudin-4. Quantitation of the ProQ/SYPRO Ruby ratio reveals that Ro32-0432 at 108 nM inhibits the TPA effect back to control level. (B) Knockdown of PKC ϵ by siRNA. The immunoblot shows whole cell lysates of OVCA433 cells transfected with siRNA for PKC ϵ or control siRNA. The cells were treated with TPA (100 ng/ml) for an

hour, 48 hrs after transfection. (C) Claudin-4 protein was immunoprecipitated from OVCA433 cells transfected with siRNA for PKC ϵ and non-targeting protein then stimulated by TPA after 48 hrs of transfection. The phosphorylated claudin-4 protein was detected using ProQ-Diamond gel stain and the total protein detected by SYPRO ruby gel stain.

Fig 4. PKC effects on barrier function in OVCA 433

(A) TPA affects the barrier function of OVCA433. Confluent cells grown on transwell membranes were treated with DMSO or TPA (100ng/ml) with or without the inhibitors Gö6850 (1 μ M), Ro32-432 (1 μ M), Gö6976 (1 μ M), and Rottlerin (6 μ M). The relative change in TER is shown at 3 hours after treatment. Data are expressed as means $\pm$ S.E. (n = 3) with significant changes with TPA (*p < 0.001). The PKC inhibitors Gö6850, Ro32-432 prevented the reduction in TER. The decrease in TER induced by TPA in OVCA433 cells is not prevented by the presence of PKC inhibitors Gö6976 and Rottlerin. (B) The role of PKC ϵ on the barrier function. Knockdown of PKC ϵ by siRNA prevented the TPA-induced decrease in TER. The accompanying immunoblot confirms the knockdown of the protein by siRNA.

Fig 5. Effects of TPA on Claudin-4 localization

(A) Localization of claudin-4 at the TJs in OVCA433 cells. Confluent cells grown on glass slides were stained with mAb anti-claudin-4 (Alexa 555, red) and pAb anti-occludin (Alexa 488, green). Shown are confocal xy individual and merged (including DAPI) showing co-localization of the two proteins. Scale bar = 20 μ m. (B) The xy plane at section 3.94 μ m and the xz and xy planes of 20 sections (each section 440 nm thick, for a total of 8.8 μ m) show co-localization of claudin-4 and occludin. (C) Claudin-4 (Alexa 488, green) is localized at the intercellular junctions of cells and absent in region without neighboring cells (arrows). Scale bar = 20 μ m. (D) Claudin-4 localization is affected by TPA treatment. Confluent OVCA433 cells were treated with TPA, in the presence or absence of PKC inhibitors followed by

immunofluorescence to show the localization of claudin-4. TPA treatment led to diffuse membrane staining of claudin-4 (arrows), with increased cytoplasmic localization. Scale bar = 5 μm . Claudin-4 immunofluorescence staining shows that PKC inhibitors Gö6850 and R032-432, both at 1 μM , prevent the TPA induced changes on claudin-4 localization, but Gö6976 (1 μM) and Rottlerin (6 μM) do not prevent TPA effects.

Fig 6. Confocal microscopy analysis of PKC ϵ and claudin-4 following TPA treatment

(A) Shown are representative confocal images of confluent OVCA433 monolayers treated with vehicle (control), TPA 100 ng/ml (1 hr) and TPA in presence of inhibitor Gö6850. Claudin-4 (green) is localized to the membrane with some cytoplasmic staining. PKC ϵ (red) is present in the nucleus with uneven distribution at the cell borders. TPA induces translocation of PKC ϵ to the membrane, and this effect is prevented by the PKC inhibitor. Scale bar = 20 μ m. (B) The z-scans of the cells provide a vertical image to show the localization of the claudin-4 and PKC ϵ . Treatment of OVCA433 with TPA cells induced co-localization of claudin-4 and PKC ϵ along the lateral membrane and more intense cytoplasmic staining of claudin-4. (C) The boxed region gives the intensity profile of the fluorophores showing that claudin-4 and

PKC ϵ consistently co-distribute. (D) In TPA treated OVCA433 cells PKC ϵ (red) is localized at the intercellular junctions along with the TJ protein occludin (green).

Fig 7. Overexpression of claudin-4 mutants affects barrier function

(A) Claudin-4 is phosphorylated at two specific sites upon TPA stimulation. HEY ovarian cancer cells (lacking endogenous claudin-4) were transfected with vectors expressing wild-type claudin-4 (WT), or mutant claudin-4 proteins (T189A, S194A, or T189-S194A). Cells were stimulated with TPA and claudin-4 was immunoprecipitated. Pro-Q Diamond/SYPRO Ruby staining shows the level of phosphorylation of the wild-type protein and the various mutants. All the ratios are normalized to the untreated WT. (B) Immunoblot shows the overexpression of wild-type claudin-4 protein and indicated mutant proteins in OVCA433 cells. (C) Localization of overexpressed claudin-4. Overexpression of claudin-4 WT and mutants T189-S194A and T189-S194D in OVCA433 cells were detected by immunofluorescence. T189-S194D overexpression results in a more diffuse staining at the intercellular junctions. (D) TER measurements of OVCA433 expressing the various constructs. TER values (expressed as means $\pm$ S.E.; $n = 7$) were taken for several days after seeding the OVCA433 cells overexpressing claudin-4 WT and mutants T189-S194A and T189-S194D. TER values shown are at day 6. TER obtained from claudin-4 T189-S194D significantly decreased compared to control, WT and T189-S194A ($P < 0.05$).