

JAM-A and aPKC

A close pair during cell-cell contact maturation and tight junction formation in epithelial cells

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Cell-cell adhesion plays a critical role in the formation of barrier-forming epithelia. The molecules which mediate cell-cell adhesion frequently act as signaling molecules by recruiting and/or assembling cytoplasmic protein complexes. Junctional adhesion molecule (JAM)-A interacts with the cell polarity protein PAR-3, a member of the PAR-3-aPKC-PAR-6 complex, which regulates the formation of cell-cell contacts and the development of tight junctions (TJs). In our recent study we found that JAM-A is localized at primordial, spot-like cell-cell junctions (pAJs) in a non-phosphorylated form. After the recruitment of the PAR-aPKC complex and its activation at pAJs, aPKC phosphorylates JAM-A at Ser285 to promote the maturation of immature junctions. In polarized epithelial cells, aPKC phosphorylates JAM-A selectively at the TJs to maintain the barrier function of TJs. Thus, through mutual regulation, JAM-A and aPKC form a functional unit that regulates the establishment of barrier-forming junctions in vertebrate epithelial cells.

Introduction

Epithelial cells and endothelial cells form sheets of cells which cover organs and body cavities. These sheets represent functional barriers that separate the different compartments in the body. To develop and maintain this function, epithelial cells interact with one another through adhesive structures including the tight junctions (TJ) at the most apical area of

the cell junction, the adherens junctions (AJ) below the TJs, and the desmosomes below the AJs. Together, these structures form the epithelial junctional complex.¹ The TJs and AJs encircle the entire cell periphery and form continuous contacts with their neighbors in a belt-like manner, whereas the desmosomes are discontinuous, button-like structures. At the ultrastructural level, the TJs can be distinguished from AJs and desmosomes by the presence of membrane fusions which involve the outer leaflets of the membranes of the two adjacent cells and in which the intercellular space is basically obliterated. In contrast, in both AJs and desmosomes the membranes of neighboring cells are between 200 and 240 Å apart from each other. These two characteristics, organization in a belt-like manner and direct membrane contact, suggested that the TJs are responsible for the barrier function of epithelia and endothelia. Functional assays with tracer substances revealed that in fact the TJs represent the junctional structure which impedes the diffusion of molecules across the epithelial sheet.¹ Thus, the TJs are considered as a “seal” with two functions: they prevent the free diffusion of solutes along the paracellular pathway, which is important for the barrier function of the epithelium, and they prevent the intermixing of intramembrane particles between the apical and the basolateral membrane domain, also called fence function, which is necessary for the establishment of apico-basal membrane polarity.²

Meanwhile, a large number of proteins have been identified at the TJs: Integral

membrane proteins including members of the JAM family, proteins with four transmembrane domains like occludin, claudins and tricellulin, and the Crumbs protein homolog 3 (CRB3); peripheral membrane proteins including scaffolding proteins like members of the zonula occludens (ZO) family and cell polarity proteins like PAR-3 and PAR-6; and finally, a large number of proteins with different functions such as adaptor proteins, signaling proteins, and kinases/phosphatases.^{3,4} Despite the substantial progress that has been made in the identification of TJ-associated molecules, the molecular mechanisms that underlie the barrier and the fence function of TJs are still incompletely understood. Most interestingly, these two functions are regulated by different mechanisms. Evidence obtained from ectopic expression of claudins in fibroblasts indicates that claudins are the molecular basis of the so-called TJ strands,⁵ the sites of membrane fusions as visualized by freeze-fracture EM.⁶ Claudins regulate the paracellular diffusion by forming charge- and size-selective pores in the paracellular space.⁷ However, claudins do not seem to regulate the intramembrane diffusion of proteins and lipids, as indicated by the normal regulation of apico-basal polarity in cells lacking both claudins at cell contacts and TJ strands.⁸ The molecular basis for the fence function is still unclear.

Cell Polarity Proteins at the TJs

A major step toward the understanding of TJ formation was the identification of cell polarity proteins which had previously been found at the subapical region (SAR) of *Drosophila* epithelial cells.⁹ The SAR localizes to the most apical part of epithelial cell-cell contacts and thus correlates with the vertebrate TJs with regard to its localization along the intercellular junction.¹⁰ The two major complexes present at the *Drosophila* SAR are the Crumbs-Stardust-DPATJ complex and the Bazooka-aPKC-DPar-6 complex.¹⁰ The mammalian orthologs of both complexes (CRB3-Pals1-PATJ complex, PAR-3-aPKC-PAR-6 complex) have been identified at the TJ of epithelial cells.¹¹ RNAi-mediated downregulation

or ectopic expression of mutant proteins impairs the development of functional TJs¹²⁻¹⁹ not discriminating between the barrier and the fence function, which implicates that both complexes play a general role in TJ formation. How the two complexes regulate TJ formation has not been clarified in detail. Studies in *Drosophila* and mammalian epithelial cells have revealed a biochemical mechanism which can explain the development of membrane asymmetry. This mechanism is based on mutual antagonistic phosphorylations between the PAR-3-aPKC-PAR-6 complex and PAR-1, a Ser/Thr kinase localized at the basolateral membrane domain. Phosphorylation of Bazooka/PAR-3 by PAR-1 results in PAR-3 exclusion from the PAR-1-containing membrane domain, and vice versa, phosphorylation of PAR-1 by aPKC inhibits PAR-1 kinase activity and results in PAR-1 exclusion from the aPKC-containing membrane domain.²⁰⁻²² This mechanism can explain how a relatively sharp border can be generated between distinct membrane domains. It is unlikely that this mechanism applies to all molecules which are asymmetrically distributed among the apical and basolateral membrane domains. However, the phosphorylation of a few key components like scaffolding proteins could be sufficient to regulate the localization of many other proteins at a particular site in the membrane.

Cell-Cell Contact Localization and Activation of the PAR-3-aPKC-PAR-6 Complex

As predicted from their evolutionary conservation, cell polarity proteins have pleiotropic functions and regulate diverse processes.²³ For example, the PAR-3-aPKC-PAR-6 complex also regulates cell migration in epithelial cells.^{24,25} In different physiological situations, the PAR-3-aPKC-PAR-6 complex is recruited to specific subcellular locations. During the formation of a multicellular epithelial sheet with polarized epithelial cells from individual non-polarized mesenchymal cells, it is necessary that the PAR-3-aPKC-PAR-6 complex is recruited to sites of early cell-cell contact formation, also called puncta or primordial, spot-like AJ (pAJs),

where it is activated to regulate the development of cell-cell contacts. This specific recruitment to early sites of cell-cell adhesion is most likely mediated by adhesion molecules which localize to the pAJs and thus mark these sites as landmarks for the localization of polarity proteins.²⁶

The process of cell-cell contact formation is characterized by a sequential recruitment of proteins to sites of cell-cell adhesion. Among the first proteins are cell adhesion molecules like E-cadherin, junctional adhesion molecule (JAM)-A and nectins, but also scaffolding proteins like ZO-1, AF-6 or α -catenin, which are able to interact with the adhesion molecules.²⁷ Occludin is recruited thereafter, and after the recruitment of occludin, claudin-1 and members of the PAR-3-aPKC-PAR-6 complex appear.⁴ After its recruitment to pAJs, the PAR complex is activated by the activity of Rho family small GTPases like Rac1 and/or Cdc42. The binding of Rac1 and/or Cdc42 to PAR-6 releases aPKC from steric inhibition, and the activated aPKC promotes the maturation of the immature cell-cell contacts by phosphorylating various target proteins including PAR-1, Numb, and PAR-3 (Fig. 1). The importance of aPKC for the maturation of pAJs into linear cell-cell contacts with an apical junctional complex has been demonstrated in cells expressing a dominant-negative form of aPKC. These cells form pAJs but are unable to develop the pAJs into mature junctions.^{16,28}

JAM-A Regulates PAR-3-aPKC-PAR-6 Localization and Serves as aPKC Target During Junction Maturation

Important questions regarding the mechanism of junctional maturation after the formation of pAJs are how the PAR-3-aPKC-PAR-6 complex is recruited to the pAJs and which proteins are phosphorylated by aPKC after its activation at pAJs. The adhesion molecule JAM-A is the primary candidate to serve as anchor for the PAR-aPKC-PAR-6 complex, and our recent studies indicate that JAM-A is also among the targets of aPKC.

JAM-A has originally been identified as a cell surface receptor on platelets.²⁹ Molecular cloning revealed that JAM-A

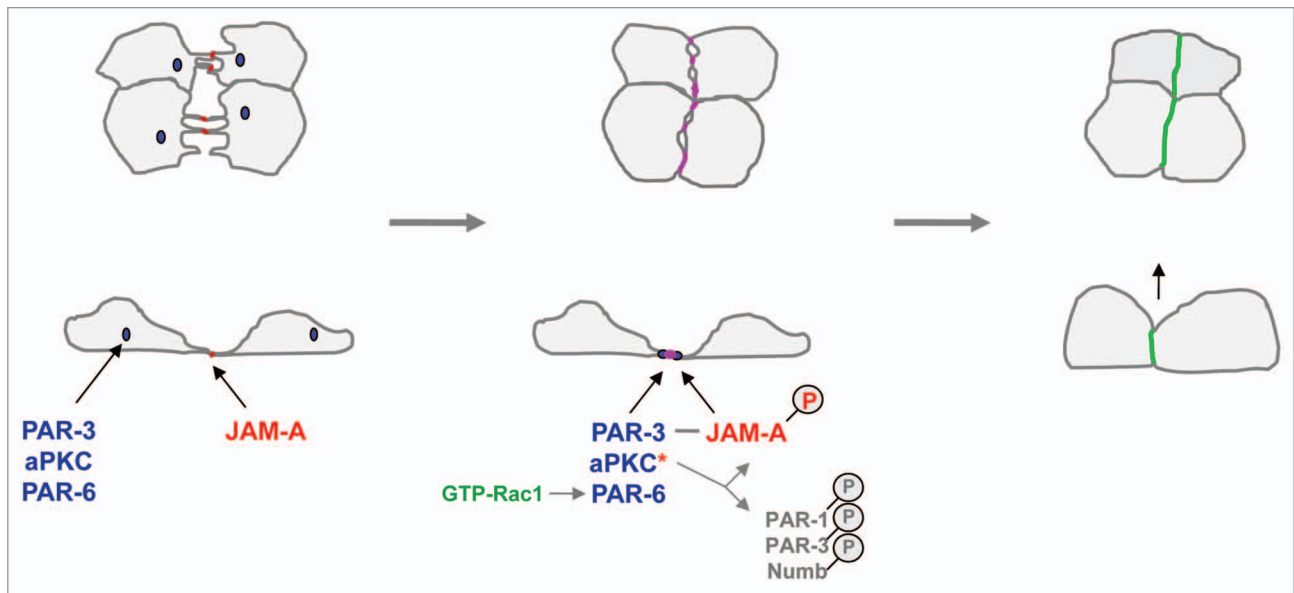


Figure 1. JAM-A regulates junctional maturation as anchor and substrate of the PAR - aPKC complex. Left panel: At early steps of junction formation, JAM-A (red) localizes to pAJs by virtue of its homophilic binding activity. At this stage, the PAR-aPKC complex (blue) localizes in the cytoplasm, and JAM-A is not phosphorylated. Middle panel: Through its PDZ domain-binding motif JAM-A interacts with PAR-3 and promotes the localization of a PAR-3-aPKC-PAR-6 complex at pAJs. Rho GTPases present at pAJs and probably activated by E-cadherin homophilic binding interact with PAR-6 allowing aPKC activation. Active aPKC (aPKC*) phosphorylates various polarity proteins like PAR-1, PAR-3, or Numb, but also JAM-A. Right panel: The phosphorylation of the cell polarity proteins PAR-1, PAR-3, and Numb regulates the formation of specific membrane domains. Ser285-phosphorylated JAM-A promotes the maturation of junction through as yet unknown mechanisms.

is expressed by a variety of cell types including epithelial and endothelial cells where it is localized at cell-cell contacts with enrichment at the TJs.^{30,31} As mentioned before, JAM-A co-localizes with E-cadherin and ZO-1 at pAJs.³² JAM-A dimerizes in cis, and cis-dimerization is required for trans-homophilic interaction.^{33,34} The trans-homophilic binding activity of JAM-A is most likely of particular importance to localize JAM-A at pAJs where only very few molecules are present which could serve as anchoring structure for other molecules. Most importantly, JAM-A contains a PDZ domain-binding motif at its C-terminus (-FLV) which directly interacts with PAR-3.^{32,35} Thus, JAM-A fulfills the criteria required for targeting the PAR-3-aPKC-PAR-6 to pAJs. In line with a role in recruiting PAR-3 to cell contacts, cells expressing of JAM-A mutants that are unable to localize at cell-cell contacts fail to develop functional TJs and normal apico-basal polarity.³⁶

Once the PAR-3-aPKC-PAR-6 complex has been recruited to pAJs and activated by Rho family small GTPases, aPKC regulates the maturation of pAJs by phosphorylating target proteins. The

close proximity of PAR-3-associated aPKC with JAM-A prompted us to test if JAM-A is phosphorylated by aPKC. Phospho-chymotryptic peptide mapping experiments revealed that JAM-A is phosphorylated in vitro by aPKC ζ with high specificity for Ser285.³⁷ A polyclonal antibody directed against Ser285-phosphorylated JAM-A allowed us to localize Ser285-phosphorylated JAM-A in cells. In scratch-wounding assays, we found that JAM-A at pAJs is not phosphorylated at Ser285. However, when pAJs have matured into more linear cell-cell contacts, JAM-A becomes phosphorylated at Ser285, and this correlates with the appearance of aPKC ζ . Inhibition of aPKC ζ using a pseudosubstrate inhibitor (PS ζ) prevents JAM-A phosphorylation at Ser285. Importantly, expression of a phosphorylation-deficient JAM-A mutant (JAM-A/S285A) impairs the maturation of cell-cell contacts, indicating that the phosphorylation of JAM-A at Ser285 by aPKC ζ is required for junction maturation. Our observations suggest that JAM-A present at pAJs serves as spatial cue for the correct localization of the PAR-3-aPKC-PAR-6 complex, and

that once the complex has been recruited and activated at pAJs, JAM-A serves as a substrate through which aPKC ζ promotes junctional maturation (Fig. 2). As a next step, it will be important to understand how JAM-A phosphorylation promotes junctional maturation. We hypothesize that the phosphorylated Ser285 residues present new binding sites for proteins that are required at nascent cell contacts to promote their further maturation.

JAM-A Phosphorylation at Ser285 Regulates the Barrier Function of Epithelial Monolayers

The second striking observation we made is that JAM-A, despite its localization along the entire intercellular junction of fully polarized epithelial cells, is Ser285-phosphorylated exclusively at the TJs. At the TJs, JAM-A co-localizes with aPKC ζ , and siRNA-mediated aPKC ζ downregulation prevents JAM-A phosphorylation, which indicates that aPKC ζ is also responsible for JAM-A Ser285 phosphorylation within the TJs. Phospho-deficient JAM-A mutants still localize to the TJs, arguing against a role of S285 phosphorylation in

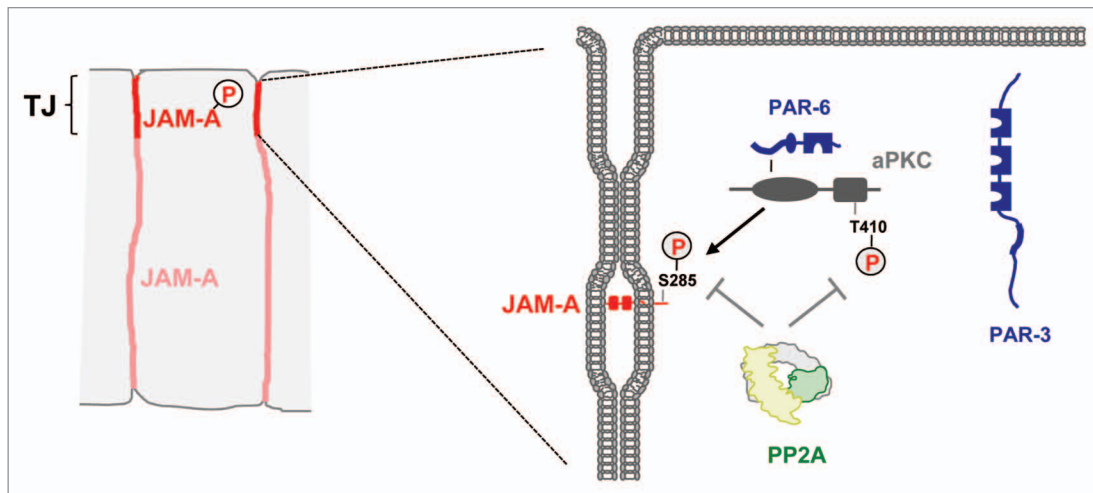


Figure 2. JAM-A is Ser285-phosphorylated exclusively at the TJs. In fully polarized epithelial cells, JAM-A is localized along the entire cell junction and enriched at the TJs. Importantly, Ser285 phosphorylation is restricted to the JAM-A population localized at the TJs. At the TJs, JAM-A is phosphorylated at Ser285 by aPKC ζ . The association of PAR-3 is not necessarily required for JAM-A to be phosphorylated by aPKC ζ since JAM-A can directly interact with aPKC ζ . PP2A antagonizes JAM-A Ser285 phosphorylation in two ways, indirectly by dephosphorylating aPKC ζ at the regulatory Thr410 residue, and directly by dephosphorylating JAM-A at Ser285.

regulating the specific localization at the TJs. Also, JAM-A/S285A-expressing cells do develop TJ strands. However, the ability of cells to prevent the free diffusion of ions and small molecular weight tracers is severely impaired in cells expressing the JAM-A/S285A mutant, which indicates that JAM-A phosphorylation at Ser285 is necessary for the development of a functional barrier. Surprisingly, the fence function does not seem to be regulated by JAM-A S285 phosphorylation, because JAM-A/S285A-expressing cells are still able to develop apico-basal polarity when grown in a three-dimensional collagen matrix. From these observations we conclude that aPKC ζ phosphorylates JAM-A at Ser285 not only during early stages of cell-cell contact formation to trigger their maturation, but also when the junction maturation process is complete, to regulate the barrier function of TJs.

As to the molecular mechanisms underlying the phosphorylation of JAM-A, we made two observations: First, at the TJs JAM-A co-localizes and interacts with protein phosphatase 2A (PP2A), which had been previously identified as TJ component.³⁸ We found that PP2A dephosphorylates the Ser285 residue of JAM-A *in vitro*³⁷ suggesting that PP2A antagonizes the activity of aPKC ζ in JAM-A S285 phosphorylation at the TJs. Interestingly, among the substrates

for PP2A is aPKC ζ itself,³⁸ and PP2A dephosphorylates aPKC at the regulatory Thr410 residue. PP2A thus can affect the levels of JAM-A Ser285 phosphorylation by directly dephosphorylating Ser285 of JAM-A, and indirectly by inhibiting the activity of aPKC ζ . Second, the interaction between JAM-A and aPKC ζ can be direct. Recombinant aPKC ζ binds to JAM-A *in vitro*, and this interaction is similarly strong as the interaction of recombinant aPKC ζ with PAR-6. In addition, aPKC ζ interacts with JAM-A deletion mutants which lack the PDZ domain-binding motif necessary for PAR-3 binding. These findings indicate that the phosphorylation of JAM-A by aPKC ζ does not require PAR-3 as scaffolding protein. This is an important observation inasmuch as it has been described that after its activation by small GTPases, PAR-3 dissociates from aPKC - PAR-6 as consequence of Ser827 phosphorylation by aPKC.³⁹ In the *Drosophila* PAR-3 homolog Bazooka this serine residue is conserved (Ser980), and aPKC-mediated phosphorylation of Ser980 excludes Bazooka from the apical membrane, confirming that PAR-3 phosphorylation by aPKC separates Bazooka/PAR-3 from aPKC - PAR-6.⁴⁰ It is unclear if JAM-A localized at the TJs is directly associated with PAR-3. The ability of aPKC ζ to interact with JAM-A directly makes PAR-3 dispensable for a continuous

Ser285 phosphorylation of JAM-A at the TJs.

Summary and Conclusions

Our previous and recent observations indicate that the role of JAM-A during the formation of cell-cell contacts is due to its intimate link with the PAR-3-aPKC-PAR-6 complex. We propose the following model. In single, contact-naïve cells, JAM-A is not stably localized at the cell surface, and the PAR-3-aPKC-PAR-6 complex is localized in the cytoplasm. Upon formation of pAJs, JAM-A is stabilized at these sites due to its homophilic binding activity resulting in a high local JAM-A concentration. At this stage, JAM-A is not phosphorylated at Ser285. In a subsequent step, PAR-3 is recruited to pAJs, most likely by a direct association with JAM-A, followed by the recruitment of PAR-6 and aPKC, which results in the formation of the PAR-3-aPKC-PAR-6 complex. This complex is still inactive. Rho family small GTPases like Rac1, which are activated at pAJs in response to E-cadherin trans-homophilic interaction,^{41,42} bind to PAR-6 and release aPKC from inhibitory constraints. Active aPKC phosphorylates various peripheral membrane proteins including PAR-3, Numb and PAR-1, but also the integral membrane protein JAM-A. Proteins which

co-localize at pAJs gradually separate from each other, specific membrane domains begin to form, and eventually TJ and AJs can be identified as structural subunits of the apical junctional complex. We speculate that S285-phosphorylated JAM-A regulates the recruitment of proteins which are specifically required for the development of a TJ-containing membrane subdomain. The identification of

JAM-A/Ser285-P-associated proteins will thus be clue to the understanding of the mechanism by which aPKC ζ -mediated phosphorylation of JAM-A regulates the maturation of immature cell junctions and the formation of a functional tissue barrier.

Disclosure of Potential Conflicts of interest

The author declares that there is no conflict of interest.

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