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# Regulation of epithelial and endothelial junctions by PAR proteins

**Klaus Ebnet[1], Sandra Iden[1], Volker Gerke[1], Atsushi Suzuki[2]**

*[1]Institute of Medical Biochemistry, ZMBE, University of Muenster, D-48149 Muenster, Germany, and [2]Department of Molecular Biology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan*

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## 1. ABSTRACT

The organization of tissues depends on intercellular junctions that connect individual cells to each other. In sheets of epithelial cells the junctions contain different components like adherens junctions or tight junctions in an asymmetric distribution along the cell-cell contacts. Tight junctions are located at the most apical region of cell junctions, act as a regulatable barrier for small solutes, and separate the apical membrane domain from the basolateral membrane domain. For a long time, the mechanisms that underly the formation of tight junctions and the development of apico-basal membrane polarity in epithelial cells have been poorly understood. Recently, strong evidence has been provided which implicates a conserved set of cell polarity proteins - the PAR proteins - in this process. Here we discuss the mechanisms by which PAR proteins regulate the formation of cell junctions with a special emphasis on vertebrate epithelial cells.

## 2. CELL POLARITY AND EPITHELIAL CELL-CELL CONTACTS

Cell polarity describes a structural asymmetry in the cell. Most cells undergo polarization, either transiently or stably, and the ability to polarize is critical to the functioning of the cell. For example, epithelial cell polarity is in part reflected by the presence of distinct membrane domains, a free membrane which faces the lumen of, for example, the intestine (apical domain), and the basolateral membrane facing neighbouring cells or the extracellular matrix (basolateral domain) (1). These domains differ in protein composition as the apical domain needs to harbor transporters for the uptake of nutrients and the basolateral domain needs to contain adhesion molecules to mediate cell-cell and cell-matrix contacts. An inability to generate or maintain this structural membrane asymmetry would impair the proper functioning of the organ.

The sites of cell-cell adhesion are organized in specialized intercellular junctional complexes including tight junctions (TJs), adherens junctions (AJs), gap junctions, and desmosomes. In polarized epithelial cells, TJs are localized at the most apical region of the lateral cell-cell contacts where they appear to form membrane fusions which involve the outer membrane leaflets of two adjacent cells (2). Two principal functions are attributed to the TJs: first, the creation of a selective barrier to diffusion along the paracellular space, and second, the formation of an intramembrane "fence" that prevents the lateral diffusion of intramembrane molecules and the intermixing of the apical and basolateral membrane domains. These separate functions highlight the role of the TJs for the individual cell as well as for the entire epithelial sheet.

During the last ten years a major progress has been made in understanding some of the basic molecular mechanisms that regulate the formation and the function of TJs as well as the development of apico-basal polarity in vertebrate epithelial cells. With the identification of the claudin family of integral membrane proteins it became clear that claudins are the molecular basis of the TJ strands and that they are responsible for the formation of ion- and size-selective aqueous pores thus constituting the barrier function (3, 4). The identification of various signaling molecules at TJs has revealed that TJs act as signaling centers which influence cell proliferation and differentiation (5). Finally, although a role for TJs in epithelial cell polarity has been appreciated since quite some time (6), the identification of members of the partitioning-defective (PAR) protein family at the TJs (7, 8) has provided a first clue on the mechanisms that regulate the development of TJs and apico-basal polarity in vertebrate epithelial cells. Here we will review the role of PAR proteins during cell-cell contact formation with a special emphasis on epithelial cell-cell contacts.

## 3. PAR POLARITY PROTEINS

### 3.1. The PAR - aPKC system as a general protein machinery for the establishment of cell polarity

The PAR proteins were firstly identified in *C.elegans* by genetic screens for mutants in which the asymmetric distribution of P granules and the asymmetric cleavage plane specification induced in the egg upon fertilization by the sperm were disrupted (9). The molecular characterization of *par* genes revealed that the encoded proteins differ in their structures and functions and include scaffolding/adaptor proteins with several protein-protein interaction domains (PAR-3, PAR-6), serine/threonine kinases (PAR-1, PAR-4), a protein containing a RING finger domain typical for E3 ubiquitin ligases (PAR-2) and a member of the 14-3-3 family of signaling proteins (PAR-5) (10, 11). The generation of antibodies for each PAR protein revealed that some of them localize asymmetrically in the *C.elegans* one-cell embryo: PAR-3 and PAR-6 localize at the anterior cortex, whereas PAR-1 and PAR-2 localize to the posterior cortex. PAR-4 and PAR-5 are distributed evenly in the cytoplasm (12). Interestingly, the analysis of PAR-protein distribution in a *par* mutant background indicated a hierarchy of their subcellular localization: PAR-3 and PAR-6 begin to localize at the anterior pole at first in response to a polarity cue, i.e. sperm entry, and direct the localization of PAR-1 and PAR-2 to the posterior pole. Simultaneously, feedbacks from PAR-1 and PAR-2 restrict the localization of PAR-3 and PAR-6. Now it is established that the resultant interdependence between the anterior PARs and the posterior PARs is the molecular basis for the formation of asymmetric membrane domains along the anterior-posterior axis. With the exception of PAR-2, all PAR proteins exist in *Drosophila* and mammals as well, and as we will see below, this mechanism turned out to be conserved and to account for the formation of distinct membrane domains in epithelial cells of *Drosophila* and vertebrates (9). The observation of a direct interaction of a mammalian serine/threonine kinase, atypical Protein Kinase C (aPKC), with the mammalian orthologue of PAR-3, originally named Atypical PKC Isotype-Specific Interacting Protein (ASIP) (7), has lead to the identification of an aPKC orthologue in *C.elegans*, named PKC-3 (13). In a similar way like aPKC in mammalian epithelial cells, PKC-3 cooperates with PAR-3 and PAR-6 to establish asymmetry in the one-cell stage of the *C.elegans* embryo. Very excitingly, the subsequent studies revealed that the physical as well as functional interactions between the PAR - aPKC system components were essentially conserved in other species such as *Drosophila* and mammals, and that the PAR system is used by different cell types and in different contexts to establish cell polarity (14-16). At this stage, the present concept of the PAR - aPKC system as a general polarity machinery was established.

### 3.2. PAR polarity proteins in epithelial cells

The essential importance of the PAR-aPKC system in epithelial polarity has been mainly revealed in *Drosophila* and mammals. In epithelial cells, the PAR proteins show a similar mutually exclusive localization as in the *C.elegans* zygote. Epithelial cells have two major membrane domains, one which is in contact with other cells or with the extracellular matrix, the basolateral domain, and one which is free of contact and normally facing the lumen of an organ, the apical domain. Proteins localized at the posterior pole in the *C.elegans* zygote (PAR-1) localize to the basolateral domain, proteins at the anterior pole (aPKC, PAR-3, PAR-6) localize apically of the basolateral domain. However, one interesting difference exists. The apical PAR complex is not evenly distributed along the apical membrane domain but is concentrated at the boundary between the basolateral and apical membrane domain. In polarized epithelial cells, this region harbors a specialized structure, the apical junctional complex (1) which contains TJ and AJ in vertebrates or the subapical region (SAR) and AJ in *Drosophila* (17). As described in more detail below, antagonistic interactions between the apical PARs and the basolateral PARs probably occur within this boundary. Studies utilizing dominant-negative mutants and RNAi-mediated knockdown approaches have established that one of the essential functions of the apical PAR complex (the PAR-3 - aPKC - PAR-6 complex) is to promote the development of TJs in response to the formation of initial cell-cell contacts whereas the basolateral PAR complex (PAR-1) functions to promote the expansion of the lateral membrane.

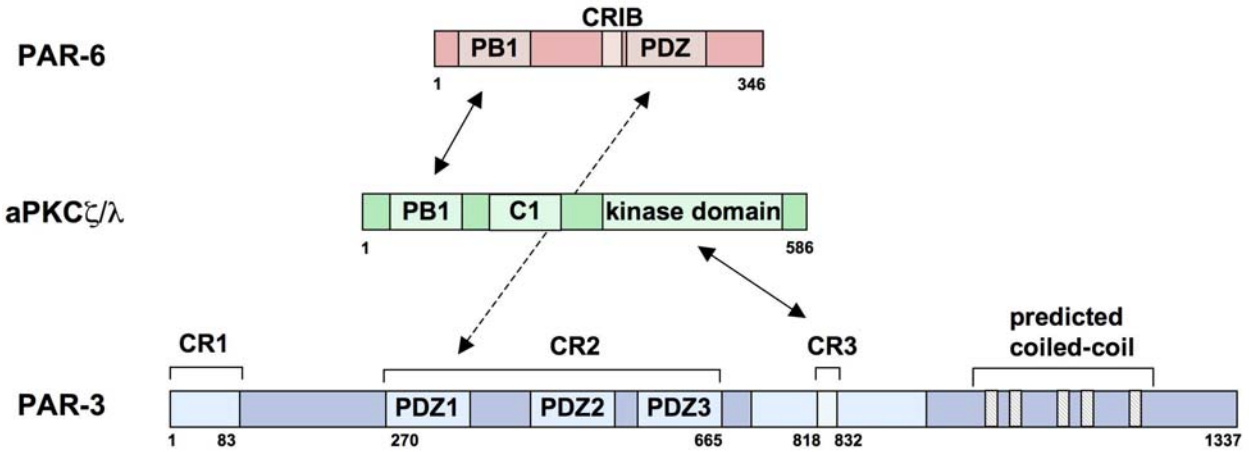


**Figure 1.** PAR-6 contains three functional domains, a PB1 (Phox and Bem1p) domain that mediates dimerization/oligomerization (147), a semi-CRIB (Cdc42/Rac1 interactive binding) domain which presents the docking site for active small GTPases (148), and a PDZ (PSD95, Dlg1, ZO-1) domain, a protein-protein interaction module (149). aPKC contains a PB1 domain, a single C1 domain which mediating the binding of diacylglycerol and phorbolesters, and the kinase domain. PAR-3 contains three PDZ domains and a region with several predicted coiled-coil domains. The conserved regions (CR) 1 to 3 are conserved between different species. Both PAR-3 and PAR-6 directly associate with aPKC through distinct domains in aPKC allowing the formation of the ternary complex. The interaction between PAR-6 and aPKC is mediated through a heterotypic PB1 - PB1 domain interaction, the interaction between PAR-3 and aPKC is mediated through the CR3 of PAR-3 and the kinase domain of aPKC. A direct interaction between PAR-6 and PAR-3 has also been described which involves the single PDZ domain of PAR-6 and PDZ domain 1 of PAR-3. The binding of active small GTPases to the semi-CRIB domain of PAR-6 activates aPKC, probably by inducing a conformational change in PAR-6.

## 4. THE PAR-3 - aPKC - PAR-6 SYSTEM IN MAMMALIAN EPITHELIAL CELLS

### 4.1. The PAR-3 - aPKC - PAR-6 complex

In vertebrates, each member of the PAR-3 - aPKC - PAR-6 complex exists in several isoforms. For PAR-6, three different proteins each encoded by a separate gene (PAR-6A, -B, -C) and one partial cDNA sequence (PAR-6D) were identified (8); for PAR-3, two genes were identified (PAR-3, PAR-3L/-beta) (18, 19), and two different aPKC genes exist (aPKClambda/iota, aPKCzeta) (20, 21). Biochemical studies have demonstrated that PAR-3 and PAR-6 form a ternary complex with aPKC, even though the formation of this ternary complex is not obligatory since several immunofluorescent studies showed that PAR-6 can co-localize with aPKC independently of PAR-3 (22-25). However, during epithelial cell-cell contact formation PAR-3, aPKC and PAR-6 function as a ternary complex with specific contributions of each molecule. In the PAR-3 - aPKC - PAR-6 complex, PAR-3 and PAR-6 associate both directly with aPKC. The interaction of PAR-6 with aPKC is mediated by a heterotypic PB1-PB1 domain interaction, the interaction of PAR-3 with aPKC by the CR3 domain of PAR-3 and the kinase domain of aPKC (16) (Figure 1). In addition, a direct interaction between PAR-3 and PAR-6 has been described by *in vitro* binding experiments (26). If this direct association is relevant for the formation of the ternary complex *in vivo* is unclear as PAR-3 with a deletion in its aPKC-binding site cannot be detected in PAR-6 immunoprecipitates (27). The binding of active small GTPases like Cdc42 or Rac1 to the CRIB domain of PAR-6 activates the ternary complex, probably by inducing a conformational change of PAR-6 which allows aPKC to become active (28). Active aPKC promotes TJ and cell polarity formation (27, 29). Interestingly, aPKC phosphorylates Ser827 of PAR-3 within the conserved aPKC-binding region and reduces thereby the affinity of PAR-3 to aPKC (30) suggesting that the composition of the complex changes upon aPKC activation.

### 4.2. The PAR-3 - aPKC - PAR-6 complex is essential for the development of TJs in mammalian epithelial cells

TJs contain various integral membrane proteins as well as a multitude of peripheral membrane proteins (5, 31). The latter proteins can be classified into scaffolding/adaptor proteins, regulatory proteins like small GTPases, G-proteins, kinases and phosphatases, and transcription factors or factors regulating RNA processing. Scaffolding proteins interact with transmembrane proteins and organize the assembly of multiprotein complexes. At the TJs, three major protein complexes exist which involve one or several scaffolding proteins: the ZO-1 - ZO-2 - ZO-3 complex (ZO complex), the CRB3 - Pals1 - PATJ complex, and the PAR-3 - aPKC - PAR-6 complex. Some scaffolding proteins also serve to link the transmembrane proteins to the cytoskeleton as exemplified by the ZO complex. All three ZO proteins ZO-1, ZO-2, and ZO-3 interact directly with various integral membrane proteins and also with F-actin, the ZO complex forms the major link to the actin cytoskeleton at TJs. Another major function of the ZO protein complex is probably to form a physical scaffold for the strand-forming proteins claudins and occludin. In agreement with this, epithelial cells which lack all three ZOs lack other TJ proteins like occludin, claudin-3 and

JAM-A, do not develop TJ strands, and completely loose their barrier function (32). Surprisingly, these cells still develop normal apico-basal membrane polarity as indicated by the asymmetric distribution of the apical and basolateral markers syntaxin and E-cadherin, respectively, indicating that the inhibition of free intramembrane diffusion is not dependent on TJ strands. The molecular nature of the intramembrane diffusion barrier is unclear. Besides its role as a scaffold for the structural organization of TJs, the ZO proteins also form a platform for signaling proteins to regulate epithelial proliferation and morphogenesis (5).

The second scaffolding protein complex consists of CRB3, which is one of three vertebrate homologues of *Drosophila* Crumbs, Protein Associated with Lin7 1 (Pals1) and Pals1-Associated Tight Junction protein (PATJ). CRB3 is a transmembrane protein, Pals1 and PATJ are cytoplasmic scaffolding proteins with several protein-protein interaction domains like L27 domains, SH3 domains, GuK domains and PDZ domains. In vertebrate epithelial cells, CRB3 localizes to the apical membrane but is concentrated at TJs where it directly interacts with Pals1 that associates with PATJ (31). Overexpression or siRNA-mediated downregulation of any of the three components leads to defects in TJ and apico-basal polarity formation (33-39) suggesting the requirement of the complex as a whole for the proper development of cell polarity. In *Drosophila*, Crumbs is an apical membrane determinant and overexpression results in an expansion of the apical membrane domain (40). Ectopic expression of CRB3 in human MCF10A epithelial cells which express little endogenous CRB3 induces the formation of functional TJs in these cells (33) suggesting an active role of CRB3 in promoting TJ formation.

The PAR-3 - aPKC - PAR-6 complex is the third scaffolding protein complex at TJs. After the identification of aPKC and PAR-3 at TJs (7) and the description of the PAR-3 - aPKC - PAR-6 complex in vertebrates (8, 26), the role of this complex in TJ formation and organization has been addressed in numerous studies. These studies mostly made use of $Ca^{2+}$-depletion to disrupt cadherin-dependent cell-cell adhesion followed by $Ca^{2+}$-repletion ($Ca^{2+}$-switch) to induce new cell-cell contact formation (41). Overexpression of a PAR-3 mutant with a mutation at Ser827 to prevent PAR-3 phosphorylation by aPKC suppresses the relocalization of ZO-1 to cell-cell contacts after $Ca^{2+}$-switch (30). Similarly, overexpression of a kinase-deficient mutant of aPKClambda prevents the relocalization of PAR-3 and ZO-1 after $Ca^{2+}$-switch and strongly impairs the formation of functional TJs as indicated by very low transepithelial electrical resistance (TER) and a strong increase in paracellular permeability (27, 29, 30). Finally, overexpression of a PAR-6 mutant which lacks the PB1 domain required for aPKC binding prevents the development of functional TJs and results in mislocalization of ZO-1, PAR-3, claudin-1 and occludin (28, 42). In complementary approaches, components of the PAR-3 - aPKC - PAR-6 were downregulated by expressing short hairpin RNAs. PAR-3 knockdown severely disrupts TJ formation as indicated by a loss of ZO-1 from cell-cell contacts and a delayed development of TER (43-45). These observation cleary indicate a critical role for the PAR-3 - aPKC - PAR-6 complex in the development of functional TJs.

Importantly, most experiments with dominant-negative mutants were performed in cells subjected to $Ca^{2+}$-switch or at low confluency when cells are still in the process of developing TJs. When the same experiments were performed with confluent cells with fully developed TJs, the overepression of the same mutants had only negligible effects (27, 30). These observations suggest that the activity of the PAR-3 - aPKC - PAR-6 complex is particularly important during early phases of junction formation, and this is further supported by the observation that aPKCkn-overxpressing cells develop primordial spot-like AJs but cannot develop mature, functional TJs (29). Once the formation of stable cell-cell contacts has been completed, the activity of the complex might be less important. A critical question is therefore how the complex is correctly recruited to early sites of cell adhesion and how it is activated.

### 4.3. The recruitment of the PAR-3 - aPKC - PAR-6 complex to early sites of cell adhesion

The process of cell-cell contact formation and cellular polarization has been studied by re-plating trypsinized cells at low densities and in wound-healing assays (46-48). These studies revealed that cells form thin protrusions filled by axial actin filaments which upon encountering protrusions of other cells form multiple transient contacts that are subsequently stabilized (49). The first sites of cell-cell contact formation are called "primordial, spot-like AJs" (pAJs) or "puncta" (46, 47). The formation of multiple pAJs between the protrusions of adjacent cells results in a zipper-like appearance of the early cell contact sites (49). Upon maturation of cell-cell contacts, the pAJs gradually fuse to form a linear contact region. Probably simultaneously, the cells start to polarize and develop cell-cell contacts in the vertical direction. During polarization, the lateral contacts gradually expand, and proteins typically localized at TJs separate from membrane domains containing AJ proteins.

Molecules which are localized at cell-cell contacts do not appear simultaneously but rather sequentially during cell-cell contact formation. The first set of molecules, characterized by the localization at pAJs, consists not only of typical AJ-associated molecules like E-cadherin, alpha-catenin, beta-catenin, nectin-2, AF-6/afadin and ponsin but also of typical TJ-associated proteins like ZO-1 and JAM-A (29, 46, 47, 50, 51). After the first set of molecules has been localized and when the pAJs fuse to form a more linear arrangement of cell-cell contacts, occludin is recruited (29, 48). Then, as a third set, claudin-1, PAR-3 and aPKClambda appear (29). This sequential recruitment of different sets of molecules probably reflects a step-wise regulatory mechanism which organizes the specific localization of these molecules at cell-cell contact sites. For example, it is conceivable that early sites of cell adhesion mediated by E-cadherin provide a landmark for the localization of other proteins like PAR proteins or occludin required for the further maturation of cell-cell

contacts (52). Once these are recruited, they need to be locally activated which is probably mediated by cell adhesion-mediated signals. When activated, they start to regulate the formation of membrane asymmetry along the nascent lateral membrane which finally leads to the formation of distinct membrane domains harboring TJs and AJs. The stepwise recruitment ensures the correct temporal and spatial activation of the polarity-promoting proteins.

It is still not completely understood if the PAR-3 - aPKC - PAR-6 complex is recruited as entire complex or if the components appear individually. Indirect evidence suggests that PAR-3 and aPKC are recruited with different kinetics and that aPKC is recruited later than PAR-3 (29). Since in most cases aPKC is complexed with PAR-6 it is likely that PAR-3 is recruited first and that aPKC and PAR-6 are recruited by PAR-3. A recent observation in keratinocytes supports this notion (53). Two integral membrane proteins with the potential to directly bind and recruit PAR-3 exist at pAJs, JAM-A and nectin-2, suggesting that they target PAR-3 to pAJs (51, 54). The overexpression of a dominant-negative JAM-A mutant as well as JAM-A knockdown during cell-cell contact formation results in mislocalization of PAR-3 and various other TJ components as well as in severe defects in TJ and cell polarity formation (51, 55) underscoring the importance of JAM-A. Once PAR-3 has been recruited to early cell contacts it might serve as scaffold for the subsequent recruitment of PAR-6 and aPKC which are probably recruited as a dimeric complex resulting in the formation of a ternary PAR-3 - aPKC - PAR-6 complex.

### 4.4. Localized activation of the PAR-3 - aPKC - PAR-6 complex at cell-cell contacts

The second critical event after the correct targeting of the PAR-3 - aPKC - PAR-6 complex to pAJs is its activation. This is most likely achieved by Rho family small GTPases. As pointed out earlier, Cdc42, Rac1 and TC10 associate in their active forms with PAR-6 (8, 26, 56-58). Furthermore, the binding of GTP-bound Cdc42 and Rac1 has been shown to lead to an activation of aPKC (28, 58-60). Besides their ability to bind to PAR-6 and to activate aPKC both Cdc42 and Rac1 have also been shown to be present at pAJs and to be activated upon initial cell-cell contact formation. The activation of the small GTPases is probably mediated by cell adhesion molecules present at pAJs. Several lines of evidence exist which support the recruitment and activation of Rac1 but not Cdc42 by E-cadherin-mediated cell adhesion (61-64). Tracking experiments using GFP-labeled Rac1 expressed in MDCK cells showed that Rac1 localizes to the initial sites of cell-cell contact formation with a concomitant loss of Rac1 from non-contacting cell surfaces (65). In addition, during contact maturation, high Rac1 levels can be observed at less mature sites whereas lower Rac1 levels are observed at more mature cell-cell contact sites with the presence of active Rac1 correlating with immature contact sites (65, 66). Finally, the Rac1-specific GEF Tiam1 directly associates with PAR-3 (43, 67). Although the role of Tiam1 in the activation is still unclear and might be cell context-dependent (43, 67), this would allow for a spatially restricted activation of Rac1 associated with the PAR-3 - aPKC - PAR-6 complex. Besides E-cadherin, nectins may also provide signals leading to the activation of both Cdc42 and Rac1. Several studies applying ectopic expression of nectins demonstrate that the trans-homophilic interaction of nectins induces the formation of filopodia and lamellipodia and the activation of Cdc42 and Rac1 (68-72). Taken together, these studies suggest that Cdc42 and Rac1 which are recruited to pAJs and activated in response to E-cadherin- and nectin-mediated cell adhesion are responsible for the localized activation of the PAR-3 - aPKC - PAR-6 complex.

The data presently available fit the following model. Initial transient contacts between membranes of opposing cells are stabilized by E-cadherin-mediated adhesion. Concomitantly with E-cadherin, cytoplasmic scaffolding molecules like ZO-1 or AF-6/afadin and the cell adhesion molecules JAM-A and nectin-2 localize at pAJs. This first set of molecules present at pAJs probably serves as landmarks for the recruitment of additional cytoplasmic molecules. For example, ZO-1 could recruit occludin that might provide signals for TJ formation (48, 73). ZO-1 probably also serves to localize claudins which appear later than occludin at nascent cell-cell contacts (29, 74). PAR-3 can localize to the still immature contacts through its interaction with JAM-A and nectin-2, and - although only indirect evidence exists for this - PAR-3 might then recruit PAR-6 - aPKC (29). The first set of molecules may also serve as activators of Rho family GTPases. The activation of Rac1 through E-cadherin and nectins in concert with PAR-3-associated Tiam1 and of Cdc42 through nectin-2 might then regulate a local activation of aPKC. Through the phosphorylation of downstream targets like PAR-1, Lgl, and probably additional unknown substrates, aPKC regulates the formation of distinct membrane domains as a prerequisite for the development of apico-basal polarity.

Clearly, additional mechanisms that are independent of the PAR complex act to regulate TJ formation and function. For example, phosphorylation and dephosphorylation of TJ proteins regulate their localization at the TJs. Occludin localization is regulated by phosphorylation on serine residues by cPKC or nPKC isoforms and by dephosphorylation on threonine residues by PP2A and PP1 (75-77). Similarly, the localization of various claudins at the TJs has been demonstrated to be regulated both positively and negatively by Ser/Thr phosphorylation (78, 79). Another mechanism involved in TJ regulation is the modulation of the levels of TJ proteins. The TJ-associated adaptor protein cingulin regulates the expression of structural components of TJs like occludin, ZO-1, ZO-3, and claudin-2 (80, 81). Finally, Rho small GTPases different from the PAR complex-associated Cdc42 and Rac1 regulate TJ function. RhoA activity which is downregulated when cells reach confluence (82, 83) seems to persist at low levels at TJs, and this activity probably is required to regulate specific aspects of TJ function. Overexpression or downregulation of the RhoA guanine nucleotide exchange factor GEF-H1/Lcf increases or decreases, respectively, the permeability of epithelial cells for small hydrophilic tracers with no change in TER

(84) suggesting a specific regulation of the paracellular permeability for small molecules but not ions by RhoA. Besides this function, RhoA also seems to be required to maintain a polarized morphology as TGFbeta signaling which is known to result in epithelial-mesenchymal transition (85) has been described to induce RhoA degradation at TJs through a occludin - PAR-6 - Smurf1-dependent pathway (86, 87).

## 5. COOPERATION OF THE PAR-3 - aPKC - PAR-6 COMPLEX WITH OTHER POLARITY PROTEIN COMPLEXES AT CELL-CELL CONTACTS

Besides the PAR-3 - aPKC - PAR-6 and the CRB3 - Pals1 - PATJ protein complexes, one additional multiprotein complex localizes to cell-cell contacts of epithelial cells which is also involved in the regulation of TJs and apico-basal polarity and which also show an asymmetric distribution, the Scribble (Scrib), Discs Large (Dlg), and Lethal Giant Larvae (Lgl) complex. In mammals, one homologue of *Drosophila* Scribble, four homologues of Dlg (Dlg1-4) and two of Lgl (Lgl1, Lgl2) exist (88). The Scrb complex localizes to the basolateral membrane domain (89). Biochemically, this complex is less well defined. Scrb exists in a biochemical complex with Lgl2 (90) but it is not clear if this interaction is direct. Mutations in any of these genes disrupt apico-basal polarity in *Drosophila* epithelial cells (91). More recently, evidence has been provided that members of the Scrb complex regulate the formation of apico-basal polarity formation in vertebrate epithelial cells as well. Although Scrb knockdown does not affect the ability of MCF10A cells to develop polarized acini when grown in 3D-cultures, it does result in a delayed TJ formation, probably through a destabilization of E-cadherin-mediated cell adhesion (92). Also, as demonstrated more recently, knockdown of Dlg1 in epithelial cells disturbs TJ formation in cells subjected to $Ca^{2+}$-switch (93). The mechansims by which members of the Scrb complex regulate TJ formation are still unknown.

### 5.1. PAR-6 interaction with the CRB3 - Pals1 - PATJ complex

PAR-6 is probably the most versatile among the PAR proteins. It can exist independently of PAR-3 to regulate centrosome polarization and directed cell movement (94, 95) or to regulate cell survival in three-dimensional epithelial culture systems (25, 96). Regarding the CRB3 - Pals1 - PATJ complex, a direct interaction between PAR-6 and Pals1 has been described (97). This interaction involves the PDZ domain of PAR-6 and a conserved internal region in Pals1, and it is regulated by active Cdc42 (97-99). Since PAR-3 is present in the Pals1-associated PAR-6 complex, and since a dominant-negative mutant of PATJ affects the localization of aPKCzeta it is likely that the entire two protein complexes are linked through the Pals1 - PAR-6 interaction (97). It is not clear yet how this link influences TJ and cell polarity formation. The fact that PAR-6 and Pals1 are involved in multiple protein-protein interactions makes it difficult to attribute a given phenotype to the direct association between PAR-6 and Pals1. PAR-6 also interacts with CRB3 (35), the transmembrane component of the CRB3 - Pals1 - PATJ complex in vertebrate epithelial cells. Notably, like the interaction with Pals1, this interaction involves the PDZ domain of PAR-6 excluding the possibility that PAR-6 associates with CRB3 and Pals1 simultaneously. It rather implicates that a CRB3 - PAR-6 complex exists independently of the Pals1 - PAR-6 complex. Interestingly, in *Drosophila* epithelial cells Crumbs has been found to be phosphorylated by aPKC, and a non-phosphorylatable Crumbs acts in a dominant-negative manner (100) suggesting that Crumbs phosphorylation is functionally relevant for epithelial cell polarity. It is not clear if CRB3 phosphorylation by aPKC is conserved as it has not been described yet in vertebrate epithelial cells.

### 5.2. PAR-6 interaction with the Scribble - Dlg1 - Lgl complex

PAR-6 also associates with Lgl, a member of the Scrb - Dlg1 - Lgl complex (23, 24). This interaction is independent of PAR-3 and involves only PAR-6 and aPKClambda. Lgl directly interacts with both PAR-6 and aPKClambda using the same interfaces in PAR-6 and aPKClambda like PAR-3 which provides a biochemical explanation for the mutually exclusive interaction of PAR-6 - aPKClambda with either PAR-3 or with Lgl (24). During early phases of cell-cell contact formation, Lgl codistributes with PAR-6 - aPKC, but in polarized epithelial cells it is separated from PAR-6 - aPKC. The early co-localization is accompanied by aPKC-mediated phosphorylation of Lgl at S653 which results in a dissociation of Lgl from the PAR-6 - aPKC complex (24). Although the molecular mechanisms are not understood in detail, these findings favor a model that explains why Lgl associates with PAR-6 - aPKC early during cell-cell contact formation but not in mature cell-cell contacts: PAR-6 and aPKC initially form a complex with Lgl which is recruited in an inactive form to cell-cell contacts. Once activated by small GTPases, Lgl is phosphorylated by aPKC and dissociates from the complex. The PAR-6 - aPKC complex might now become available for PAR-3 which might have a higher affinity for the PAR-6 - aPKC complex than Lgl. In the newly formed PAR-3 - aPKC - PAR-6 complex, PAR-3 serves as scaffold to regulate the specific localization and activation of the PAR-6 - aPKC complex which in turn promotes the development of apico-basal polarity. Two observations provide support for this model: Overexpression of Lgl in MDCK cells impaired the ability of the cells to form functional TJs, and downregulation of endogenous Lgl resulted in increased amounts of PAR-3 associated with PAR-6 - aPKC as well as in increased amounts of Cdc42 associated with PAR-6 (24, 101).

## 6. THE FORMATION OF DISTINCT MEMBRANE DOMAINS THROUGH RECIPROCAL INHIBITORY INTERACTIONS

The early studies using *C.elegans* embryos indicated a mutually exclusive localization of certain PAR proteins at distinct cortical domains with PAR-1 and PAR-2 localizing at the posterior cortex of the fertilized egg and PAR-3, PKC3, and PAR-6 at the anterior cortex (12). An important piece of information regarding the mechanism underlying the mutual exclusion of PAR proteins from the

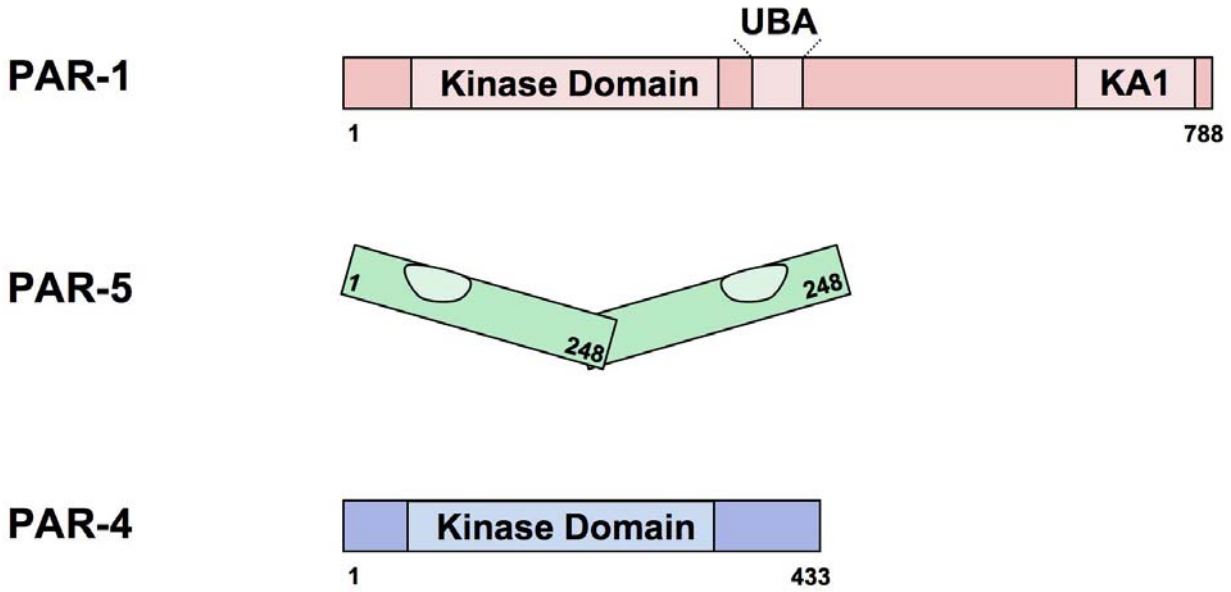


**Figure 2.** PAR-1 is a Ser/Thr kinase with a N-terminal kinase domain, a UBA (ubiquitin-associated) domain, a sequence motif present in enzymes of the ubiquitination pathway (150), and a KA1 (kinase-associated domain 1) domain, a conserved motif at the C-terminus of unknown function (151). PAR-5 is a member of the 14-3-3 family of proteins which form constitutive dimers and bind to their target proteins in a phosphorylation-dependent manner (152). PAR-4 is a Ser/Thr kinase without typical domains. In contrast to the PAR-3 - aPKC - PAR-6 complex, PAR-1, PAR-4 and PAR-5 do not form a complex but undergo transient interactions in response to specific signals (153).

same membrane compartment has been obtained from studies in epithelial cells. In *Drosophila* epithelial cells, the PAR-3/Bazooka - aPKC - PAR-6 complex localizes to the most apical region of the lateral membrane, the marginal zone (which would correspond to the localization TJs in vertebrate epithelial cells) whereas other polarity proteins like PAR-1 or Lgl localize to the basolateral membrane domain. PAR-1 phosphorylates PAR-3/Bazooka at two serine residues, S151 and S1085 (S144 and S889 in rat PAR-3), leading to the formation of binding sites for PAR-5/14-3-3 (102, 103). The binding of PAR-5/14-3-3 masks two residues critical for PAR-3 function: S151 is located close to the CR1 region that is required for oligomerization and function of PAR-3 (104, 105); S1085 is located close to the CR3 region that is involved in aPKC binding and contains a serine residue (S980 in *Drosophila*) which negatively regulates the interaction between PAR-3 and aPKC (30, 102). Importantly, the binding of 14-3-3/PAR-5 to S1085 of PAR-3 blocks the ability of aPKC to interact with PAR-3 (102). Thus, PAR-1-mediated phosphorylation of PAR-3 blocks PAR-3 function in two ways: first, it inhibits PAR-3 oligomerization, and second, it blocks the ability of PAR-3 to assemble a functional PAR-3 - aPKC - PAR-6 complex (Figure 3). As a consequence, the membrane domain containing active PAR-1 is devoid of a functional PAR-3 - aPKC - PAR-6 complex.

Interestingly, the antagonistic interaction between the PAR-3 - aPKC - PAR-6 complex and a mammalian PAR-1 isoform, PAR-1b, has also been demonstrated in mammalian epithelial cells: aPKC phosphorylates PAR-1 on T595, and this again represents a binding site for 14-3-3/PAR-5 (106, 107). As a consequence of 14-3-3/PAR-5 binding, the kinase activity of PAR-1 is reduced and PAR-1 dissociates from the membrane (106, 107) resulting in a PAR-1-free membrane in the apical domain where an active PAR-3 - aPKC - PAR-6 complex is localized. A similar mechanism regulates the formation of distinct membrane domains in the *C.elegans* zygote (108).

## 7. PAR-1, PAR-4 AND PAR-5

As pointed out in the previous section, PAR-1b is a Ser/Thr kinase which localizes to the basolateral membrane domain and regulates membrane asymmetry by phosphorylating PAR-3. Whether PAR-1b directly contributes to junction formation in mammalian epithelial cells is less clear but evidence exists that it enhances cell adhesion by promoting the association of E-cadherin with the cytoskeleton (109). Since PAR-1b is recruited to the lateral membrane at the later stage of cell polarization, and since PAR-1b-depleted cells develop functional TJs rather normally, it might regulate membrane asymmetry predominantly after the establishment of the junctional structures are matured (107, 110).

Mammalian PAR-4/LKB1 was identified as the responsible gene for the Peutz-Jeghers syndrome, and has been shown to regulate cell growth, proliferation, metabolism (111). Interestingly, the overexpression of PAR-4/LKB1 in single intestinal epithelial cells is sufficient to induce certain aspects of cell polarity like actin reorganization-dependent brush border formation or the polarized sorting of apical and basolateral membrane

markers (112), establishing that this Ser/Thr kinase also works as a cell polarity protein in mammalian epithelial cells. Recent evidence indicates that PAR-4/LKB1 might be also involved in the assembly of epithelial cell-cell contacts and of TJs. PAR-4/LKB1 phosphorylates and activates PAR-1a, PAR-1b and PAR-1c (113) suggesting that it works upstream of PAR-1 in mammalian epithelial cells. However, PAR-4/LKB1 is not the specific upsteam kinase of PAR-1, but is a master kinase that activates 13 kinases of the AMPK subfamily, to which PAR-1 belongs. Therefore, the function of PAR-4/LKB1 is thought to be mediated by not only PAR-1 but also other members of AMPK subfamily, and recent evidence in fact indicates that PAR-4/LKB1 is involved in the assembly of epithelial cell-cell contacts and TJs by activating the AMP-activated kinase (AMPK) during $Ca^{2+}$-switch-induced cell-cell contact formation (114, 115). Thus, although PAR-4 is not distributed in a polarized fashion and has not been detected at epithelial cell-cell contacts (112, 116), it might exert its activity indirectly by activating PAR-1 and/or AMPK.

PAR-5 is a member of the highly conserved and ubiquitously expressed 14-3-3 family of proteins (117). 14-3-3 proteins constitutively form homo- or heterodimers, and can interact with a wide variety of proteins. In most cases they interact in a Ser/Thr phosphorylation-dependent manner with their target proteins (118), and this binding can regulate the interaction of the target protein with other proteins. As mentioned above, PAR-5/14-3-3 regulates the activity and subcellular localization of PAR-3 and PAR-1 and thus contributes to their asymmetric localization at the lateral cell-cell contacts (see also Figure 3).

## 8. PAR PROTEINS AND CELL CONTACT FORMATION IN OTHER CELLULAR SYSTEMS

### 8.1. The PAR-3 - aPKC - PAR-6 complex and endothelial cell-cell contacts

Endothelial cell-cell contacts are principally similarly organized like epithelial cell-cell contacts with TJs formed by occludin and claudins and AJs formed by vascular endothelial cell (VE-) cadherin and nectins and the associated catenin and afadin - ponsin compexes (119, 120). However, as opposed to epithelial cells, the lateral membrane domains connecting adjacent cells are largely unpolarized. TJs are localized along the entire lateral membrane and are intermingled with AJs and Gap Junctions (121, 122). Accordingly, proteins which in polarized epithelial cells localize to a specific area like TJs or AJs are in close proximity and localize along the entire intercellular contact region (123, 124). Interestingly, endothelial cells have two distinct PAR protein complexes: A "conventional" PAR complex consisting of PAR-3, aPKC and PAR-6 which probably localizes to TJs through the association of PAR-3 with a JAM family member (51, 125), and a second, more recently identified PAR protein complex, which localizes to AJs through an association with VE-cadherin (126). In this complex, both PAR-3 and PAR-6 associate directly with VE-cadherin through independent domains. Most strikingly, this complex lacks aPKC and therefore is unable to promote cellular polarization by way of aPKC. The function of this PAR complex is still unclear. Ectopic expression of VE-cadherin in MDCK II epithelial cells leads to a mislocalization of the TJ proteins ZO-1 and occludin (126) strongly supporting that VE-cadherin influences the organization of TJs in endothelial cells. One could imagine that the sequestration of PAR-3 and PAR-6 to AJs might be inhibitory for the polarization process which in epithelial cells leads to the formation of distinct membrane domains harboring TJs and AJs.

### 8.2. PAR proteins and leukocyte cell-cell contacts

The high conservation of PAR proteins during evolution and their involvement in many different aspects of cell polarity suggests that their role in cell-cell contact formation might not be restricted to homotypic cell-cell contacts between epithelial cells or endothelial cells. In fact, recent evidence indicates that PAR proteins also regulate heterotypic cell-cell interactions which are rather transient in nature. The activation of the adaptive immune system provides examples for transient interactions between different cell types. When T cells encounter antigen-presenting cells (APC), like dendritic cells or B cells, they undergo a morphological change from a spherical into a highly polarized cell. This is accompanied by a massive actin polymerization at the cell-cell contact zone to increase the interactive T-cell - APC surface area as well as by a reorientation of the MTOC, the microtubule cytoskeleton and the Golgi (127, 128). Simultaneously, a highly organized network of adhesion molecules, receptors and cytoplasmic proteins, the supramolecular activation cluster (SMAC) or immunological synapse, is established at the interface between the T cell and the APC (129). The formation of the immunological synapse is necessary both for the activation of the T cell and for the targeted delivery of cytokines and other effector molecules towards the APC or towards the target cell (128). Recently, it turned out that members of all three major cell polarity complexes of epithelial cells are involved in T cell polarization during immunological synapse formation (130-133). PAR-3 and CRB3 are distributed in the cytoplasm at early stages of T-cell - APC contact formation but co-localize with markers for the T cell receptor at the T cell - APC interface after a stable immunological synapse has been formed (133). Interestingly, Dlg1 and Scrb are rapidly recruited to the immunological synapse after T cell receptor stimulation but are excluded from more matured immunological synapses and localize to the distal pole of T-cells engaged in T cell - APC interactions. This suggests a similar role for polarity proteins during heterotypic T cell - APC interactions as observed at homotypic epithelial cell interactions, namely the generation of distinct membrane domains that serve as platforms for cell surface receptors and signaling proteins.

### 8.3. PAR proteins and Schwann cell autotypic and heterotypic contacts

In the nervous system, reciprocal interactions exist between neurons and glial cells (oligodendrocytes in the central nervous system, Schwann cells in the peripheral nervous system) to form the myelin sheath that allows for membrane depolarization only at specific sites of the axon, the nodes of Ranvier. The result of this membrane insulation is a rapid nerve conduction. The formation of the

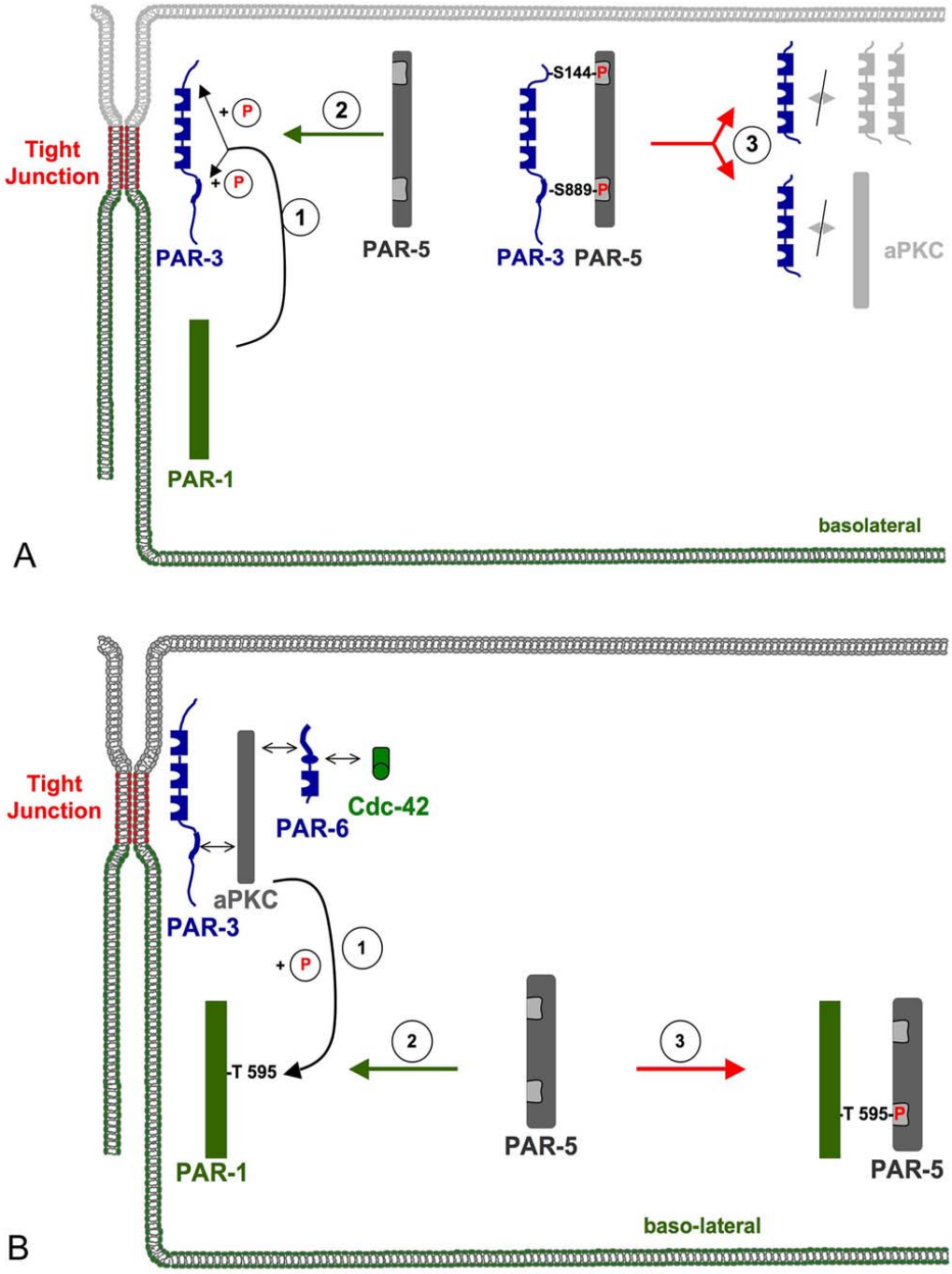


**Figure 3.** Reciprocal inhibitory interactions between PAR-1 and the PAR-3 - aPKC - PAR-6 complex. A: PAR-1 inactivates the PAR-3 - aPKC - PAR-6 complex. PAR-1 phosphorylates PAR-3 at two serine residues, S144 and S889 (1), and as a consequence, PAR-5/14-3-3 binds to phosphorylated PAR-3 (2). The binding of PAR-5 to phosphorylated PAR-3 functionally inactivates PAR-3 by a) inhibiting its oligomerization through the CR1 domain (3, top) and b) inhibiting its association with aPKC (3, bottom). As a consequence, PAR-3 is excluded from the PAR-1-containing membrane domain (102). B: aPKC inactivates PAR-1. aPKC phosphorylates PAR-1 at threonine residue T595 (1) which leads to the binding of PAR-5/14-3-3 to phosphorylated PAR-1 (2). As a consequence, the kinase activity of PAR-1 is reduced and PAR-1 is removed from the membrane (3). As final result of the two inhibitory interactions, PAR-1 is excluded from the TJ region, and the PAR-3 - aPKC - PAR-6 complex is excluded from the basolateral membrane domain leading to two membrane domains with distinct identities.

myelin sheath involves a unidirectional wrapping of multiple membrane layers around the axon, initiated by heterotypic axon - glial cell membrane interactions and continued by autotypic glial cell membrane interactions (134, 135). The myelin can be divided into a compact myelin where the cytoplasm is restricted to a thin band and a noncompact myelin where the volume of the cytoplasm is increased (134). Areas of noncompact myelin, the paranodal loops, Schmidt-Lanterman incisures, and the inner and outer mesaxons, contain junctional structures similar to those found at epithelial cell-cell contacts, like TJs, AJs and gap junctions (136). Thus, it is not surprising that molecules localized at epithelial TJs were found at Schmidt-Lanterman incisures and paranodal loops of Schwann cells, e.g. claudins, MUPP1, PATJ, ZO-1, ZO-2 and also PAR-3 (137). It is not clear yet if these proteins form the same complexes as in epithelial cells or, for example, if PAR-3 acts on its own or exists in a complex with aPKC and PAR-6. Interestingly, PAR-3 has recently been identified at the interface between Schwann cells and axons along the internodal region (138). PAR-3 is expressed by the Schwann cells and is required to recruit the p75 neurotrophin receptor to the glial-axon junction to regulate myelination of the axon. As overexpression of PAR-6 in this system has a similar effect like overexpression of PAR-3, it is likely that the entire PAR-3 - aPKC - PAR-6 complex is involved in the regulation of myelination (138).

### 8.4. PAR proteins and Sertoli cell - spermatid junctions

The development of male germ cells from diploid spermatogonia through tetraploid spermatocytes to haploid spermatids and finally to spermatozoa involves a complex series of cellular and molecular changes including mitosis, meiosis and spermiogenesis (139). During all these steps, the developing germ cells are engaged in intimate interactions with Sertoli cells, the cell type which is responsible for structural and nutritional support. The intimate cell-cell interaction ensures that via paracrine factors and signaling molecules Sertoli cells can provide developing germ cells with the nutrients needed. In addition to their heterotypic interactions with germ cells, Sertoli cells form homotypic interactions including a TJ-like structure, the blood-testis-barrier (BTB), which separates the systemic circulation from the process of germ cell development to prevent autoimmune reactions. Not surprisingly, proteins that are found at TJs of epithelial cells, e.g. occludin, claudins and JAMs as well as their cytoplasmically associated protein including ZO-1, ZO-2 and cingulin, are expressed by Sertoli cells and localize to the BTB (139, 140). Very little is known about cell polarity proteins like those of the PAR-3 - aPKC - PAR-6, the CRB3 - Pals - PATJ or the Scrb - Dlg - Lgl complexes in Sertoli cells. However, recent evidence strongly suggests that some of these proteins participate in germ cell development. Germ cells express various TJ-associated cell adhesion molecules including JAM-C (141), coxsackie and adenovirus receptor (CAR) (142) and JAM4 (143). The inactivation of the JAM-C gene in mice leads to male sterility due to a developmental arrest of developing spermatids at the transition from round spermatids into elongated spermatids (141). This arrest is probably caused by mislocalization of cell polarity proteins including PAR-6, aPKClambda and PATJ (141). The binding of spermatid-expressed JAM-C to its putative counter-receptor JAM-B on Sertoli cells is obviously required for the polarized localization of these proteins at the head of spermatids and the further maturation of round spermatids into elongated spermatids. Interestingly, PAR-3 does not co-localize with JAM-C at the head of wildtype spermatids and is not mislocalized in JAM-C-deficient spermatids despite the ability of PAR-3 to directly interact with JAM-C (125) indicating that the polarity-promoting PAR complex in spermatids consists of PAR-6 and aPKC but lacks PAR-3. It is not clear yet if PAR-6 - aPKC regulates the formation of distinct membrane domains and/or the re-orientation of the MTOC during polarization in developing spermatids.

## 9. CONCLUSIONS

During the last few years, PAR proteins have turned out to be major players in cell polarity. They are highly conserved during evolution and are involved in diverse aspects of cell polarity such as the development of anterior-posterior polarity in the egg after fertilization, the reorganization of the MTOC during directed cell migration, the development of apico-basal polarity in epithelial cells, the regulation of asymmetric cell division in neuroblasts or the specification of single axons from multiple neurites. A common principle that has emerged is that reciprocal inhibitory interactions regulate the establishment of complementary membrane domains, and this principle holds true for various cell types including the *C.elegans* zygote and *Drosophila* and vertebrate epithelial cells. With regard to the formation of TJs and the development of apico-basal polarity in vertebrate epithelial cells there are still many open questions. For example, what are the downstream targets of aPKC besides PAR-1 and Lgl? What regulates the apical localization of TJs and the localization of AJs underneath? What is the functional interrelationship of the three major polarity complexes? Genetic evidence in *Drosophila* suggests a functional hierarchy in which the PAR-3 - aPKC - PAR-6 complex is placed at the top (144) but it is unclear if a similar hierachy exists in vertebrate epithelial cells. Finally, it will be important to understand the role of PAR polarity proteins in human diseases. Recent evidence indicates that PAR proteins can be targets for oncogenic microorganisms and for cellular oncogenes (25, 145) as well as targets for TGFβ during TGFβ-induced epithelial-mesenchymal transition (87) suggesting a critical role for PAR proteins in tissue homeostasis (146). Clearly, more surprising roles of PAR polarity proteins are expected to be discovered in the near future.

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**Note:** Present address of Dr Sandra Iden: The Netherlands Cancer Institute, Division of Cell Biology, Amsterdam, The Netherlands

**Abbreviations:** AJ, adherens junction; aPKC, atypical protein kinase C; JAM, junctional adhesion molecule; PAR, partitioning-defective; TJ, tight junction

**Key Words:** Cell polarity, PAR proteins, Cell-Cell Contacts, Tight Junctions, Epithelial Cells, Membrane Asymmetry, JAM, Review

**Send correspondence to:** Klaus Ebnet, Institute of Medical Biochemistry, Center of Molecular Biology of Inflammation (ZMBE), University Muenster, Germany**,** Tel.: 4902518352127**,** Fax: 4902518356748**,** E-mail: ebnetk@uni-muenster.de

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