aPKC phosphorylates JAM-A at Ser285 to promote cell contact maturation and tight junction formation

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The PAR-3–atypical protein kinase C (aPKC)–PAR-6 complex has been implicated in the development of apicobasal polarity and the formation of tight junctions (TJs) in vertebrate epithelial cells. It is recruited by junctional adhesion molecule A (JAM-A) to primordial junctions where aPKC is activated by Rho family small guanosine triphosphatases. In this paper, we show that aPKC can interact directly with JAM-A in a PAR-3–independent manner. Upon recruitment to primordial junctions, aPKC phosphorylates JAM-A at S285 to promote the maturation of immature cell–cell contacts. In fully polarized cells, S285-phosphorylated JAM-A is localized exclusively at the TJs, and S285 phosphorylation of JAM-A is required for the development of a functional epithelial barrier. Protein phosphatase 2A dephosphorylates JAM-A at S285, suggesting that it antagonizes the activity of aPKC. Expression of nonphosphorylatable JAM-A/S285A interferes with single lumen specification during cyst development in three-dimensional culture. Our data suggest that aPKC phosphorylates JAM-A at S285 to regulate cell–cell contact maturation, TJ formation, and single lumen specification.

Introduction

In multicellular organisms, epithelial cells cover organs and body cavities to generate a selective barrier between distinct compartments. Epithelial cells develop apicobasal polarity reflected by a defined organization of intercellular junctional complexes, the existence of distinct plasma membrane domains, and the asymmetric distribution of molecules. The epithelium is sealed by tight junctions (TJs), which form at the most apical part of cell–cell contacts (Tsukita et al., 2001). TJs are crucial for the barrier function of epithelial cells because they restrict the diffusion of ions and macromolecules along the intercellular cleft (paracellular diffusion barrier; Van Itallie and Anderson, 2004). In addition, TJs prevent the free diffusion of proteins and lipids between the apical and the basolateral membrane domain (intramembrane diffusion barrier; van Meer and Simons, 1986), implicating them in the regulation of apicobasal membrane polarity.

TJs are composed of various integral membrane proteins, cytoplasmic scaffolding proteins, and adaptor proteins as well as regulatory proteins, including kinases and phosphatases, small GTPases, and guanine nucleotide exchange factors (Matter and Balda, 2003b; Ebnet, 2008). Two major cytoplasmic scaffolding protein complexes are the PAR-3–atypical PKC (aPKC)–PAR-6 complex and the Pals1–PATJ complex (Macara, 2004). Both complexes are required for TJ formation as inferred from knockdown studies and from ectopic expression of dominant-negative mutant proteins (Shin et al., 2006; Suzuki and Ohno, 2006). PAR-3 and PAR-6 serve as scaffolding proteins to regulate the localization and Cdc42/Rac1-mediated activation of aPKC, respectively. The Pals1–PATJ complex consists of the two scaffolding proteins Pals1 and PATJ, which have no catalytic activity. However, this complex can be physically linked to the PAR–aPKC–PAR-6 complex (Hurd et al., 2003). In addition, it is linked to the Cdc42-specific Rho GTPase-activating protein

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Abbreviations used in this paper: AJ, adherens junction; aPKC, atypical PKC, CS, Ca^{2+} switch; JAM-A, junctional adhesion molecule A; Lgl, Lethal giant larvae; pAJ, primordial, spotlike AJ; shRNA, small hairpin RNA; TER, trans-epithelial electrical resistance; TJ, tight junction; WT, wild type.

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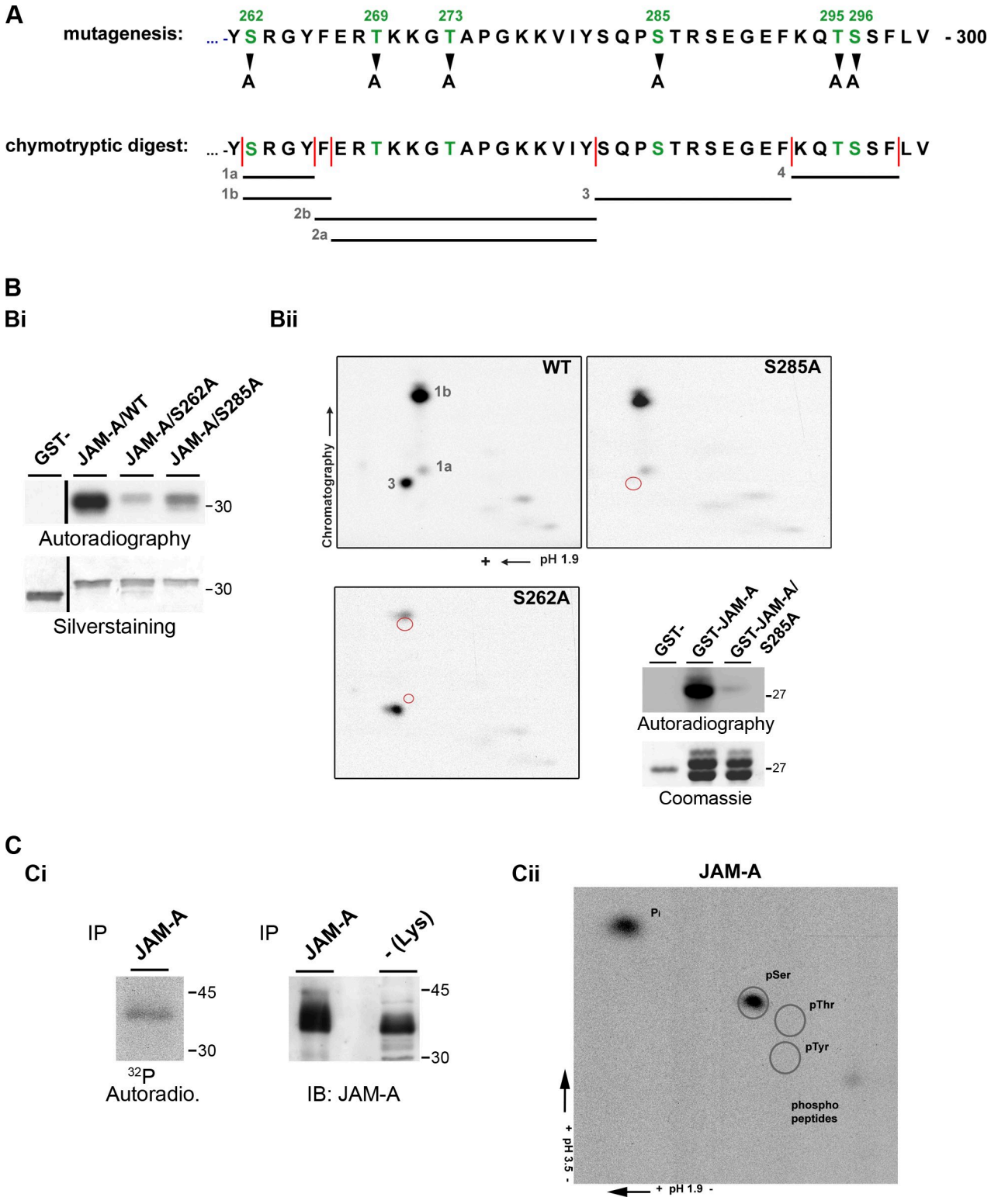

Figure 1. **JAM-A is phosphorylated by aPKCζ at S285.** (A, top) Amino acid sequence of the entire cytoplasmic domain of murine JAM-A (aa 261–300). Green letters indicate S and T residues, which were mutated to A based on evolutionary conservation and match to PKC consensus sites (Kennelly and Krebs, 1991). S262 represents a PKC phosphorylation consensus site only in combination with the adjacent GST residues. (bottom) JAM-A peptides predicted to be generated by chymotrypsin cleavage. Red vertical bars indicate predicted cleavage sites. At Y265 and F266, two chymotrypsin cleavage sites are juxtaposed. As the enzymatic hydrolysis requires two additional amino acids C terminally to the cleavage site, peptides 1 and 2 may each exist in two variants, 1a/1b and 2a/2b, respectively, depending on which residue is targeted first. (B) JAM-A is phosphorylated by aPKCζ at S285. (Bi) GST–JAM-A fusion proteins with the entire cytoplasmic domain of JAM-A (aa 261–300) either wild type (WT) or mutated (S262A and S285A) were phosphorylated with aPKCζ. (top) Phosphorylation was analyzed by SDS-PAGE and ^{32}P autoradiography. (bottom) Equal amounts of proteins were controlled by silver staining of 5% of the eluted proteins (input). (Bii) Phosphopeptide mapping of phosphorylated JAM-A by two-dimensional electrophoresis and thin-layer chromatography. Chymotryptic digest of aPKCζ-phosphorylated GST–JAM-A/WT results in two major phosphopeptide spots (labeled 1b and 3; top left). One of the

Rich1, through which it may indirectly influence the activity of the PAR–aPKC complex (Wells et al., 2006). Together, these observations place aPKC at the center of a protein network that regulates the formation and integrity of TJs in epithelial cells.

During cell–cell contact formation, aPKC interacts with different scaffolding proteins and phosphorylates various target proteins. At early phases of cell–cell contact formation, it forms a ternary complex with PAR-6 and Lethal giant larvae (Lgl; Yamanaka et al., 2003). The association of Lgl with aPKC–PAR-6 prevents the interaction of aPKC–PAR-6 with PAR-3. aPKC activation leads to Lgl phosphorylation and its segregation from the aPKC–PAR-6 complex (Yamanaka et al., 2003), allowing aPKC–PAR-6 to associate with cell–cell contact-associated PAR-3 and to form an active PAR-3–aPKC–PAR-6 complex at those sites. Active aPKC then phosphorylates a defined set of target proteins, such as PAR-1 or Numb, leading to their exclusion from the aPKC-containing membrane domain (Hurov et al., 2004; Suzuki et al., 2004; Smith et al., 2007; Morais-de-Sá et al., 2010). In turn, PAR-1 phosphorylates PAR-3, which prevents PAR-3 oligomerization and its stable localization at the membrane (Benton and St Johnston, 2003a,b; Mizuno et al., 2003). These mutual phosphorylations regulate the formation of distinct membrane domains. Once TJs are formed, the activity of aPKC at TJs is most likely continuously required to maintain their functional integrity. Furthermore, it is likely that aPKC activity is not only used to exclude basolateral membrane markers, such as Lgl or PAR-1, from the apical contact region but also to regulate the function or activity of other components within the TJs (Aono and Hirai, 2008).

A putative candidate protein subject to phosphorylation by aPKC is the Ig superfamily member junctional adhesion molecule A (JAM-A). In polarized epithelial cells, JAM-A localizes to lateral cell–cell contacts and is enriched at TJs (Martìn-Padura et al., 1998; Liu et al., 2000). During cell–cell contact formation, JAM-A localizes to the earliest sites of cell–cell interactions, the primordial, spotlike adherens junctions (AJs; pAJs), in which it serves to recruit PAR-3 and to assemble an active PAR-3–aPKC–PAR-6 complex (Ebnet et al., 2001; Itoh et al., 2001). Cells expressing JAM-A mutants that do not localize at cell–cell contacts fail to develop functional TJs and apicobasal membrane polarity (Rehder et al., 2006). These observations prompted us to analyze whether phosphorylation of JAM-A by aPKC is part of the molecular mechanism by which aPKC regulates the formation of TJs and the development of apicobasal polarity.

Results

aPKCζ phosphorylates JAM-A at S285 in vitro

We analyzed JAM-A phosphorylation by incubating GST–JAM-A fusion proteins containing the entire cytoplasmic domain of JAM-A (Y_{261}–V_{300}; Fig. 1 A) with aPKCζ in the presence of radiolabeled ATP followed by either conventional SDS-PAGE or by chymotryptic digest and two-dimensional analysis of the resulting peptides. These experiments indicated that aPKCζ phosphorylates JAM-A and that S285 is a major aPKCζ phosphorylation site (Fig. S1). The relatively strong autoradiography signals remaining in all single, double, or triple mutants prompted us to also mutate S262, which reflects an artificial PKC consensus site resulting from the fusion of GST sequences with JAM-A sequences. Conventional SDS-PAGE indicated that JAM-A phosphorylation was reduced after mutating either S262 or S285 (Fig. 1 Bi). Chymotrypsin treatment and phosphopeptide analysis revealed two major phosphopeptide spots (Fig. 1 Bii, top left, 1b and 3). Mutating S285 to Ala abolished spot 3 (Fig. 1 Bii, red circles). Mutating S262 abolished spot 1b as well as one of the minor spots labeled 1a (Fig. 1 Bii, red circles). To verify that S262 is phosphorylated because of the presence of an artificial PKC consensus site, we generated new fusion proteins in which three additional JAM-A–specific amino acids were inserted between the GST sequence and S262 of JAM-A. This construct showed almost no ^{32}P incorporation when S285 was mutated to Ala (Fig. 1 Bii, bottom right). Thus, these observations indicate that aPKCζ phosphorylates JAM-A strongly and exclusively at S285 in vitro.

To address whether JAM-A is phosphorylated in cells, we metabolically labeled confluent KLN205 cells using [^{32}P]orthophosphate, immunoprecipitated endogenous JAM-A, and performed phosphoamino acid analyses. SDS-PAGE and autoradiography indicated a phosphorylated band that was identified as JAM-A by Western blotting (Fig. 1 Ci). This band was excised from the membrane and subjected to hydrolysis followed by two-dimensional separation on thin-layer cellulose plates. Autoradiography revealed a prominent signal for phosphorylated serine but not threonine or tyrosine residues (Fig. 1 Cii), indicating that in the cell, JAM-A is phosphorylated exclusively on serine residues.

aPKCζ exists in a complex with JAM-A in epithelial cells

To analyze whether aPKCζ and JAM-A form a complex, we performed coimmunoprecipitation and pull-down experiments.

spots (3) is lost in the S285A mutant (top right, red circle). The second major spot (1b) as well as one minor spot (1a) are lost in the S262A mutant (indicated by red circles), which is consistent with the prediction of chymotrypsin generating two peptides harboring S262 (bottom left). Because S262 was known to represent an artificial PKC consensus generated at the fusion point between GST and JAM-A (GS**R**HYS_{262}RGY, with GST sequence underlined and the basic residue at position −3 of the phosphoacceptor site in bold), a newly generated GST fusion protein with three additional JAM-A–specific amino acids between GST and the JAM-A sequence (RGSWFAYS_{262}RGY, with the GST sequence underlined) was analyzed by in vitro phosphorylation and conventional SDS-PAGE (bottom right). Note that the strong phosphorylation of this construct by aPKC is almost completely abolished after mutating S285 to Ala, suggesting that S285 is the only major phosphorylation site for aPKC in vitro. Black lines indicate that intervening lanes have been spliced out. (C) JAM-A is serine phosphorylated in cells. (Ci, left) Autoradiography of JAM-A immunoprecipitated from [^{32}P]orthophosphate-labeled KLN205 cells. (right) Immunoblot analysis of lysates and of immunoprecipitated JAM-A was performed as controls. (Cii) Phosphoamino acid analysis of JAM-A. Immunoprecipitated JAM-A shown in Ci (^{32}P autoradiography [Autoradio.]) was isolated from the membrane, subjected to acid hydrolysis, and analyzed by two-dimensional thin-layer electrophoresis. Relative positions of pSer, pThr, and pTyr spots are indicated. JAM-A is exclusively phosphorylated at Ser residues. pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine; Pi, orthophosphate. Molecular markers are given in kilodaltons.

JAM-A was detectable in aPKCζ immunoprecipitates obtained from transiently transfected HEK293T cells (Fig. 2 A) as well as in immunoprecipitates obtained from Caco-2 epithelial cells, a human colonic epithelial cell line that adopts a polarized morphology with well-developed TJs (Fig. 2 B). In vitro translated aPKCζ bound to recombinant JAM-A as strong as to its direct binding partner PAR-6 (Joberty et al., 2000), indicating that the interaction between aPKCζ and JAM-A can be direct and does not require PAR-3 as a scaffold (Fig. 2 C). In line with this observation, JAM-A deletion mutants lacking the PDZ domain binding motif required for the interaction with PAR-3 (Ebnet et al., 2001) were readily detectable in aPKCζ immunoprecipitates (Fig. 2 D). Together, these findings indicate that aPKCζ forms a complex with JAM-A in epithelial cells and that this interaction can be direct and does not require PAR-3.

JAM-A is phosphorylated at S285 in epithelial cells

To analyze the relevance of JAM-A phosphorylation at S285 in cells, we generated phospho-S285 (S285-P)–specific pAbs (Fig. S2). Using these antibodies, we analyzed JAM-A S285 phosphorylation in MTD-1A epithelial cells, a polarizing epithelial cell line with well-developed TJs (Suzuki et al., 2002). MTD-1A cells grown to confluence showed a strong S285-P signal at cell–cell contacts (Fig. 3 A). When the cells were grown under low Ca^{2+} to disrupt cell–cell contacts (Gumbiner and Simons, 1986), JAM-A appeared at the periphery of rounded cells. Interestingly, the S285-P signal was completely absent in these contact-naive cells. When cells were replenished with Ca^{2+} (Ca^{2+} switch [CS]) for 3 or 8 h to induce new cell–cell contact formation, the S285-P signal colocalized with total JAM-A at maturing cell–cell contacts (Fig. 3 A). These findings indicate that JAM-A is phosphorylated at S285 in confluent cells, is dephosphorylated during disruption of cell–cell contacts, and is rephosphorylated during CS-induced new cell–cell contact formation.

aPKC phosphorylates JAM-A at S285 early during cell–cell contact formation

During cell–cell contact formation E-cadherin, ZO-1, and JAM-A are the first proteins that appear at so-called pAJs (also called puncta), whereas PAR-3, aPKC, and PAR-6 appear later (Yonemura et al., 1995; Suzuki et al., 2002). The sequential appearance of JAM-A and aPKCζ suggests that JAM-A phosphorylation at S285 occurs only after aPKCζ has been recruited. To address this question, MTD-1A cells were scratch wounded to induce new junction formation (Fig. 3 B). Total JAM-A was detected in the center of the wounds in a spotlike pattern typical for proteins present at pAJs (Fig. 3 B, top). S285 phosphorylation of JAM-A was not detected at pAJs but was detected only when pAJs had matured into more linear cell–cell contact structures. These observations indicate that JAM-A phosphorylation at S285 occurs with a significant delay after its localization at pAJs. As observed previously (Suzuki et al., 2002), aPKCζ was absent at pAJs. It appeared at more mature cell–cell contact sites with a delay similar to JAM-A S285 phosphorylation (Fig. 3 B, bottom). The similarity in the kinetics of appearance of aPKCζ and of S285 phosphorylation of JAM-A suggested that aPKCζ phosphorylates JAM-A after its recruitment to pAJs. To address this question, we inhibited aPKCζ activity using the aPKC-specific inhibitor PSζ (pseudosubstrate ζ). Incubation of wounded cells with PSζ almost completely abolished the S285 phosphorylation signal and resulted in a diffuse intracellular staining (Fig. 3 C). Together, these observations suggest the following hierarchy of events: JAM-A localizes to pAJs in the absence of S285 phosphorylation, in which it serves to recruit PAR-3. Subsequently, after assembly of the PAR-3–aPKC–PAR-6 complex at pAJs and activation of aPKC by Rac1/Cdc42 small GTPases, aPKC phosphorylates JAM-A at S285. The direct and PAR-3–independent interaction of aPKCζ with JAM-A allows a continuous S285 phosphorylation of JAM-A despite the dissociation of aPKCζ from PAR-3 as a result of aPKC-mediated S827 phosphorylation of PAR-3 (Nagai-Tamai et al., 2002; Morais-de-Sá et al., 2010).

Cell–cell contact maturation is promoted by S285 phosphorylation of JAM-A

To test whether the early phosphorylation of JAM-A/S285 is required for cell–cell contact formation, we analyzed the recruitment of ZO-1 as early marker for nascent cell–cell contacts during CS-induced new contact formation (Ando-Akatsuka et al., 1999). MDCK II cells stably expressing JAM-A/wild type (WT) or JAM-A/S285A under a doxycycline-regulated promoter (MDCK II Tet-Off cells; Rehder et al., 2006) were subjected to CS to induce new contact formation. 2 h after readdition of Ca^{2+}, ZO-1 immunoreactivity significantly increased in cells overexpressing JAM-A/WT (Fig. 4 A), indicating that JAM-A promotes junctional maturation. In contrast, overexpression of JAM-A/S285A significantly decreased ZO-1 immunoreactivity (Fig. 4 A). The delay in ZO-1 recruitment in JAM-A/S285A–expressing cells was less pronounced 4 h after CS, suggesting that JAM-A/S285 phosphorylation is important during the early steps of junctional maturation (Fig. 4 B). A similar pattern was observed for β-catenin and occludin (Fig. 4 B). Together, these observations indicate that aPKC-mediated S285 phosphorylation of JAM-A promotes cell–cell contact maturation at early time points of contact formation.

S285-phosphorylated JAM-A localizes exclusively to TJs of polarized epithelial cells

Because JAM-A localizes along the entire cell–cell contact of epithelial cells (Liu et al., 2000), we analyzed JAM-A/S285 phosphorylation in highly polarized MTD-1A cells. In contrast to total JAM-A, S285-P JAM-A showed a restricted distribution at the apical cell–cell contact area (Fig. 5 A). Stainings for the two TJ-specific proteins occludin (Fig. 5 A) or ZO-1 (Fig. S3 A) showed a complete overlap of S285-P JAM-A with both proteins, indicating that S285-P JAM-A localizes exclusively to the TJs. In addition, S285-P JAM-A completely overlaps with aPKCζ and PAR-3 (Fig. S3 B). Treatment of polarized MTD-1A cells for 2 h with PSζ inhibitor (Fig. 5 B) or knockdown of aPKCζ using siRNA (Fig. 5 C) abolished the pAb S285-P immunoreactivity, indicating that S285 phosphorylation of

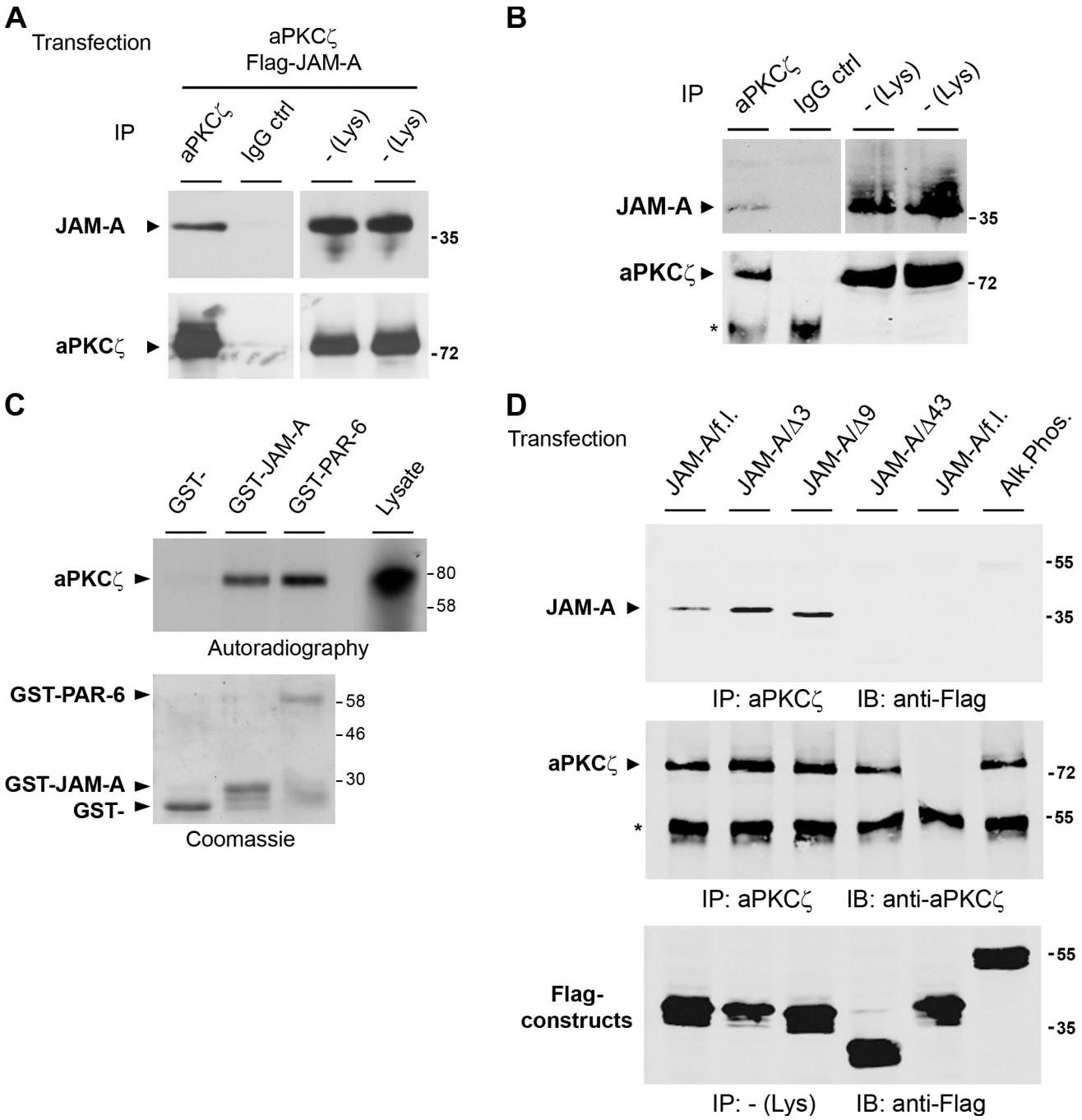

Figure 2. **JAM-A directly interacts with aPKCζ.** (A) JAM-A interacts with aPKCζ in HEK293T cells. aPKCζ immunoprecipitates (IP) obtained from HEK293T cells transiently transfected with aPKCζ and Flag–JAM-A were immunoblotted (IB) with Flag tag antibodies. In lanes labeled - (Lys), 0.75% of the input was loaded. (B) JAM-A associates with aPKCζ in Caco-2 epithelial cells. Immunoprecipitates obtained with antibodies against aPKCζ were analyzed for the presence of JAM-A (top) or aPKCζ (bottom). Lanes labeled - (Lys) contain 1.1% of the input. (C) The interaction of aPKCζ with JAM-A is direct. (top) GST–JAM-A was incubated with recombinant, [^{35}S]methionine-labeled aPKCζ. GST–PAR-6C served as a positive control (ctrl). (bottom) Equal loading of GST fusion proteins was analyzed by Coomassie brilliant blue staining. The lysate lane contains 10% of the input. Note that aPKCζ interacts with JAM-A as strongly as with PAR-6. (D) The association of JAM-A with aPKCζ does not require a PDZ domain protein. HEK293T cells were transfected with Flag–JAM-A constructs, either full-length JAM-A (JAM-A/f.l.), C-terminal deletion mutants lacking three (JAM-A/Δ3) or nine (JAM-A/Δ9) amino acids, which include the PDZ domain binding motif, or the entire cytoplasmic domain (JAM-A/Δ43). (top) Immunoprecipitates obtained with aPKCζ antibodies (first through fourth and sixth lanes) or with isotype-matched control antibodies (fifth lane) were analyzed for the presence of Flag-tagged constructs. (middle) The specificity of the aPKCζ immunoprecipitation was analyzed by immunoblotting 10% of the precipitated material with antibodies against aPKCζ. (bottom) Equal expression of the transfected constructs was verified by immunoblotting cell lysates with Flag tag antibodies. Asterisks indicate Ig heavy chains. Note that the absence of the PDZ domain binding motif in JAM-A does not abolish aPKCζ binding. - (Lys), lysate (no immunoprecipitation, with postnuclear supernatant loaded); Alk.Phos., alkaline phosphatase. Molecular markers are given in kilodaltons.

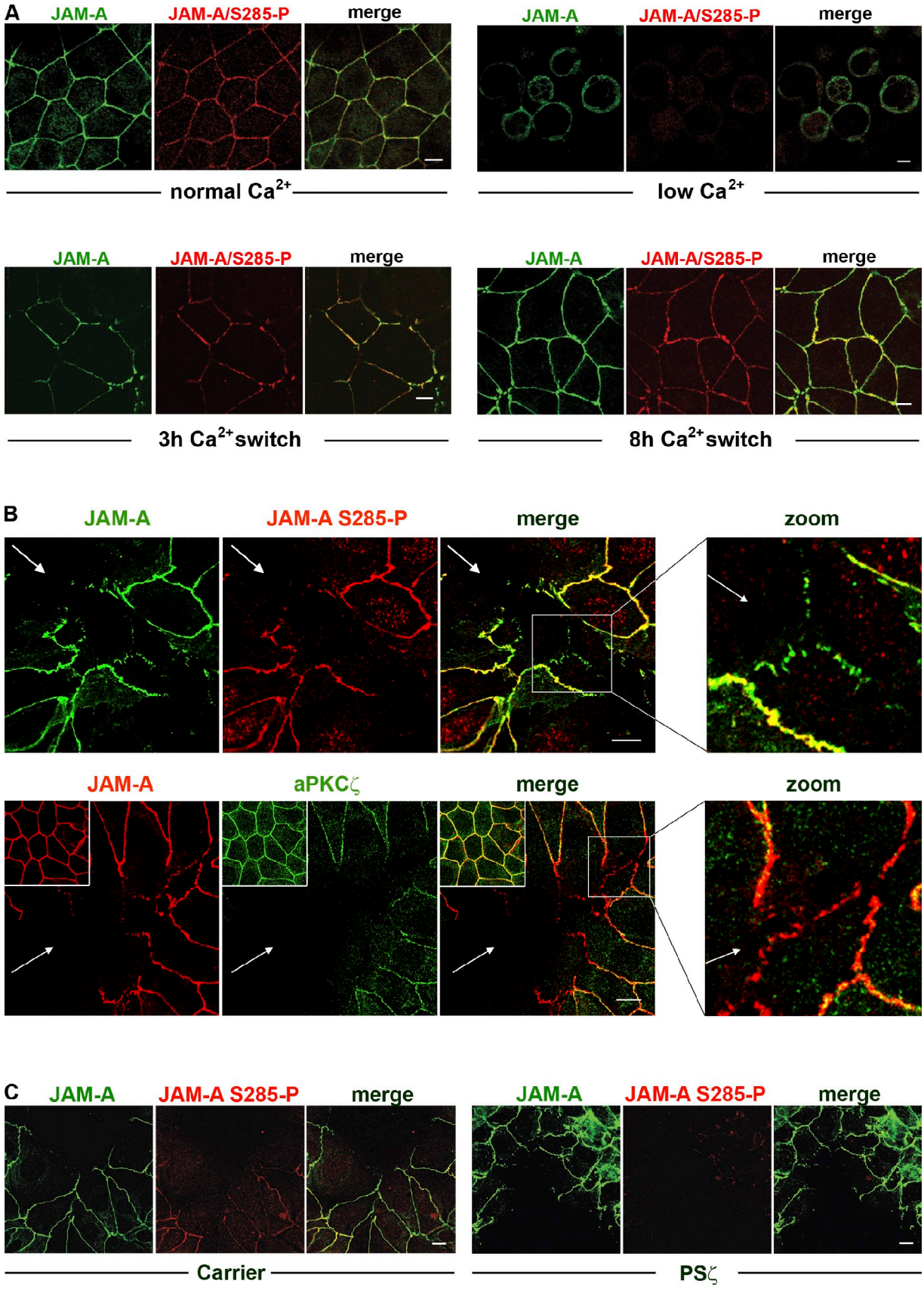

Figure 3. **JAM-A is phosphorylated at S285 by aPKCζ during cell–cell contact formation.** (A) JAM-A is S285 phosphorylated in polarized MTD-1A cells. MTD-1A cells grown to confluence were either kept in normal medium (normal Ca^{2+}), grown under low Ca^{2+} conditions for 18 h (low Ca^{2+}), or grown under low Ca^{2+} followed by Ca^{2+} replenishment for 3 or 8 h to induce new contact formation (CS). Cells were stained with antibodies against total JAM-A and

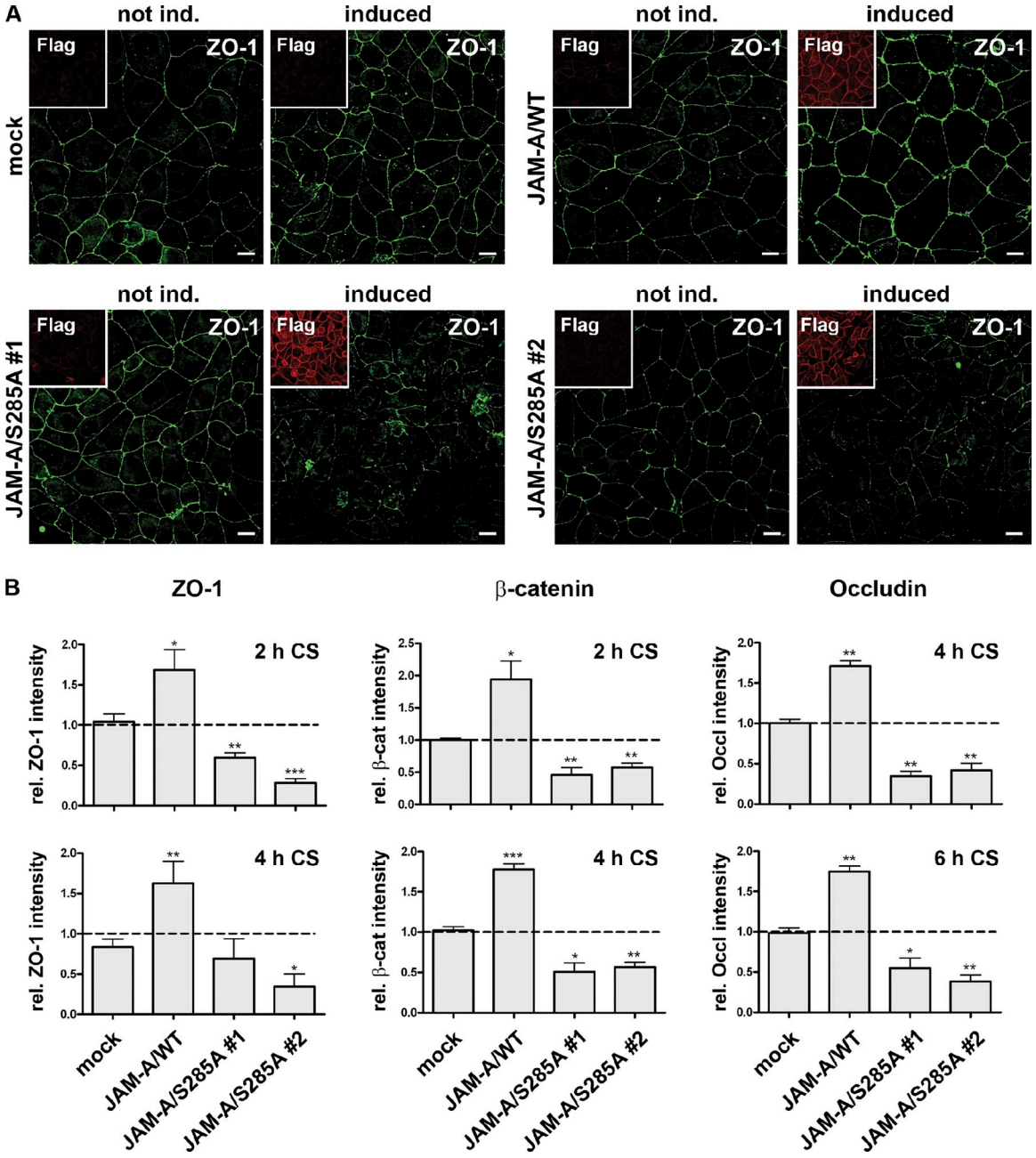

Figure 4. **JAM-A S285 phosphorylation promotes junction maturation.** MDCK II Tet-Off cells stably transfected with either empty vector (mock) or wild-type JAM-A (JAM-A/WT) or S285A JAM-A (JAM-A/S285A; two independent clones, #1 and #2) were subjected to Ca^{2+} switch (CS) for 2 or 4 h and then fixed and stained with antibodies against ZO-1, β-catenin, or occludin. (A) Representative ZO-1 immunofluorescence pictures taken 2 h after CS. Insets show expression of the Flag-tagged JAM-A constructs. (B) Statistical analysis of ZO-1 (left), β-catenin (middle), and occludin (right) immunofluorescence at cell–cell contacts at different time points after CS as indicated. The intensity of the immunofluorescence signals at cell–cell contacts was analyzed using ImageJ software. Data are given as ratios of the mean fluorescence intensities per cell after and before induction of expression of the respective constructs. Error bars denote the means ± SEM from three separate experiments. Statistical significance was evaluated using unpaired *t* tests; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. not ind., not induced; rel., relative; β-cat, β-catenin; Occl, occludin. Bars, 10 µm.

S285-P JAM-A. JAM-A is S285 phosphorylated at mature cell–cell contacts, is dephosphorylated upon cell–cell contact disruption, and is rephosphorylated during cell–cell contact formation. (B) JAM-A phosphorylation at S285 correlates with the appearance of aPKCζ at early sites of cell–cell contact. Wound-scratched MTD-1A cells were stained with antibodies against total JAM-A and S285-P JAM-A (top) or with antibodies against total JAM-A and aPKCζ (bottom). S285 phosphorylation of JAM-A correlates with the appearance of aPKCζ and is only observed after pAJs have matured into more linear cell–cell contacts. Arrows indicate the direction of the woundings. Insets show confluent areas of the monolayers. Boxes in the merge pictures mark the area shown in the zoomed in pictures. (C) Inhibition of aPKCζ activity blocks JAM-A S285 phosphorylation during cell–cell contact formation. Confluent MTD-1A cells were scratch wounded, grown in the presence of 25 µM PSζ, and then stained with antibodies against total JAM-A and S285-P JAM-A. JAM-A phosphorylation at S285 is abolished in the presence of PSζ. Bars: (A) 5 µm; (B and C) 10 µm.

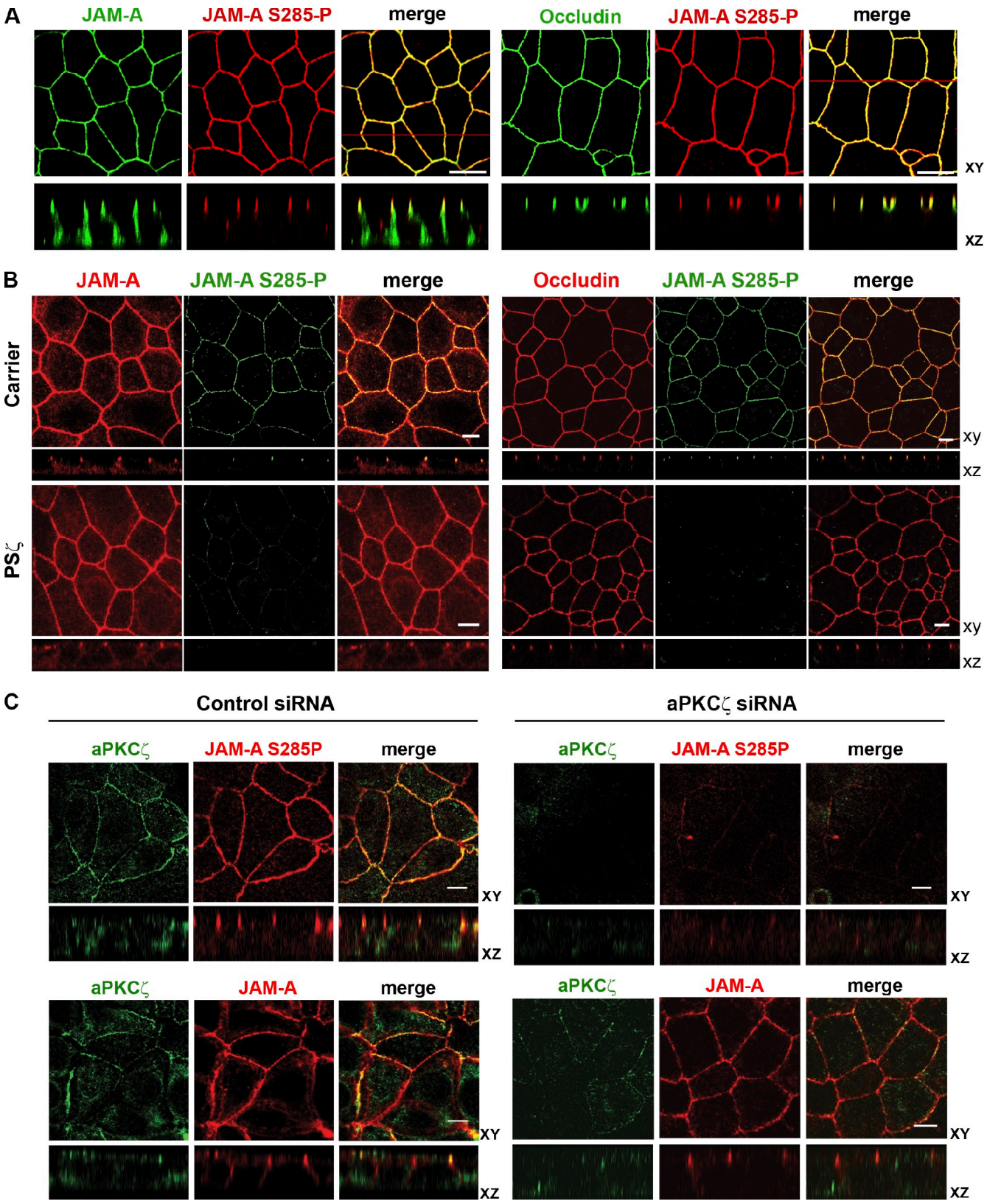

Figure 5. **JAM-A S285 phosphorylation is restricted to TJs in polarized epithelial cells.** (A) Polarized MTD-1A epithelial cells were stained with antibodies against S285-P JAM-A and total JAM-A (left) or against S285-P JAM-A and occludin (right). S285 phosphorylation is detectable exclusively at the TJs. Red lines indicate the position of the xz sections. (B) Polarized MTD-1A cells were incubated for 2 h with 25 µM PSζ inhibitor and then stained as indicated. (C) MTD-1A cells were transfected with either control siRNAs or aPKCζ-specific siRNAs and then stained as indicated. PSζ incubation or aPKCζ down-regulation abolished the S285 phosphorylation of JAM-A. Bars: (A) 10 µm; (B and C) 5 µm.

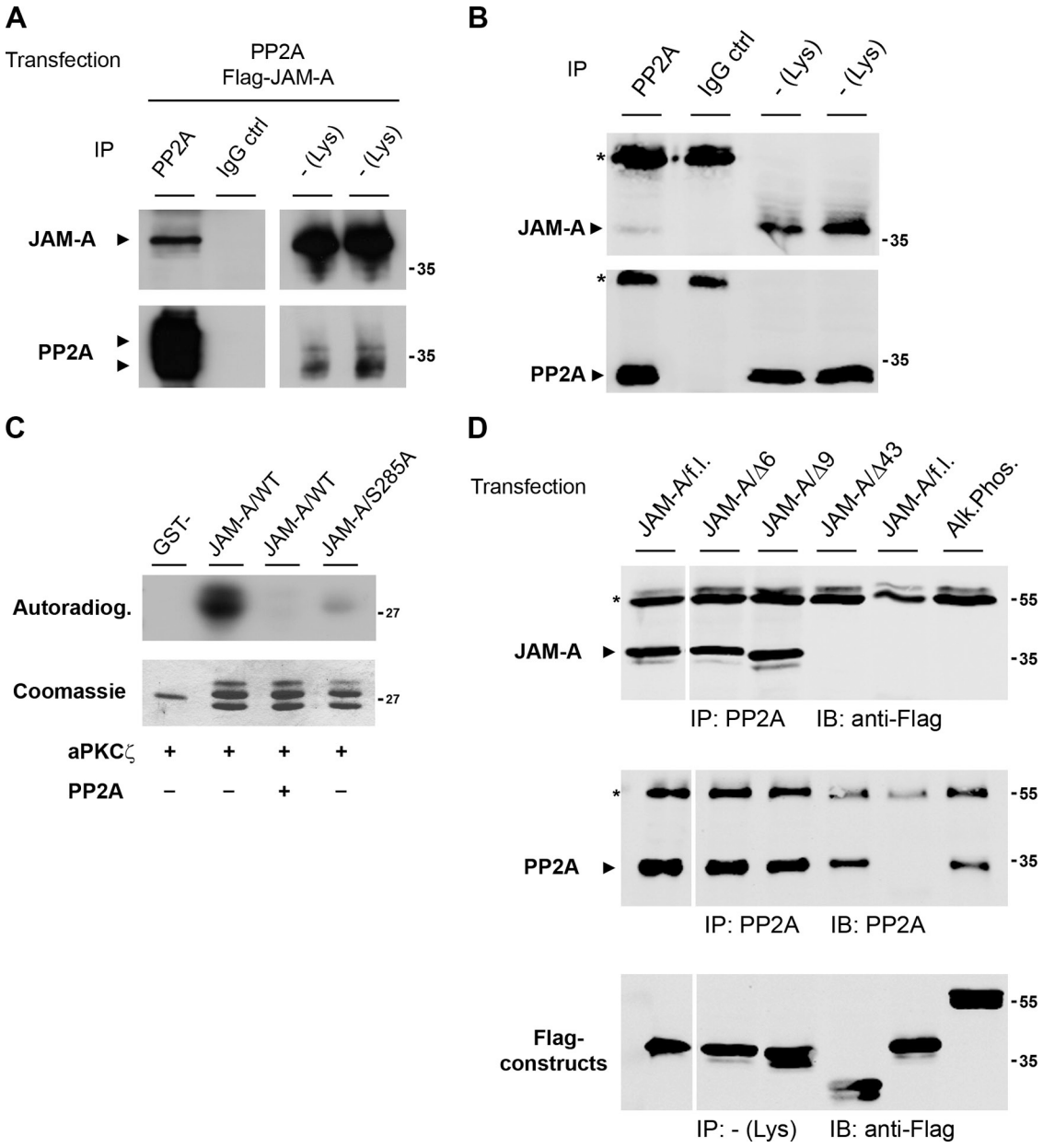

Figure 6. **PP2A interacts with JAM-A, and dephosphorylates JAM-A at Ser285.** (A) PP2A interacts with JAM-A in HEK293T cells. PP2A immunoprecipitates (IP) obtained from HEK293T cells transfected with HA-tagged PP2A-Cα and Flag-tagged JAM-A were immunoblotted (IB) with antibodies against the Flag tag. The arrowheads in PP2A immunoblots indicate endogenous PP2A-C and ectopically expressed HA-tagged PP2A-Cα. Lanes labeled - (Lys) contain 0.75% of the input. (B) PP2A exists in a complex with JAM-A in Caco-2 epithelial cells. Immunoprecipitates obtained with antibodies against PP2A were analyzed for the presence of JAM-A (top) or for the presence of PP2A (bottom). Isotype-matched control antibodies (IgG ctrl) were used as controls. Lanes labeled - (Lys) contain 1.1% of the input. (C) PP2A dephosphorylates JAM-A at Ser285. GST–JAM-A/WT and GST–JAM-A/S285A phosphorylated with aPKCζ in the presence of γ-[^{32}P]ATP were incubated with PP2A and then analyzed by SDS-PAGE and autoradiography (Autoradiog.). PP2A completely reversed aPKCζ-mediated phosphorylation. (D) The association of JAM-A with PP2A is not mediated by the PDZ domain binding motif of JAM-A. HEK293T cells were transfected with various Flag–JAM-A constructs as indicated. (top) Immunoprecipitates obtained with PP2A antibodies (first through fourth and sixth lanes) or isotype-matched control antibodies (fifth lane) were analyzed for the presence of Flag constructs. (middle) The specificity of the PP2A immunoprecipitation was verified by immunoblotting with PP2A antibodies. (bottom) Equal expression of the transfected constructs was verified by immunoblotting cell lysates with Flag antibodies. Asterisks denote Ig heavy chains. White lines indicate that intervening lanes have been spliced out. - (Lys), lysate; Alk. Phos., alkaline phosphatase; JAM-A/f.l., full-length JAM-A. Molecular markers are given in kilodaltons.

TJ-associated JAM-A is mediated by aPKCζ. To test whether S285 phosphorylation regulates the targeting of JAM-A to the TJs, we ectopically expressed a phosphodeletion mutant (S285A) and a phosphomimicking mutant (S285D) of JAM-A in MDCK II cells. Both mutants colocalized with ZO-1 at the apical region of the cell–cell contacts (Fig. S3 C), indicating that S285 phosphorylation does not regulate the localization of JAM-A to TJs. In support of this, blocking JAM-A S285 phosphorylation with PSζ or by aPKCζ knockdown did not change the localization of JAM-A at TJs (Fig. 5, B and C). Together, these findings suggest a TJ-specific function of aPKC-mediated JAM-A S285 phosphorylation.

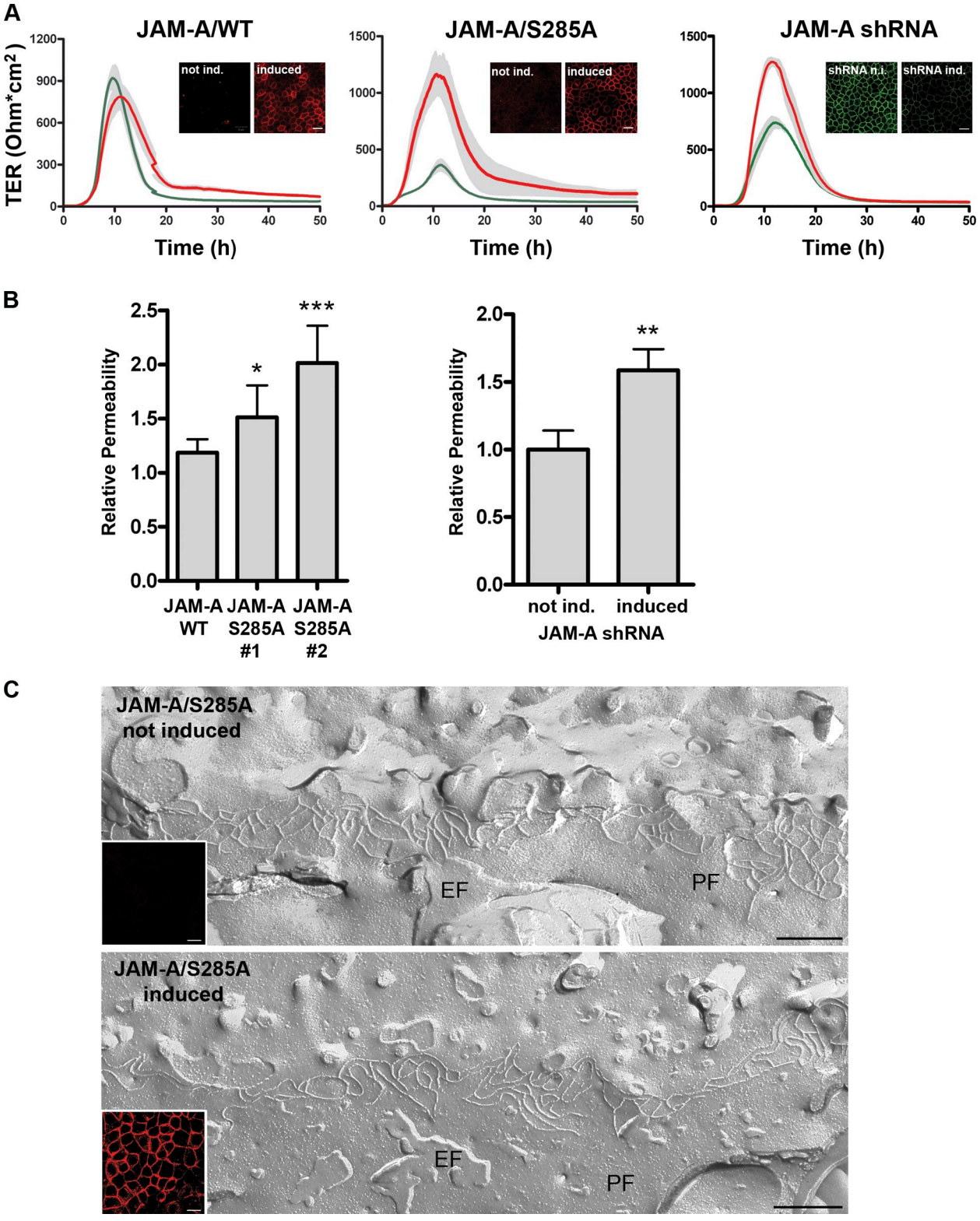

Figure 7. **JAM-A S285 phosphorylation is required for the formation of functional TJs.** (A) MDCK II cells expressing WT JAM-A (left), S285A JAM-A (middle), or JAM-A shRNAs (right) under a tetracycline-regulated promoter were left uninduced (red graphs) or were induced (green graphs). At confluence, cells were subjected to CS, and the development of TER was monitored over a period of 50 h. The gray area indicates deviation of triplicates. Experiments were performed at least three times for each clone. (insets) Immunofluorescence analysis of ectopic JAM-A constructs (left and middle) or endogenous JAM-A (right). (B) MDCK cells used in A (two independent clones for JAM-A/S285A, #1 and #2) were analyzed for 4.3-kD TRITC-dextran permeability. Data are given as a ratio of the fluorescence intensities present in the lower compartments with and without expression of the respective constructs (left) or as relative fluorescence intensities after shRNA expression (right). Error bars denote the means ± SD from three separate experiments. Statistical significance was evaluated using unpaired *t* tests; *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. (C) MDCK II Tet-Off cells expressing JAM-A/S285A were analyzed by freeze fracture electron microscopy. The TJs are composed of approximately two to three strands with a few interconnections running parallel to the cell

PP2A (protein phosphatase 2A) cooperates with aPKC to balance S285 phosphorylation levels of JAM-A

As JAM-A is S285 phosphorylated exclusively at TJs, we investigated whether aPKCζ-mediated phosphorylation of JAM-A is antagonized by PP2A, a protein phosphatase, which localizes at TJs and regulates TJ formation (Nunbhakdi-Craig et al., 2002). JAM-A was detectable in PP2A immunoprecipitates obtained from transiently transfected HEK293T cells as well as from nontransfected Caco-2 cells (Fig. 6, A and B), indicating that JAM-A associates with PP2A in polarized epithelial cells. Incubation of aPKCζ-phosphorylated GST–JAM-A with recombinant PP2A almost completely abolished JAM-A S285 phosphorylation (Fig. 6 C). Deleting either six or nine C-terminal amino acid residues of JAM-A did not abolish the association with PP2A (Fig. 6 D), indicating that PP2A binding is not mediated through a PDZ domain protein associated with JAM-A. Together, these observations indicate that PP2A interacts with JAM-A and dephosphorylates JAM-A at S285. The findings suggest that PP2A antagonizes aPKCζ-mediated phosphorylation of JAM-A at TJs.

aPKCζ-mediated JAM-A S285 phosphorylation regulates the barrier function of polarized epithelial cells

The development of trans-epithelial electrical resistance (TER) is a hallmark of barrier-forming epithelial cells (Matter and Balda, 2003a). To study the role of S285 phosphorylation of JAM-A for TER development, we used MDCK II Tet-Off cells stably expressing JAM-A/WT or JAM-A/S285A. Induced expression of WT JAM-A did not significantly affect the development of TER (Fig. 7 A) as observed previously (Rehder et al., 2006). Expression of JAM-A/S285A resulted in a strong decrease in TER, indicating that the barrier function of TJs is severely impaired when JAM-A cannot be phosphorylated at S285. Expression of JAM-A/S285A also significantly increased the permeability for a 4.3-kD TRITC-dextran when compared with JAM-A/WT expression (Fig. 7 B), indicating that the barrier for small molecular mass hydrophilic molecules is also impaired in the absence of JAM-A S285 phosphorylation. The defect in the barrier function cannot be explained by an inability to form TJ strands, as expression of JAM-A/S285A did not affect the formation of TJ strands (Fig. 7 C). In addition, the defect in TJ function is unlikely to be caused by a mislocalization of PAR-3 because recruitment of PAR-3 to cell–cell contacts and to TJs is not affected by the S285A mutation (Fig. S4, A and B). Together, these observations suggest that aPKCζ-mediated phosphorylation of JAM-A at S285 is critical for the formation of a functional barrier for ions and small hydrophilic molecules in epithelial cells.

JAM-A S285 phosphorylation is not required for apicobasal membrane polarity but is critical for epithelial morphogenesis

The maintenance of apicobasal membrane polarity requires a diffusion barrier that prevents the intermixing of apical and basolateral membrane components. This intramembrane diffusion barrier colocalizes with the paracellular diffusion barrier at TJs but is regulated by a different molecular mechanism (Umeda et al., 2006). To analyze whether JAM-A S285 phosphorylation is required for the development of apicobasal polarity, we expressed JAM-A/S285A in MDCK II epithelial cells and analyzed lumen formation in three-dimensional cyst assays (O'Brien et al., 2002). In the absence of JAM-A/S285A expression, MDCK cells developed normal cysts with a single lumen surrounded by a monolayer of epithelial cells (Fig. 8). Expression of JAM-A/S285A resulted in the formation of cysts, which, instead of a single lumen, contained multiple lumens. Interestingly, apicobasal polarity of individual cells was not disturbed, as indicated by the apical localization of F-actin in cells surrounding the lumens (Fig. 8). These observations suggest that despite the role of JAM-A S285 phosphorylation in the regulation of the epithelial barrier function, S285 phosphorylation of JAM-A is not involved in the regulation of the intramembrane diffusion barrier.

JAM-A is hyperphosphorylated at S285 during mitosis

During cell division, individual mitotic cells within the epithelial monolayer round up, but retain extensive contacts to the surrounding interphase cells (Baker and Garrod, 1993; Théry and Bornens, 2008). Apicobasal membrane polarity, as well as TJs, is maintained during all stages of mitosis (Reinsch and Karsenti, 1994; Kim and Raphael, 2007), and the barrier function of epithelial cells may even increase during mitosis (Soler et al., 1993). When stained for S285 phosphorylation, mitotic cells show a much stronger signal than the surrounding nondividing cells (Fig. 9). The increased phosphorylation of JAM-A persists to telophase and cytokinesis (Fig. 9, middle), suggesting that it is maintained throughout mitosis. The increase in signal intensity in mitotic cells was not observed with a polyclonal JAM-A antibody directed against the nonphosphorylated peptide (Fig. 9, bottom), indicating that it reflects increased S285 phosphorylation but not altered JAM-A recruitment to cell–cell contacts. Our findings thus indicate that during mitosis, JAM-A is hyperphosphorylated at S285, which may contribute to a stabilization of cell–cell contacts and barrier function in mitotic cells.

Discussion

The formation of an apical junction complex, including TJs and AJs, in vertebrate epithelial cells is regulated by highly conserved cell polarity proteins, including aPKC. Once recruited to

surface and are surrounded by a looser network comprising often anastomosing strands of variable orientation. No significant differences were observed between control cells (top) and JAM-A/S285A–expressing cells (bottom). (insets) Immunofluorescence analysis of JAM-A/S285A expression. not ind., not induced; EF, exoplasmic face; PF, protoplasmic face. Bars: (C, main images) 0.5 µm; (A and C, insets) 20 µm.

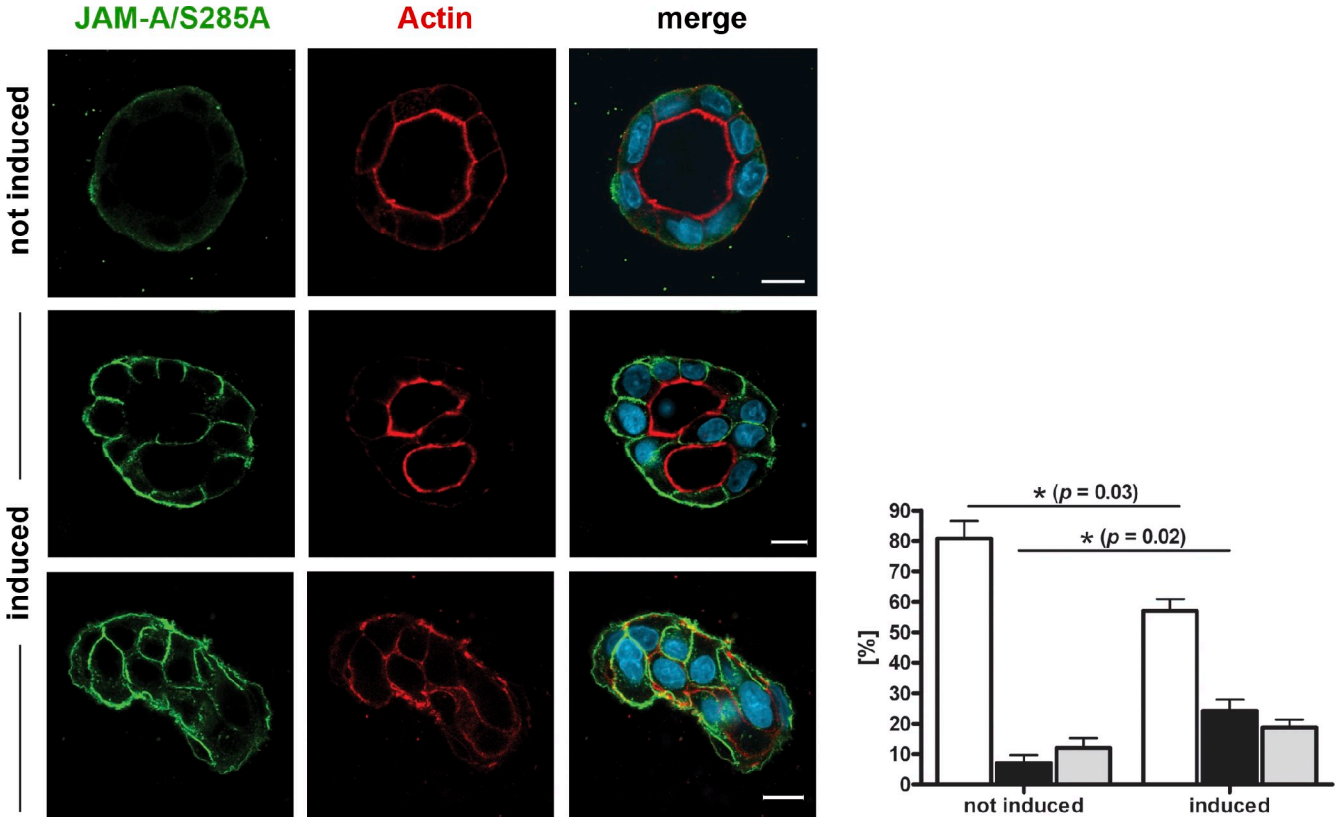

Figure 8. **JAM-A S285 phosphorylation is not required for the development of the apical membrane domain but for single lumen specification.** MDCK II Tet-Off cells inducibly expressing wild-type Flag–JAM-A (JAM-A/WT) or Flag–S285A JAM-A (JAM-A/S285A) were grown in a three-dimensional collagen matrix for 5–7 d and then fixed and stained with antibodies against JAM-A (anti-Flag) and with rhodamine-phalloidin to detect apical F-actin. Cysts were classified as normal (spherical with single lumen, white columns), multiluminal (spherical with more than one lumen, black columns), and disorganized (loss of spherical morphology without lumen, gray columns). Expression of JAM-A/S285A significantly increased the number of multiluminal cysts in which apicobasal polarity was retained. Error bars denote the means ± SD from three separate experiments. Statistical analysis was performed with unpaired *t* tests. Bars, 10 µm.

pAJs through its association with JAM-A–bound PAR-3 and activated through Cdc42/Rac1 small GTPases, aPKC promotes the maturation of primordial junctions into apical cell junctions, with TJs being separated from AJs (Suzuki et al., 2002). In this study, we find that shortly after the formation of pAJs, aPKCζ phosphorylates JAM-A at S285 to promote junctional maturation. In fully polarized epithelial cells, aPKCζ-phosphorylated JAM-A localizes specifically at the TJs, and this activity is most likely antagonized by PP2A. JAM-A S285 phosphorylation is required for the development of a functional epithelial barrier and for normal epithelial morphogenesis. We also find that JAM-A is hyperphosphorylated at S285 in mitotic cells. Our findings identify S285 of JAM-A as a novel mediator of aPKC activity during cell–cell contact maturation as well as during barrier function development in epithelial cells.

JAM-A S285 phosphorylation during cell–cell contact formation

Cell–cell contact formation is a stepwise process that is characterized by the sequential localization of integral membrane and cytoplasmic proteins to sites of cell–cell adhesion. JAM-A is among the first cell adhesion molecules that appear at pAJs, and PAR-3 and aPKC are recruited later (Ebnet et al., 2001; Suzuki et al., 2002). Consistent with the predicted role for cell adhesion molecules to act as positional cues for the correct localization of cell polarity proteins (Nelson, 2003), the early localization of JAM-A most likely serves to recruit PAR-3 and promote the formation of an active PAR-3–aPKC–PAR-6 complex at these sites. Interestingly, we find that JAM-A is present at pAJs in a non-S285–phosphorylated state. S285 phosphorylation of JAM-A occurs after aPKC has been recruited to pAJs. These findings thus suggest that nonphosphorylated JAM-A present at pAJs recruits PAR-3 and promotes the assembly of the PAR-3–aPKC–PAR-6 complex. Through the binding of Rho family small GTPases to PAR-6, aPKC is activated to phosphorylate JAM-A at S285. JAM-A phosphorylation at S285 promotes the maturation of cell–cell contacts. These findings thus identify JAM-A as a novel target through which aPKC regulates junctional maturation after the establishment of pAJs.

Ectopic expression of a kinase-negative mutant of aPKC (aPKCkn) results in a more drastic phenotype than expression of JAM-A/S285A. As opposed to JAM-A/S285A–expressing cells, aPKCkn-expressing cells fail to develop the pAJs into mature cell–cell contacts, with TJs segregated from AJs (Suzuki et al., 2002). This suggests that contact maturation requires additional phosphorylation of proteins other than JAM-A. aPKC has been shown to phosphorylate PAR-1 as well as Numb, and in both cases, the phosphorylation leads to the exclusion of the proteins from the

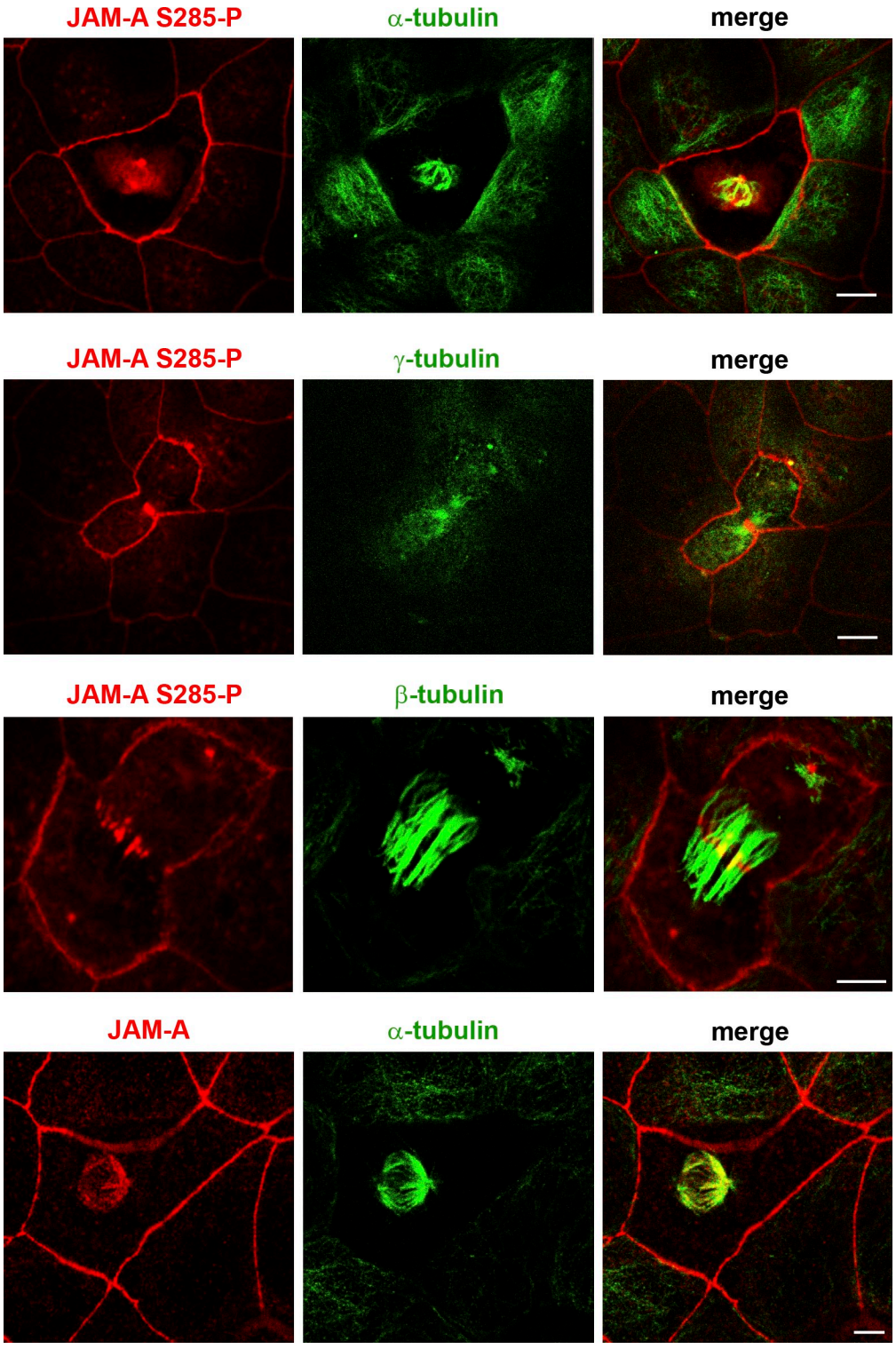

Figure 9. **JAM-A is hyperphosphorylated at S285 during mitosis.** Confluent monolayers of MTD-1A cells were stained with antibodies against S285-P JAM-A (top and middle rows) or total JAM-A (bottom row) and antibodies against α-tubulin, β-tubulin, or γ-tubulin as indicated. Note that JAM-A phosphorylation at S285 is strongly increased in dividing cells when compared with surrounding nondividing cells. Bars, 5 µm.

aPKC-containing membrane domain, which is part of the mechanism of how aPKC regulates the development of membrane asymmetry (Benton and St Johnston, 2003b; Hurov et al., 2004; Suzuki et al., 2004; Smith et al., 2007). In the case of JAM-A, however, phosphorylation by aPKC does not lead to its exclusion from the aPKC-containing membrane domain, indicating that JAM-A S285 phosphorylation serves a different purpose. In line with the role of JAM-A as a positional cue, we speculate that aPKC-mediated S285 phosphorylation of JAM-A generates new binding sites for the recruitment of proteins that regulate cell–cell contact

maturation after pAJs have been formed. Pull-down experiments using phosphorylated JAM-A peptides combined with mass spectrometry will help to identify these proteins.

JAM-A S285 phosphorylation regulates the barrier function of TJs

JAM-A is enriched at TJs, but a substantial amount of JAM-A molecules is localized along the lateral membrane (Liu et al., 2000; Rehder et al., 2006). We observed that in polarized epithelial cells, S285 phosphorylation of JAM-A is detected exclusively at TJs. Because a phosphodeficient JAM-A mutant (JAM-A/S285A) was not excluded from TJs, S285 phosphorylation does not regulate the localization of JAM-A at TJs. Therefore, it is more likely that the TJ-associated subpopulation of JAM-A, which is characterized by S285 phosphorylation, is either phosphorylated exclusively within TJs by aPKCζ or, alternatively, is phosphorylated by aPKCζ within a different subcellular compartment and is then transported to the TJs. This JAM-A population is continuously phosphorylated by aPKCζ as indicated by the loss of the S285 phosphorylation when fully polarized epithelial cells are incubated for 2 h with the PSζ inhibitor. Phosphorylation of JAM-A at S285 does probably not contribute to the overall maintenance of TJs as indicated by the apical restriction of occludin in PSζ-treated cells and by the apical localization of PAR-3 and the presence of TJ strands in JAM-A/S285A–expressing cells. JAM-A S285 phosphorylation by aPKC rather regulates specifically the barrier function of TJs. The mechanism by which JAM-A phosphorylation regulates the barrier function is presently unclear. We can rule out the possibility that it regulates the barrier by changing the expression levels of proteins involved in the formation of TJ strands because we observed no difference between JAM-A/WT– and JAM-A/S285A–expressing cells with regard to their influence on the expression of claudin-1, claudin-2, claudin-10, claudin-15, occludin, or ZO-1 (Fig. S5). It is possible that S285 phosphorylation serves to assemble a signaling module, which operates at the level of TJs to maintain their functional integrity. The presence of a Cdc42-dependent signaling module that regulates the maintenance of TJs has recently been described (Wells et al., 2006).

We have previously shown that ectopic expression of JAM-A cytoplasmic domain deletion mutants in MDCK II renal epithelial cells disrupts the epithelial barrier (Rehder et al., 2006). JAM-A knockout mice have a barrier and a morphological defect in the intestine and in the cornea but not in the kidney (Kang et al., 2007; Laukoetter et al., 2007). Most likely, tissue-specific factors contribute to the tissue-specific requirements in the barrier function. In support of this, we observed that JAM-A regulates the levels of claudin-2, but not claudin-10 and claudin-15, in MDCK II cells (Fig. S5), whereas the opposite has been observed in intestinal SK-CO15 cells (Laukoetter et al., 2007). These findings suggest that JAM-A contributes to the barrier function in a tissue-specific manner, via a S285 phosphorylation-independent mechanism involving the selective regulation of claudins.

aPKC and PP2A cooperate to regulate TJ function and JAM-A S285 phosphorylation

PP2A antagonizes aPKC-mediated S285 phosphorylation in vitro. PP2A has been described to be localized at TJs, and a study using pharmacological inhibition or reduced expression of the catalytic subunit of PP2A indicated that PP2A negatively affects TJ formation and barrier function (Nunbhakdi-Craig et al., 2002). This activity of PP2A probably resides in its ability to dephosphorylate ZO-1 and occludin (Nunbhakdi-Craig et al., 2002; Seth et al., 2007). Besides these structural components of TJs, PP2A interacts with and dephosphorylates aPKCζ at T410 (Nunbhakdi-Craig et al., 2002), resulting in down-regulation of aPKC activity (Hirai and Chida, 2003). Thus, by targeting T410 of aPKCζ, PP2A can also indirectly contribute to the reduced phosphorylation of structural components of TJs, such as occludin, ZO-1, and claudin-4 (Nunbhakdi-Craig et al., 2002; Seth et al., 2007; Aono and Hirai, 2008). We identified PP2A in a complex with JAM-A in cells, and we found that PP2A dephosphorylates JAM-A at S285 in vitro. These findings point to PP2A as a negative regulator of JAM-A S285 phosphorylation. Although it is possible that other protein phosphatases, such as PP1, are involved in regulating JAM-A phosphorylation (Seth et al., 2007; Traweger et al., 2008), and although we cannot exclude the possibility that protein kinases, such as PKCα, regulate JAM-A phosphorylation at S285 under certain conditions in epithelial cells (Ozaki et al., 2000), the loss of the S285 phosphorylation in aPKCζ knockdown and PSζ-treated cells, the identification of PP2A and JAM-A in the same protein complex, and the ability of PP2A to regulate the activity of aPKCζ and to dephosphorylate JAM-A in vitro suggest that aPKCζ and PP2A cooperate in regulating the TJ-specific phosphorylation of JAM-A.

JAM-A is hyperphosphorylated during mitosis

We also demonstrate that JAM-A is hyperphosphorylated at S285 during mitosis. Previous studies indicate that cell–cell contacts, including TJs, between mitotic and interphase cells are maintained during mitosis and cytokinesis (Baker and Garrod, 1993; Soler et al., 1993). The increased S285 phosphorylation of JAM-A in mitotic cells could be required to stabilize and maintain the functional integrity of TJs between mitotic and interphase cells to avoid a loss of the barrier function during mitosis. On the other hand, our observation that ectopic expression of JAM-AS/S285A in epithelial cells leads to a multiluminal phenotype when grown in a three-dimensional collagen matrix points to a role for JAM-A phosphorylation that is distinct from its role in regulating the barrier function. Recent evidence correlates mitotic spindle orientation with single lumen specification during cystogenesis, and several proteins associated with JAM-A, including aPKC, PAR-3, and PAR-6, have been implicated in this process (Jaffe et al., 2008; Mitsushima et al., 2009; Hao et al., 2010; Durgan et al., 2011), which opens the possibility that JAM-A is involved in spindle orientation. JAM-A has also been described to regulate epithelial proliferation (Laukoetter et al., 2007; Nava et al., 2011) and to be phosphorylated during mitosis at several serine residues, including the S285-corresponding S284 residue in HeLa cells (Dephoure et al., 2008; Gauci et al., 2009). Together, these observations point to an important role of JAM-A S285 phosphorylation during cell division, which may be independent of its function in regulating the cell–cell contact and epithelial barrier formation.

Materials and methods

Cell culture and transfections

Caco-2 cells (American Type Culture Collection), HEK293T cells, KLN205 cells (Kaneko et al., 1980), MDCK II Tet-Off cells (BD), and MTD-1A cells (provided by M. Takeichi, Kyoto University, Japan) were grown in DME with 10% FCS, 2 mM glutamine, and 100 U/ml penicillin/streptomycin. MDCK II Tet-Off cells expressing Flag-tagged JAM-A/S285A were generated by transfecting MDCK II Tet-Off cells with pTRE2hyg expression vectors (BD). Transfected cells were selected in DME medium supplemented with 100 µg/ml G418, 1 µg/ml puromycin, 150 µg/ml hygromycin, and 50 ng/ml doxycycline. Flag–JAM-A/S285A expression was induced by growing cells in medium lacking doxycycline. CHO cells were maintained in α-MEM supplemented with 10% FCS, 2 mM glutamine, and 100 U/ml penicillin/streptomycin. Transient transfections of plasmids using either GeneJammer (Agilent Technologies) or FuGENE 6 (Roche) transfection reagents were performed according to the manufacturer's instructions.

Antibodies and reagents

The following antibodies were used in this study: rat pAb anti–ZO-1, mouse mAb anti-PP2A (Millipore), mouse mAb antioccludin (Invitrogen), anti-Flag tag mouse mAb M2 and rabbit pAb (Sigma-Aldrich), rabbit pAb anti-aPKCζ, rabbit anti–human JAM-A (Santa Cruz Biotechnology, Inc.), and mouse anti–human JAM-A mAb (BD). mAbs and pAbs directed against murine JAM-A (H2O2-106-7-4 and pAb JAM-A extracellular domain [Affi828], respectively) have been previously described (Malergue et al., 1998; Rehder et al., 2006). A pAb against Ser285-phosphorylated JAM-A was generated by immunizing rabbits with a synthetic peptide comprising aa 282–294 (SQP[pSer]TRSEGEFKQ) of murine JAM-A coupled to ovalbumin. For affinity purification, specific antibodies were adsorbed against the phosphorylated peptide coupled to BSA (pAb S285-P). Antibodies against the remaining part of the peptide were removed by adsorption against the nonphosphorylated peptide, resulting in a pAb recognizing the same peptide in a nonphosphorylated form (pAb S285-N). The following reagents were used in this study: PKCζ PS peptide (PSζ; Sigma-Aldrich), recombinant aPKCζ, and PP2A (Millipore).

DNA constructs and site-directed mutagenesis

For transient expression of Flag-tagged JAM-A constructs, murine JAM-A cDNA (aa 25–300) or human JAM-A cDNA (aa 26–299) and C-terminal deletion constructs (hJAM-A/Δ3 [aa 26–296], hJAM-A/Δ6 [aa 26–293], hJAM-A/Δ9 [aa 26–290], and hJAM-A/Δ43 [aa 26–256, lacks entire cytoplasmic domain]) were cloned into the pFlag-CMV1 vector (Ebnet et al., 2001). For inducible expression in MDCK II cells, a pTRE2hyg-based vector encoding Flag-tagged JAM-A fusion proteins was used (Rehder et al., 2006). aPKCζ was cloned in pcDNA4-V5His (Invitrogen). The PP2A cDNA was provided by E. Sontag (University of Newcastle, Callaghan, Australia). For recombinant protein expression in *Escherichia coli*, the pGEX-4T-1 vector containing the entire cytoplasmic tail of murine JAM-A (aa 261–300 and aa 258–300; Ebnet et al., 2000) was used. GST–PAR-6C was generated by cloning full-length mouse PAR-6C in pGEX-6P-2. All point mutations were generated by a PCR-based approach using mismatch primer pairs.

RNA interference

To deplete JAM-A and aPKCζ in epithelial cells, mixtures of four siRNA duplexes (ON-TARGETplus SMARTpool siRNAs; Thermo Fisher Scientific) were used. 2×10^6 cells were transfected with 100 pmol (JAM-A) or 20 pmol (aPKCζ) siRNAs by electroporation (Lonza) according to the manufacturer's instructions. To obtain high knockdown efficiencies, the cells were harvested after 24 h and subjected to a second electroporation using the same siRNAs. After 48 h, the cells were fixed for immunofluorescence analysis. MDCK cells stably expressing JAM-A small hairpin RNAs (shRNAs) under a tetracycline-regulated promoter have been previously described (Rehder et al., 2006).

In vitro phosphorylation and dephosphorylation assays and phosphopeptide analysis

Phosphorylation experiments were performed essentially as previously described (Ebnet et al., 2003). GST fusion proteins were coupled to glutathione Sepharose (GE Healthcare) using buffer B (10 mM Hepes-NaOH, pH 7.4, 100 mM KCl, 1 mM $MgCl_2$, and 0.1% Triton X-100) and subjected to in vitro kinase reactions with either recombinant active aPKCζ or PKCα (Enzo Life Sciences). aPKCζ incubations were performed for 30 min at 30°C with 10 ng enzyme in the presence of 5 µCi γ-[^{32}P]ATP in PKCζ kinase buffer (18 mM Hepes, pH 7.4, 0.3 mM EDTA, 2 mM EGTA, 1% BSA, 400 µM DTT, 400 µM sodium pervanadate, 30 mM $MgCl_2$, 200 µM of cold ATP, 10 mM β-glycerophosphate, 50 µg/ml phosphatidylserine, 5 µg/ml diacylglycerides, and 10 µg/ml leupeptin). Similarly, kinase assays with PKCα were performed with 10 ng recombinant enzyme and 5 µCi γ-[^{32}P]ATP in PKCα kinase buffer (14 mM Hepes, pH 7.4, 700 µM DTT, 700 µM sodium pervanadate, 700 µM $CaCl_2$, 900 µM sodium fluoride, 15 mM $MgCl_2$, 100 µM of cold ATP, 18 mM β-glycerophosphate, 50 µg/ml phosphatidylserine, 5 µg/ml diacylglycerides, and 12 µg/ml leupeptin) for 30 min at 30°C. For in vitro dephosphorylation assays, the phosphorylated GST fusion proteins were washed and then incubated for 30 min at 30°C with recombinant PP2A (Millipore). The GST fusion proteins were washed and subsequently eluted from the beads by boiling in SDS sample buffer. Supernatants were separated by SDS-PAGE and analyzed by autoradiography.

For phosphopeptide mapping, the bands corresponding to JAM-A were eluted from the polyacrylamide gels and prepared as previously described (Boyle et al., 1991). In brief, the protein was precipitated using trichloroacetic acid in the presence of RNase A (Sigma-Aldrich), oxidized with freshly prepared performic acid, and digested with 0.5 U chymotrypsin (Worthington Biochemical Corporation) for 3–5 h at 37°C. An additional 0.5 U enzyme was added and incubated as in the previous paragraph to ensure complete digestion. The separation of chymotryptic peptides in the first dimension was performed in pH 1.9 buffer (2.5% [vol/vol] formic acid and 7.8% [vol/vol] glacial acetic acid) using a thin-layer electrophoresis apparatus (Hunter HTLE-7000; C.B.S. Scientific Company, Inc.) followed by thin-layer chromatography along the second dimension in phosphochromatography buffer (37.5% [vol/vol] *n*-butanol, 25% [vol/vol] pyridine, and 30% [vol/vol] glacial acetic acid). Phosphopeptides were visualized by autoradiography after 10 d of exposure.

In vivo labeling and phosphoamino acid analysis

Confluent KLN205 cells were washed in phosphate-free DME and subsequently metabolically labeled for 20 h in phosphate-free DME supplemented with 0.5 mCi/ml [^{32}P]orthophosphate and 0.5% dialyzed FCS. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% [vol/vol] Triton X-100, 10 µg aprotinin/ml, 1 mM Pefabloc, 10 µg leupeptin/ml, 1 µg Pepstatin A/ml, and 1 mM DTT), and endogenous JAM-A was immunoprecipitated using affinity-purified polyclonal rabbit antibodies. Phosphorylated proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After excision of the bands corresponding to JAM-A, amino acids were released by acid hydrolysis in constantly boiling hydrochloric acid for 1 h at 100°C and separated by two-dimensional electrophoresis on thin-layer cellulose plates using the thin-layer electrophoresis apparatus (Hunter HTLE-7000) according to the manufacturer's protocol. Separation of phosphoamino acids was controlled by addition of phosphoserine, phosphothreonine, and phosphotyrosine standards, which were visualized by ninhydrin staining. Radioactively labeled phosphoamino acids originating from the sample were visualized by autoradiography after 8 d of exposure.

CS experiments and analysis of junction formation

For CS experiments, adherent cells were incubated in low Ca^{2+} medium (Ca^{2+}-free DME; Invitrogen; 5% dialyzed FCS, 4 mM L-glutamine, and 3 µM $CaCl_2$) for 16–20 h. To induce new cell contact formation, the medium was replaced by medium supplemented with 1.8 mM $CaCl_2$ (CS). The rate of junction assembly was determined by measuring the accumulation of ZO-1 at cell–cell contacts using ImageJ software (National Institutes of Health). In brief, the maximal intensity of the ZO-1 signal (projections from confocal stacks) at cell–cell contacts within a given field of view (between 70 and 100 cells) was measured using the NeuronJ tracing tool of the ImageJ software. The resulting mean intensity value for the given field of view was multiplied with the total length of ZO-1–positive cell contact sites to account for ZO-1–negative cell contacts and divided by the number of cells, resulting in the mean ZO-1 intensity per cell. For each clone and each experiment, at least three fields of view were analyzed. Each experiment was performed at least three times. Mean values and SDs were calculated from three independent experiments.

Immunofluorescence microscopy

Immunofluorescence analyses were performed with cells grown on either fibronectin-coated chamber slides (Lab-Tek: Thermo Fisher Scientific) or on fibronectin-coated filters (0.4-µm pore size; Transwell; Corning). Cells were fixed in either 4% paraformaldehyde for 10 min followed by incubation in PBS/0.5% Triton X-100 for 15 min at RT or in ice-cold EtOH for 30 min and acetone for 3 min at RT followed by rehydration in blocking buffer (PBS/10% FCS). After blocking for 1 h, cells were incubated with primary antibodies in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01%

Tween 20, and 0.1% BSA for 1 h at RT or overnight at 4°C. After washing, cells were incubated with fluorochrome-conjugated, highly cross-adsorbed secondary antibodies for 1 h at RT. As fluorochromes, either Cy2 and Cy3 conjugates (Jackson ImmunoResearch Laboratories, Inc.) or Alexa Fluor 488 and Alexa Fluor 568 conjugates (Invitrogen) were used. After washing, cells were mounted in fluorescence mounting medium (Dako) and stored at 4°C. Nuclear staining was performed with DNA dye (DRAQ5; Biostatus, Ltd.). Immunofluorescence microscopy was performed using a confocal microscope (LSM 510 Meta; Carl Zeiss) equipped with Plan-Neofluar lenses (Plan-Neofluar: air, 20× magnification, numerical aperture 0.5; Plan-Neofluar differential interference contrast: oil, 40× magnification, numerical aperture 1.3; Carl Zeiss) or Plan-Apochromat lenses (Plan-Apochromat differential interference contrast: oil, 63× magnification, numerical aperture 1.4; Carl Zeiss).

TER

The TER of MDCK II cells was analyzed by seeding cells on fibronectin-coated polycarbonate filters (0.4-µm pore size; CoStar) in a 24-well device. After reaching confluence, cells were grown in low Ca^{2+} medium for 18 h and then subjected to a CS. TER was monitored online over a period of 60–80 h using an automated multiwell device (cellZscope; nanoAnalytics). This device was placed within the CO_2 incubator, and online recordings of the TER were taken in 15-min intervals, resulting in a smooth curve when plotted versus time over a period of 50 h. Data points are expressed as mean values of three different filters measured simultaneously.

Analysis of paracellular diffusion

The paracellular diffusion of TRITC-dextran was analyzed as described previously (Rehder et al., 2006). In brief, cells grown to confluence were subjected to a CS to induce new cell contact formation. 4 h after CS, 4.3-kD TRITC-dextran was added to the upper compartment of the filters at 1 mg/ml and allowed to diffuse for 2 h. The fluorescence in the lower compartment was analyzed using a fluorescence reader (excitation at 485 nm and emission at 530 nm; Lambda Fluoro 320; MWG Biotech). Each experiment was performed at least three times. Mean values and SDs were calculated from three independent experiments.

MDCK cyst assays

MDCK cyst assays were performed essentially as previously described (Rehder et al., 2006). In brief, MDCK cells were seeded as single-cell suspension in 0.18% rat tail type I collagen (BD). After 5–6 d, the gels were washed, treated with 100 U/ml collagenase VII (Sigma-Aldrich) for 15 min at RT, and then fixed. Cells were permeabilized by treatment with 0.25% Triton X-100/PBS (for 1 h at RT), washed with 2% goat serum/PBS (for 1 h at RT), and then incubated with primary and fluorochrome-conjugated secondary antibodies in 2% goat serum/PBS for a minimum of 12 h at 4°C each. After extensive washing, the gels were mounted on glass coverslips using Kaiser's glycerol gelatin (Merck). Cysts were analyzed using a confocal microscope as specified in the Immunofluorescence microscopy paragraph.

Freeze fracture electron microscopy

MDCK cells were fixed with 2% glutaraldehyde in PBS for 2 h at RT and then gently scraped from the culture vessels. Cells incubated in 30% glycerol for 2 h were mounted on gold-nickel carriers and immediately rapidly frozen in Freon 22 cooled with liquid nitrogen. The samples were fractured using a freeze fracture unit (BA 310; Balzers, AG) at −100°C. Replicas of the fractured cells were made by electron beam evaporation of platinum-carbon and carbon at angles of 38° and 90° and to a thickness of 2 and 20 nm, respectively. The replicas were incubated overnight in household bleach at RT to remove the cells from the replicas. The replicas were washed in distilled water, mounted on grids, and examined in a transmission electron microscope (410; Philips).

Online supplemental material

Fig. S1 shows initial characterization of JAM-A phosphorylation by aPKCζ in vitro. Fig. S2 shows the characterization of a pAb specific for S285-phosphorylated JAM-A. Fig. S3 shows that S285-phosphorylated JAM-A colocalizes with ZO-1, PAR-3, and aPKCζ at TJs. Fig. S4 shows that S285 phosphorylation of JAM-A does not influence the localization of PAR-3. Fig. S5 shows that JAM-A regulates claudin-2 expression independent of S285 phosphorylation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201104143/DC1.

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References

Ando-Akatsuka, Y., S. Yonemura, M. Itoh, M. Furuse, and S. Tsukita. 1999. Differential behavior of E-cadherin and occludin in their colocalization with ZO-1 during the establishment of epithelial cell polarity. *J. Cell. Physiol.* 179:115–125. http://dx.doi.org/10.1002/(SICI)1097-4652(199905)179:2<115::AID-JCP1>3.0.CO;2-T

Aono, S., and Y. Hirai. 2008. Phosphorylation of claudin-4 is required for tight junction formation in a human keratinocyte cell line. *Exp. Cell Res.* 314:3326–3339. http://dx.doi.org/10.1016/j.yexcr.2008.08.012

Baker, J., and D. Garrod. 1993. Epithelial cells retain junctions during mitosis. *J. Cell Sci.* 104:415–425.

Benton, R., and D. St Johnston. 2003a. A conserved oligomerization domain in *Drosophila* Bazooka/PAR-3 is important for apical localization and epithelial polarity. *Curr. Biol.* 13:1330–1334. http://dx.doi.org/10.1016/S0960-9822(03)00508-6

Benton, R., and D. St Johnston. 2003b. *Drosophila* PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. *Cell.* 115:691–704. http://dx.doi.org/10.1016/S0092-8674(03)00938-3

Boyle, W.J., P. van der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* 201:110–149. http://dx.doi.org/10.1016/0076-6879(91)01013-R

Dephoure, N., C. Zhou, J. Villén, S.A. Beausoleil, C.E. Bakalarski, S.J. Elledge, and S.P. Gygi. 2008. A quantitative atlas of mitotic phosphorylation. *Proc. Natl. Acad. Sci. USA.* 105:10762–10767. http://dx.doi.org/10.1073/pnas.0805139105

Durgan, J., N. Kaji, D. Jin, and A. Hall. 2011. Par6B and atypical PKC regulate mitotic spindle orientation during epithelial morphogenesis. *J. Biol. Chem.* 286:12461–12474. http://dx.doi.org/10.1074/jbc.M110.174235

Ebnet, K. 2008. Organization of multiprotein complexes at cell-cell junctions. *Histochem. Cell Biol.* 130:1–20. http://dx.doi.org/10.1007/s00418-008-0418-7

Ebnet, K., C.U. Schulz, M.K. Meyer Zu Brickwedde, G.G. Pendl, and D. Vestweber. 2000. Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J. Biol. Chem.* 275:27979–27988.

Ebnet, K., A. Suzuki, Y. Horikoshi, T. Hirose, M.K. Meyer Zu Brickwedde, S. Ohno, and D. Vestweber. 2001. The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). *EMBO J.* 20:3738–3748. http://dx.doi.org/10.1093/emboj/20.14.3738

Ebnet, K., M. Aurrand-Lions, A. Kuhn, F. Kiefer, S. Butz, K. Zander, M.K. Meyer zu Brickwedde, A. Suzuki, B.A. Imhof, and D. Vestweber. 2003. The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein PAR-3: a possible role for JAMs in endothelial cell polarity. *J. Cell Sci.* 116:3879–3891. http://dx.doi.org/10.1242/jcs.00704

Gauci, S., A.O. Helbig, M. Slijper, J. Krijgsveld, A.J. Heck, and S. Mohammed. 2009. Lys-N and trypsin cover complementary parts of the phosphoproteome in a refined SCX-based approach. *Anal. Chem.* 81:4493–4501. http://dx.doi.org/10.1021/ac9004309

Gumbiner, B., and K. Simons. 1986. A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide. *J. Cell Biol.* 102:457–468. http://dx.doi.org/10.1083/jcb.102.2.457

Hao, Y., Q. Du, X. Chen, Z. Zheng, J.L. Balsbaugh, S. Maitra, J. Shabanowitz, D.F. Hunt, and I.G. Macara. 2010. Par3 controls epithelial spindle orientation by aPKC-mediated phosphorylation of apical Pins. *Curr. Biol.* 20:1809–1818. http://dx.doi.org/10.1016/j.cub.2010.09.032

Hirai, T., and K. Chida. 2003. Protein kinase Czeta (PKCzeta): activation mechanisms and cellular functions. *J. Biochem.* 133:1–7. http://dx.doi.org/10.1093/jb/mvg017

Hurd, T.W., L. Gao, M.H. Roh, I.G. Macara, and B. Margolis. 2003. Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nat. Cell Biol.* 5:137–142. http://dx.doi.org/10.1038/ncb923

Hurov, J.B., J.L. Watkins, and H. Piwnica-Worms. 2004. Atypical PKC phosphorylates PAR-1 kinases to regulate localization and activity. *Curr. Biol.* 14:736–741. http://dx.doi.org/10.1016/j.cub.2004.04.007

Itoh, M., H. Sasaki, M. Furuse, H. Ozaki, T. Kita, and S. Tsukita. 2001. Junctional adhesion molecule (JAM) binds to PAR-3: a possible mechanism for the recruitment of PAR-3 to tight junctions. *J. Cell Biol.* 154:491–497. http://dx.doi.org/10.1083/jcb.200103047

Jaffe, A.B., N. Kaji, J. Durgan, and A. Hall. 2008. Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. *J. Cell Biol.* 183:625–633. http://dx.doi.org/10.1083/jcb.200807121

Joberty, G., C. Petersen, L. Gao, and I.G. Macara. 2000. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat. Cell Biol.* 2:531–539. http://dx.doi.org/10.1038/35019573

Kaneko, T., G.A. LePage, and T.K. Shnitka. 1980. KLN205—a murine lung carcinoma cell line. *In Vitro*. 16:884–892. http://dx.doi.org/10.1007/BF02619426

Kang, L.I., Y. Wang, A.T. Suckow, K.J. Czymmek, V.G. Cooke, U.P. Naik, and M.K. Duncan. 2007. Deletion of JAM-A causes morphological defects in the corneal epithelium. *Int. J. Biochem. Cell Biol.* 39:576–585. http://dx.doi.org/10.1016/j.biocel.2006.10.016

Kennelly, P.J., and E.G. Krebs. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* 266:15555–15558.

Kim, Y.H., and Y. Raphael. 2007. Cell division and maintenance of epithelial integrity in the deafened auditory epithelium. *Cell Cycle*. 6:612–619. http://dx.doi.org/10.4161/cc.6.5.3929

Laukoetter, M.G., P. Nava, W.Y. Lee, E.A. Severson, C.T. Capaldo, B.A. Babbin, I.R. Williams, M. Koval, E. Peatman, J.A. Campbell, et al. 2007. JAM-A regulates permeability and inflammation in the intestine in vivo. *J. Exp. Med.* 204:3067–3076. http://dx.doi.org/10.1084/jem.20071416

Liu, Y., A. Nusrat, F.J. Schnell, T.A. Reaves, S. Walsh, M. Pochet, and C.A. Parkos. 2000. Human junction adhesion molecule regulates tight junction resealing in epithelia. *J. Cell Sci.* 113:2363–2374.

Macara, I.G. 2004. Parsing the polarity code. *Nat. Rev. Mol. Cell Biol.* 5:220–231. http://dx.doi.org/10.1038/nrm1332

Malergue, F., F. Galland, F. Martin, P. Mansuelle, M. Aurrand-Lions, and P. Naquet. 1998. A novel immunoglobulin superfamily junctional molecule expressed by antigen presenting cells, endothelial cells and platelets. *Mol. Immunol.* 35:1111–1119. http://dx.doi.org/10.1016/S0161-5890(98)00102-3

Martìn-Padura, I., S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, et al. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J. Cell Biol.* 142:117–127. http://dx.doi.org/10.1083/jcb.142.1.117

Matter, K., and M.S. Balda. 2003a. Functional analysis of tight junctions. *Methods*. 30:228–234. http://dx.doi.org/10.1016/S1046-2023(03)00029-X

Matter, K., and M.S. Balda. 2003b. Signalling to and from tight junctions. *Nat. Rev. Mol. Cell Biol.* 4:225–236. http://dx.doi.org/10.1038/nrm1055

Mitsushima, M., F. Toyoshima, and E. Nishida. 2009. Dual role of Cdc42 in spindle orientation control of adherent cells. *Mol. Cell. Biol.* 29:2816–2827. http://dx.doi.org/10.1128/MCB.01713-08

Mizuno, K., A. Suzuki, T. Hirose, K. Kitamura, K. Kutsuzawa, M. Futaki, Y. Amano, and S. Ohno. 2003. Self-association of PAR-3-mediated by the conserved N-terminal domain contributes to the development of epithelial tight junctions. *J. Biol. Chem.* 278:31240–31250. http://dx.doi.org/10.1074/jbc.M303593200

Morais-de-Sá, E., V. Mirouse, and D. St Johnston. 2010. aPKC phosphorylation of Bazooka defines the apical/lateral border in *Drosophila* epithelial cells. *Cell*. 141:509–523. http://dx.doi.org/10.1016/j.cell.2010.02.040

Nagai-Tamai, Y., K. Mizuno, T. Hirose, A. Suzuki, and S. Ohno. 2002. Regulated protein-protein interaction between aPKC and PAR-3 plays an essential role in the polarization of epithelial cells. *Genes Cells*. 7:1161–1171. http://dx.doi.org/10.1046/j.1365-2443.2002.00590.x

Nava, P., C.T. Capaldo, S. Koch, K. Kolegraff, C.R. Rankin, A.E. Farkas, M.E. Feasel, L. Li, C. Addis, C.A. Parkos, and A. Nusrat. 2011. JAM-A regulates epithelial proliferation through Akt/β-catenin signalling. *EMBO Rep.* 12:314–320. http://dx.doi.org/10.1038/embor.2011.16

Nelson, W.J. 2003. Adaptation of core mechanisms to generate cell polarity. *Nature*. 422:766–774. http://dx.doi.org/10.1038/nature01602

Nunbhakdi-Craig, V., T. Machleidt, E. Ogris, D. Bellotto, C.L. White III, and E. Sontag. 2002. Protein phosphatase 2A associates with and regulates atypical PKC and the epithelial tight junction complex. *J. Cell Biol.* 158:967–978. http://dx.doi.org/10.1083/jcb.200206114

O'Brien, L.E., M.M. Zegers, and K.E. Mostov. 2002. Opinion: Building epithelial architecture: insights from three-dimensional culture models. *Nat. Rev. Mol. Cell Biol.* 3:531–537. http://dx.doi.org/10.1038/nrm859

Ozaki, H., K. Ishii, H. Arai, H. Horiuchi, T. Kawamoto, H. Suzuki, and T. Kita. 2000. Junctional adhesion molecule (JAM) is phosphorylated by protein kinase C upon platelet activation. *Biochem. Biophys. Res. Commun.* 276:873–878. http://dx.doi.org/10.1006/bbrc.2000.3574

Rehder, D., S. Iden, I. Nasdala, J. Wegener, M.K. Brickwedde, D. Vestweber, and K. Ebnet. 2006. Junctional adhesion molecule-a participates in the formation of apico-basal polarity through different domains. *Exp. Cell Res.* 312:3389–3403. http://dx.doi.org/10.1016/j.yexcr.2006.07.004

Reinsch, S., and E. Karsenti. 1994. Orientation of spindle axis and distribution of plasma membrane proteins during cell division in polarized MDCKII cells. *J. Cell Biol.* 126:1509–1526. http://dx.doi.org/10.1083/jcb.126.6.1509

Seth, A., P. Sheth, B.C. Elias, and R. Rao. 2007. Protein phosphatases 2A and 1 interact with occludin and negatively regulate the assembly of tight junctions in the CACO-2 cell monolayer. *J. Biol. Chem.* 282:11487–11498. http://dx.doi.org/10.1074/jbc.M610597200

Shin, K., V.C. Fogg, and B. Margolis. 2006. Tight junctions and cell polarity. *Annu. Rev. Cell Dev. Biol.* 22:207–235. http://dx.doi.org/10.1146/annurev.cellbio.22.010305.104219

Smith, C.A., K.M. Lau, Z. Rahmani, S.E. Dho, G. Brothers, Y.M. She, D.M. Berry, E. Bonneil, P. Thibault, F. Schweisguth, et al. 2007. aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. *EMBO J.* 26:468–480. http://dx.doi.org/10.1038/sj.emboj.7601495

Soler, A.P., K.V. Laughlin, and J.M. Mullin. 1993. Effects of epidermal growth factor versus phorbol ester on kidney epithelial (LLC-PK1) tight junction permeability and cell division. *Exp. Cell Res.* 207:398–406. http://dx.doi.org/10.1006/excr.1993.1207

Suzuki, A., and S. Ohno. 2006. The PAR-aPKC system: lessons in polarity. *J. Cell Sci.* 119:979–987. http://dx.doi.org/10.1242/jcs.02898

Suzuki, A., C. Ishiyama, K. Hashiba, M. Shimizu, K. Ebnet, and S. Ohno. 2002. aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization. *J. Cell Sci.* 115:3565–3573. http://dx.doi.org/10.1242/jcs.00032

Suzuki, A., M. Hirata, K. Kamimura, R. Maniwa, T. Yamanaka, K. Mizuno, M. Kishikawa, H. Hirose, Y. Amano, N. Izumi, et al. 2004. aPKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity. *Curr. Biol.* 14:1425–1435. http://dx.doi.org/10.1016/j.cub.2004.08.021

Théry, M., and M. Bornens. 2008. Get round and stiff for mitosis. *HFSP J.* 2:65–71. http://dx.doi.org/10.2976/1.2895661

Traweger, A., G. Wiggin, L. Taylor, S.A. Tate, P. Metalnikov, and T. Pawson. 2008. Protein phosphatase 1 regulates the phosphorylation state of the polarity scaffold Par-3. *Proc. Natl. Acad. Sci. USA*. 105:10402–10407. http://dx.doi.org/10.1073/pnas.0804102105

Tsukita, S., M. Furuse, and M. Itoh. 2001. Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* 2:285–293. http://dx.doi.org/10.1038/35067088

Umeda, K., J. Ikenouchi, S. Katahira-Tayama, K. Furuse, H. Sasaki, M. Nakayama, T. Matsui, S. Tsukita, M. Furuse, and S. Tsukita. 2006. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell*. 126:741–754. http://dx.doi.org/10.1016/j.cell.2006.06.043

Van Itallie, C.M., and J.M. Anderson. 2004. The molecular physiology of tight junction pores. *Physiology (Bethesda)*. 19:331–338. http://dx.doi.org/10.1152/physiol.00027.2004

van Meer, G., and K. Simons. 1986. The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* 5:1455–1464.

Wells, C.D., J.P. Fawcett, A. Traweger, Y. Yamanaka, M. Goudreault, K. Elder, S. Kulkarni, G. Gish, C. Virag, C. Lim, et al. 2006. A Rich1/Amot complex regulates the Cdc42 GTPase and apical-polarity proteins in epithelial cells. *Cell*. 125:535–548. http://dx.doi.org/10.1016/j.cell.2006.02.045

Yamanaka, T., Y. Horikoshi, Y. Sugiyama, C. Ishiyama, A. Suzuki, T. Hirose, A. Iwamatsu, A. Shinohara, and S. Ohno. 2003. Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Curr. Biol.* 13:734–743. http://dx.doi.org/10.1016/S0960-9822(03)00244-6

Yonemura, S., M. Itoh, A. Nagafuchi, and S. Tsukita. 1995. Cell-to-cell adherens junction formation and actin filament organization: similarities and differences between non-polarized fibroblasts and polarized epithelial cells. *J. Cell Sci.* 108:127–142.