EMBO reports

scientific report

A distinct PAR complex associates physically with VE-cadherin in vertebrate endothelial cells

Sandra Iden[1,2], Daniela Rehder[1,2], Benjamin August[2], Atsushi Suzuki[3], Karen Wolburg-Buchholz[4], Hartwig Wolburg[5], Shigeo Ohno[3], Jürgen Behrens[6], Dietmar Vestweber[2,4] & Klaus Ebnet[1,2+]

[1]Institute of Medical Biochemistry, ZMBE, and [2]Institute of Cell Biology, ZMBE, University of Münster, Münster, Germany, [3]Department of Molecular Biology, Yokohama City University School of Medicine, Kanazawa-ku, Yokohama, Japan, [4]Max-Planck-Institute for Molecular Biomedicine, Münster, Germany, [5]Institute of Pathology, University of Tübingen, Tübingen, Germany, and [6]Nikolaus-Fiebiger-Center for Molecular Medicine, University Erlangen-Nürnberg, Nürnberg, Germany

A cell polarity complex consisting of partitioning defective 3 (PAR-3), atypical protein kinase C (aPKC) and PAR-6 has a central role in the development of cell polarity in epithelial cells. In vertebrate epithelial cells, this complex localizes to tight junctions. Here, we provide evidence for the existence of a distinct PAR protein complex in endothelial cells. Both PAR-3 and PAR-6 associate directly with the adherens junction protein vascular endothelial cadherin (VE-cadherin). This association is direct and mediated through non-overlapping domains in VE-cadherin. PAR-3 and PAR-6 are recruited independently to cell–cell contacts. Surprisingly, the VE-cadherin-associated PAR protein complex lacks aPKC. Ectopic expression of VE-cadherin in epithelial cells affects tight junction formation. Our findings suggest that in endothelial cells, another PAR protein complex exists that localizes to adherens junctions and does not promote cellular polarization through aPKC activity. They also point to a direct role of a cadherin in the regulation of cell polarity in vertebrates.

Keywords: cell polarity; endothelial cells; membrane asymmetry; PAR proteins; VE-cadherin

EMBO reports (2006) **7**, 1239–1246. doi:10.1038/sj.embor.7400819

[1]Institute of Medical Biochemistry, ZMBE, and [2]Institute of Cell Biology, ZMBE, University of Münster, Von-Esmarch-Str. 56, D-48149 Münster, Germany
[3]Department of Molecular Biology, Yokohama City University School of Medicine, 3-9, Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan
[4]Max-Planck-Institute for Molecular Biomedicine, Von-Esmarch-Str. 56, D-48149 Münster, Germany
[5]Institute of Pathology, University of Tübingen, Liebermeisterstr. 8, D-72076 Tübingen, Germany
[6]Nikolaus-Fiebiger-Center for Molecular Medicine, University Erlangen-Nürnberg, Glueckstr. 6, D-91054, Germany
[+]Corresponding author. Tel: +49 251 8352127; Fax: +49 251 8356748; E-mail: ebnetk@uni-muenster.de

Received 1 June 2006; revised 24 August 2006; accepted 4 September 2006; published online 20 October 2006

INTRODUCTION

Vertebrate epithelial cells are characterized by apico-basal polarity with two distinct domains, the apical and the basolateral membrane domains. The formation of these domains depends on the presence of tight junctions, an intramembrane diffusion barrier that restricts the apical–basolateral diffusion of membrane components. Recent evidence indicates a crucial role of a protein complex consisting of the partitioning defective (PAR) proteins PAR-3 and PAR-6 and atypical protein kinase C (aPKC) in cell polarity formation in various cell types and organisms (Macara, 2004; Suzuki & Ohno, 2006). In this complex, PAR-3 acts as a scaffolding protein, which through direct interactions assembles PAR-6, aPKC and the Rac1 guanine nucleotide exchange factor Tiam1 in close proximity (Chen & Macara, 2005; Mertens *et al*, 2005). The activation of aPKC promotes the polarization process through the phosphorylation of aPKC targets such as lethal giant larvae (Lgl) or PAR-1, which results in the segregation of these proteins from the aPKC-containing membrane domain and ultimately in the formation of membrane asymmetry (Suzuki & Ohno, 2006). PAR-6 can associate with constituents of other cell polarity complexes, such as protein associated with Lin-7 (Pals1) and crumbs3 (CRB3) from the CRB3–Pals1–PATJ (Pals1-associated tight junction protein) complex and Lgl from the Scrib–Dlg–Lgl (for scribble–discs large–Lgl) complex (Hurd *et al*, 2003; Plant *et al*, 2003; Yamanaka *et al*, 2003; Lemmers *et al*, 2004). This indicates that various cell polarity complexes are physically linked and suggests a functional interdependence between these complexes. Genetic studies in *Drosophila* place the PAR-3–aPKC–PAR-6 complex at the top of the hierarchy in a complex series of events regulating the formation of epithelial cell polarity (Bilder *et al*, 2003; Tanentzapf & Tepass, 2003). In vertebrate epithelial cells, the PAR-3–aPKC–PAR-6 complex localizes to tight junctions (Macara, 2004). Dominant-negative mutants and short interfering RNA-mediated downregulation of individual components of the complex result in defects in tight junction and cell polarity formation (Suzuki & Ohno, 2006), indicating an important role of this complex in epithelial cell polarity.

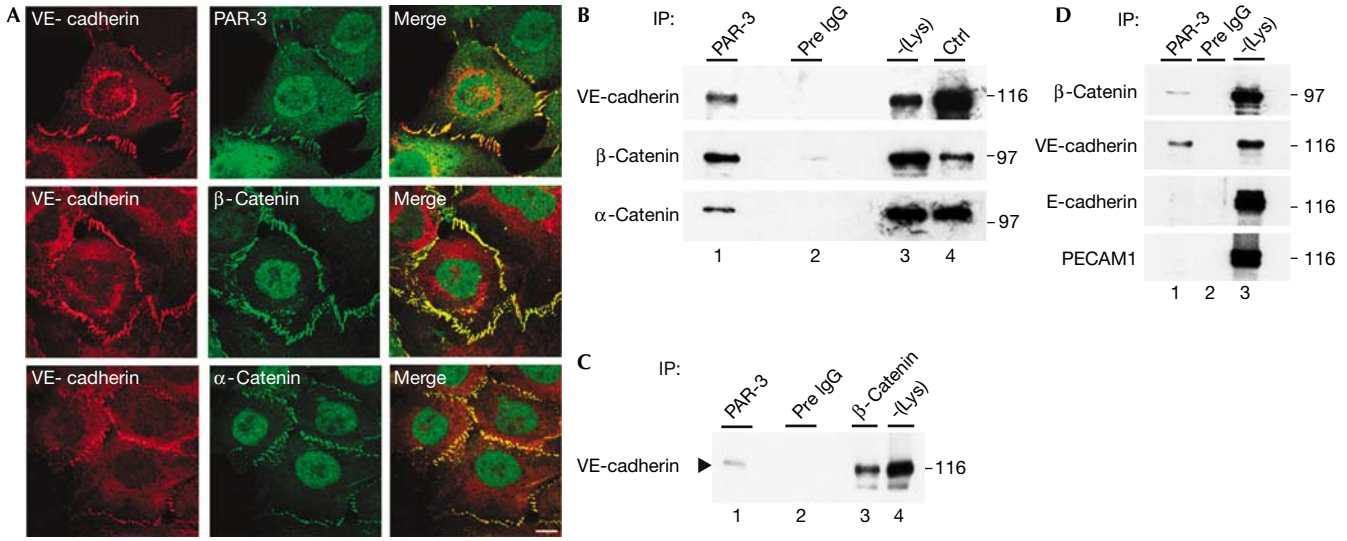

Fig 1 | PAR-3 associates with the VE-cadherin–catenin complex in cells. (**A**) PAR-3 is recruited to VE-cadherin-based cell–cell contacts. Chinese hamster ovary (CHO) cells stably expressing VE-cadherin (VE-cad/CHO) were stained for endogenous PAR-3, β-catenin and α-catenin. Scale bar, 10 μm. (**B**) PAR-3 is associated with the VE-cadherin–catenin complex in VE-cad/CHO cells. PAR-3 immunoprecipitates were blotted for VE-cadherin, β-catenin and α-catenin. In lane 3, postnuclear supernatant (cell lysate, 1% of input) was loaded. Control immunoprecipitations (lane 4) were performed with antibodies against β-catenin (top panel), α-catenin (middle panel) or VE-cadherin (bottom panel). (**C**) PAR-3 and VE-cadherin interact endogenously. PAR-3 was immunoprecipitated from myEnd endothelial cells and immunoprecipitates were blotted with antibodies against VE-cadherin (lane 4: postnuclear supernatant, 1% of input). (**D**) PAR-3 associates with VE-cadherin but not E-cadherin. PAR-3 was immunoprecipitated from human embryonic kidney 293T cells transiently transfected with PAR-3 and β-catenin, VE-cadherin, E-cadherin or platelet–endothelial cell adhesion molecule 1 (PECAM1). Immunoprecipitates were blotted with antibodies indicated at the left. Lane 3: postnuclear supernatant, 1% of input. Lys, lysate; Ctrl, control.

In this study, we provide evidence that in endothelial cells, besides the PAR-3–aPKC–PAR-6 complex, a distinct PAR-3–PAR-6 complex exists. This latter complex localizes to adherens junctions by associating with vascular endothelial cadherin (VE-cadherin). We find that both PAR-3 and PAR-6 can directly and independently associate with VE-cadherin, and that both are recruited to cell–cell contacts independently of each other. Interestingly, the VE-cadherin-associated PAR complex is not associated with aPKC. Our findings have implications for the mechanisms that regulate apico-basal polarity in endothelial cells.

RESULTS AND DISCUSSION

PAR-3 exists in a complex with VE-cadherin

In wild-type Chinese hamster ovary (CHO) cells, PAR-3 is localized diffusely in the cytoplasm and not enriched at cell–cell contacts unless cells are transfected with junctional adhesion molecule (JAM)-A, which has previously been found to associate with PAR-3 (Ebnet *et al*, 2001). We first observed that ectopically expressed VE-cadherin recruits endogenous PAR-3 to cell–cell contacts in CHO cells (Fig 1A). These observations prompted us to analyse whether PAR-3 is associated physically with VE-cadherin. In fact, PAR-3 immunoprecipitates obtained from VE-cad/CHO lysates contained VE-cadherin, β-catenin and α-catenin (Fig 1B). VE-cadherin was also detected in PAR-3 immunoprecipitates obtained from endothelial cells (Fig 1C), indicating the existence of a VE-cadherin–PAR-3 complex in endothelial cells. In addition to VE-cadherin, PAR-3 immunoprecipitated β-catenin but not E-cadherin or platelet–endothelial cell adhesion molecule 1 (PECAM1) after ectopic expression in human embryonic kidney (HEK)293T cells (Fig 1D), suggesting that PAR-3 interacts specifically with VE-cadherin among the integral membrane proteins tested.

PAR-3 associates directly with VE-cadherin

We next addressed the possibility of a direct interaction between PAR-3 and VE-cadherin. In glutathione *S*-transferase (GST)-pull-down assays, we found that VE-cadherin precipitated a PAR-3 construct consisting of PSD-95–discs large–zonula occludens 1 (PDZ) domains 1 to 3 (PAR-3/PDZ1–3) in a similar manner as JAM-A, JAM-B and JAM-C (Fig 2A), indicating that the interaction between VE-cadherin and PAR-3 is direct. This interaction is mediated through the carboxy-terminal five amino acids of VE-cadherin, as deletion of these residues completely abrogated binding to PAR-3 and replacing the C-terminal five amino acids of E-cadherin by those of VE-cadherin rendered E-cadherin functional in PAR-3/PDZ1–3 binding (Fig 2B). GST-pulldown experiments with recombinant individual PDZ domains of PAR-3 showed that it binds to VE-cadherin predominantly through its third PDZ domain (Fig 2C). Notably, this interaction is specific for VE-cadherin, as no binding was observed with E-cadherin or N-cadherin (Fig 2A–C). As suggested by our experimental evidence, VE-cadherin contains a type II PDZ-domain-binding motif at its C terminus, which is unique to VE-cadherin (Fig 2D). Transient transfection experiments confirmed that the recruitment of PAR-3 by VE-cadherin in living cells is mediated through the PDZ-domain-binding motif of VE-cadherin and PDZ domain 3 of PAR-3 (supplementary Fig 1 online).

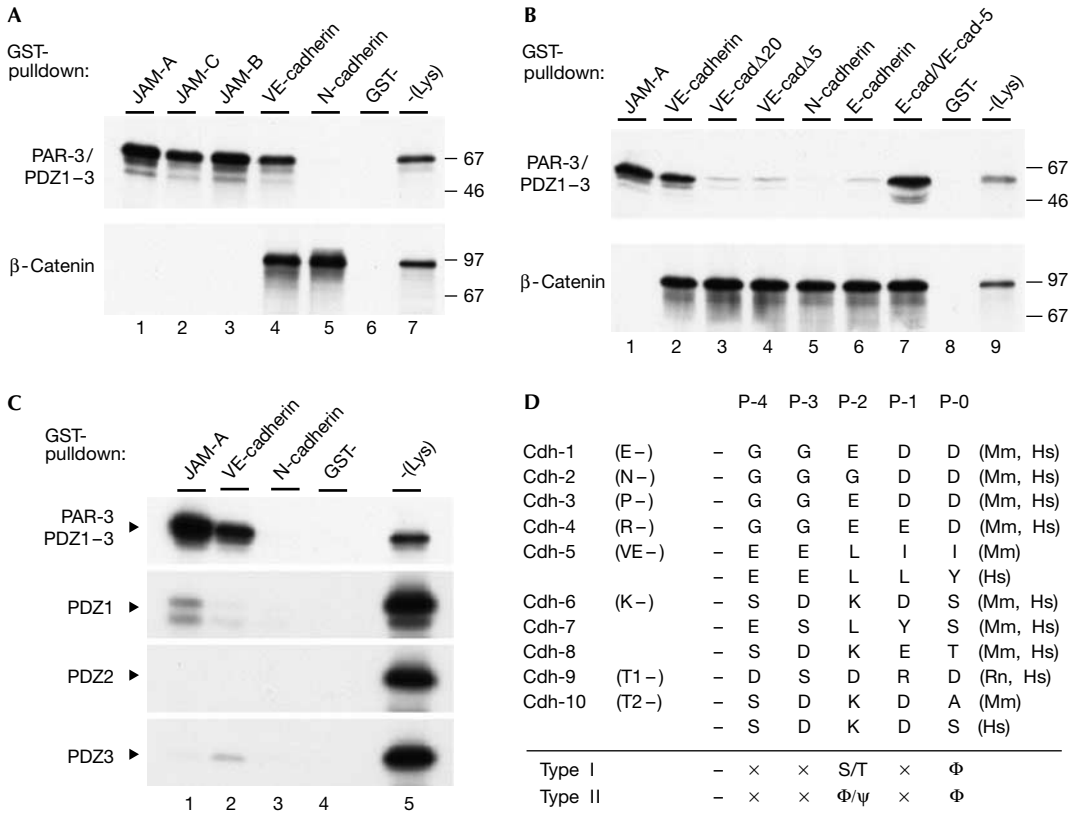

Fig 2 | PAR-3 associates directly with VE-cadherin. (**A**) Glutathione *S*-transferase (GST)-pulldown experiments were performed with GST-fusion proteins and *in vitro*-translated, ^{35}S-methionine-labelled PAR-3/PDZ1–3 (top panel) or β-catenin (bottom panel). (**B**) The interaction between PAR-3 and VE-cadherin is dependent on the PDZ domain. GST-fusion proteins comprising the cytoplasmic domain of junctional adhesion molecule A (JAM-A; lane 1), VE-cadherin (lane 2), VE-cadherin lacking the carboxy-terminal 20 amino acids (lane 3), VE-cadherin lacking the C-terminal five amino acids (lane 4), N-cadherin (lane 5), E-cadherin (lane 6) and E-cadherin with the C-terminal five amino acids replaced by the C-terminal five amino acids of VE-cadherin (lane 7) were used in GST-pulldown experiments, as described in (**A**). (**C**) The third PDZ domain of PAR-3 mediates the association with VE-cadherin. GST-pulldown experiments were performed with GST-fusion proteins and recombinant PAR-3 proteins comprising either PDZ domains 1–3 or individual PDZ domains. (**D**) Schematic representation of C-terminal five amino-acid residues present in cadherins 1–10. VE-cadherin (Cdh-5) contains a canonical type II PDZ-domain-binding motif. Φ, hydrophobic residue; Ψ, aromatic residue; Cdh, cadherin; Hs, *Homo sapiens*; Mm, *Mus musculus*; P, amino-acid position relative to the C-terminal amino acid; Rn, *Rattus norvegicus*; VE-cad, VE-cadherin.

PAR-6 associates directly with VE-cadherin

To address the question whether PAR-6 is part of the VE-cadherin-based PAR-3 complex, we carried out co-immunoprecipitation experiments after transient transfection of PAR-6α or PAR-6γ in stable VE-cad/CHO cells. VE-cadherin was present in PAR-6 immunoprecipitates, indicating that PAR-6 is also associated with it (Fig 3A). Surprisingly, GST-pulldown experiments showed a direct interaction between them (Fig 3B). This interaction is not mediated through the PDZ-domain-binding motif of VE-cadherin, but through the remaining part of the cytoplasmic domain (Fig 3B). Mapping experiments showed a region encompassing amino acids 621–689 of VE-cadherin responsible for PAR-6 binding (Fig 3C; supplementary Fig 2B,C online). Despite an overlap of the PAR-6-binding region with the juxtamembrane domain (amino acids 627–664) including the p120^{ctn} core binding site (amino acids 647–663 of VE-cadherin), p120^{ctn} and PAR-6 can simultaneously bind to VE-cadherin (Fig 3D), suggesting that PAR-6 does not compete with p120^{ctn} for VE-cadherin binding. The association of PAR-6 is specific for VE-cadherin, as no association was observed with E-cadherin, N-cadherin or JAM-A (Fig 3B). Co-immunoprecipitation experiments using human umbilical vein endothelial cell (HUVEC) lysates showed that PAR-6 is present in VE-cadherin immunoprecipitates, indicating that it is complexed with VE-cadherin in endothelial cells (Fig 3E). These results indicate that PAR-6 can associate with VE-cadherin directly and independently of PAR-3.

The VE-cadherin-based PAR complex lacks aPKC

In vertebrate epithelial cells, both PAR-3 and PAR-6 are directly associated with aPKC to form a ternary complex in which small GTPases such as Cdc42 and Rac1 regulate aPKC activity (Suzuki & Ohno, 2006). This complex is probably recruited to cell–cell contacts through the association of PAR-3 with JAM-A (Ebnet *et al*, 2001; Itoh *et al*, 2001). Accordingly, JAM-A-based cell–cell contacts were positive for aPKC in JAM-A/CHO cells (Fig 4A,B). Surprisingly, in VE-cad/CHO cells, aPKC was not present at VE-cadherin-based cell contacts (Fig 4A,B) despite the presence of PAR-3 (Fig 1A). As aPKC can be associated with PAR-6 in the

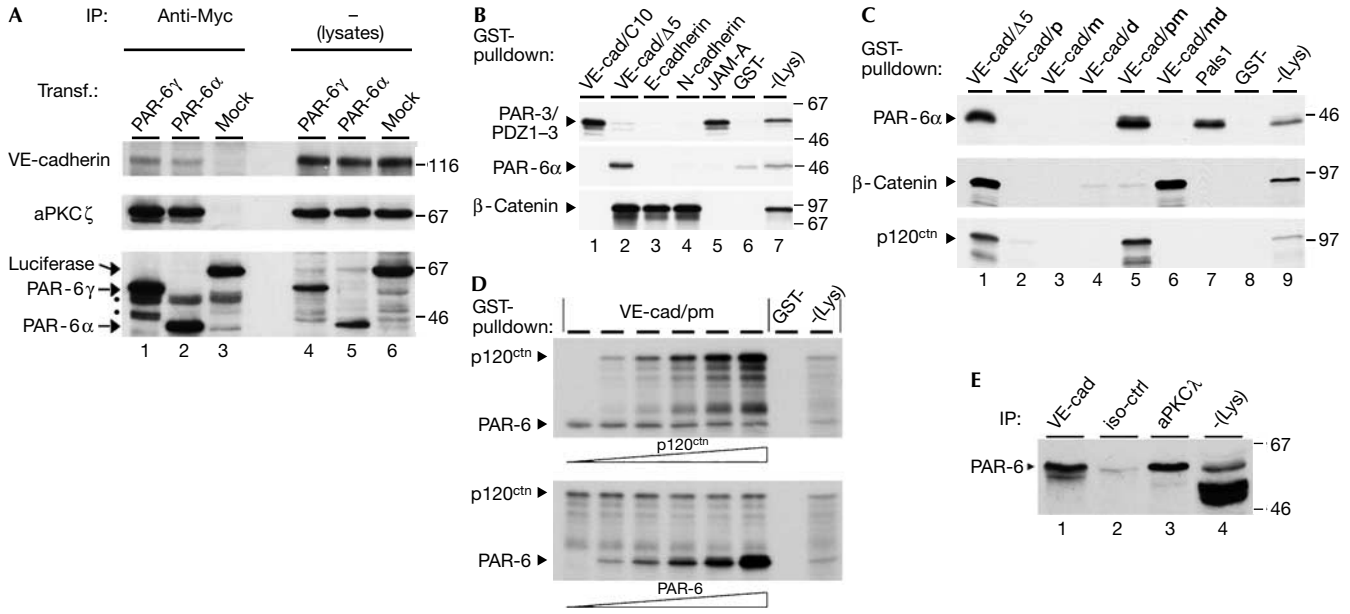

Fig 3 | PAR-6 associates with VE-cadherin. (**A**) VE-cad/CHO cells were transiently transfected (Transf.) with Myc-tagged PAR-6γ or PAR-6α. Transfected proteins were immunoprecipitated (IP) with Myc polyclonal antibodies and immunoprecipitates were analysed for the presence of VE-cadherin (top panel), atypical protein kinase Cζ (aPKCζ; middle panel) or PAR-6 proteins (bottom panel). VE-cadherin is present in both PAR-6γ and PAR-6α immunoprecipitates. (**B**) The association between PAR-6 and VE-cadherin is direct. Glutathione *S*-transferase (GST)-pulldown experiments were performed with GST-fusion proteins comprising the carboxy-terminal ten amino acids of VE-cadherin (lane 1), the cytoplasmic domain of VE-cadherin lacking the C-terminal PDZ-domain-binding motif (lane 2), the cytoplasmic domains of E-cadherin (lane 3), N-cadherin (lane 4) or junctional adhesion molecule A (JAM-A; lane 5) and recombinant, ^{35}S-labelled PAR-3/PDZ1–3 or PAR-6α or β-catenin, as indicated. (**C**) GST-fusion proteins comprising the cytoplasmic tail without the PDZ motif (lane 1), the membrane-proximal (lane 2), middle (lane 3) or membrane-distal (lane 4) region, the membrane-proximal plus the middle region (lane 5) or the middle plus the membrane-distal region (lane 6), or amino acids 1–182 of Pals1 (lane 7) were incubated with *in vitro*-translated PAR-6α (top panel), β-catenin (middle panel) or p120^{ctn} (bottom panel). PAR-6α binds to a region encompassing the membrane-proximal and middle region of VE-cadherin and overlaps with the binding site for p120^{ctn}. (**D**) A GST–VE-cadherin fusion protein (VE-cad/pm, amino acids 621–730) was incubated with *in vitro*-translated PAR-6α and p120^{ctn} with one protein kept at a constant amount and the other protein at an increasing amount. The association of PAR-6 with VE-cadherin is not changed by increasing amounts of p120^{ctn} (top panel) and vice versa (bottom panel). (**E**) PAR-6 and VE-cadherin form a complex in endothelial cells. Lysates from confluent human umbilical vein endothelial cells were immunoprecipitated with antibodies indicated at the top, and immunoprecipitates were blotted with antibodies against PAR-6 (T20; SantaCruz Biotechnology Inc., Heidelberg, Germany). In lane 4, postnuclear supernatant (cell lysate, 1% of input) was loaded. iso-ctrl, isotype control; VE-cad, VE-cadherin.

absence of PAR-3 (Yamanaka *et al*, 2003), we analysed the recruitment of endogenous aPKC to cell–cell contacts after ectopic expression of PAR-6 in VE-cad/CHO and JAM-A/CHO cells. Despite the presence of PAR-6 at cell–cell contacts of both JAM-A/CHO and VE-cad/CHO cells, aPKC was recruited to PAR-6-positive sites only in JAM-A/CHO cells (Fig 4C). Biochemical experiments from endothelial cell lysates confirmed that aPKC is not associated with the VE-cadherin-associated PAR complex. A GST–VE-cadherin fusion protein consisting of the C-terminal 10 amino-acid residues of VE-cadherin precipitated PAR-3 but not aPKCζ, whereas a GST–JAM-A fusion protein precipitated both PAR-3 and aPKC (Fig 4D). Also, VE-cadherin immunoprecipitates were positive for PAR-3 and PAR-6 but negative for aPKC (Fig 4E), indicating that the VE-cadherin-associated PAR complex lacks aPKC. Interestingly, VE-cadherin precipitates contained predominantly the 100 kDa isoform of PAR-3, which lacks the aPKC binding site (Fig 4D,E). This might partly explain the absence of aPKC in the VE-cadherin-based PAR complex.

PAR-6 is recruited late to cell–cell contacts

In confluent HUVEC monolayers, PAR-6 was absent at cell–cell contacts (Fig 5A). We carried out wounding assays to analyse the localization of PAR-6 at early cell–cell contacts. Immature cell–cell contacts at the leading edge of cells migrating into the wound were positive for VE-cadherin and PAR-3 but negative for PAR-6 (Fig 5B, left panels). Also, in areas in the same samples where no wounding was performed, cell–cell contacts were positive for PAR-3 but negative for PAR-6 (Fig 5B, right panels). When cells were grown for 1 day after reaching confluency, cell contacts were positive for PAR-3 but negative for PAR-6; however, PAR-6 appeared at cell–cell contacts when cells were cultured for 3 days after reaching confluency (Fig 5C). These findings indicate that PAR-3 and PAR-6 are recruited independently to cell–cell contacts and that the localization of PAR-6 at cell junctions requires fully matured cell–cell contacts. To address the possibility that PAR-3 and PAR-6 exist independently of each other in endothelial cells, we carried out immunoprecipitation experiments. PAR-6 immunoprecipitates contained PAR-3, indicating that they coexist

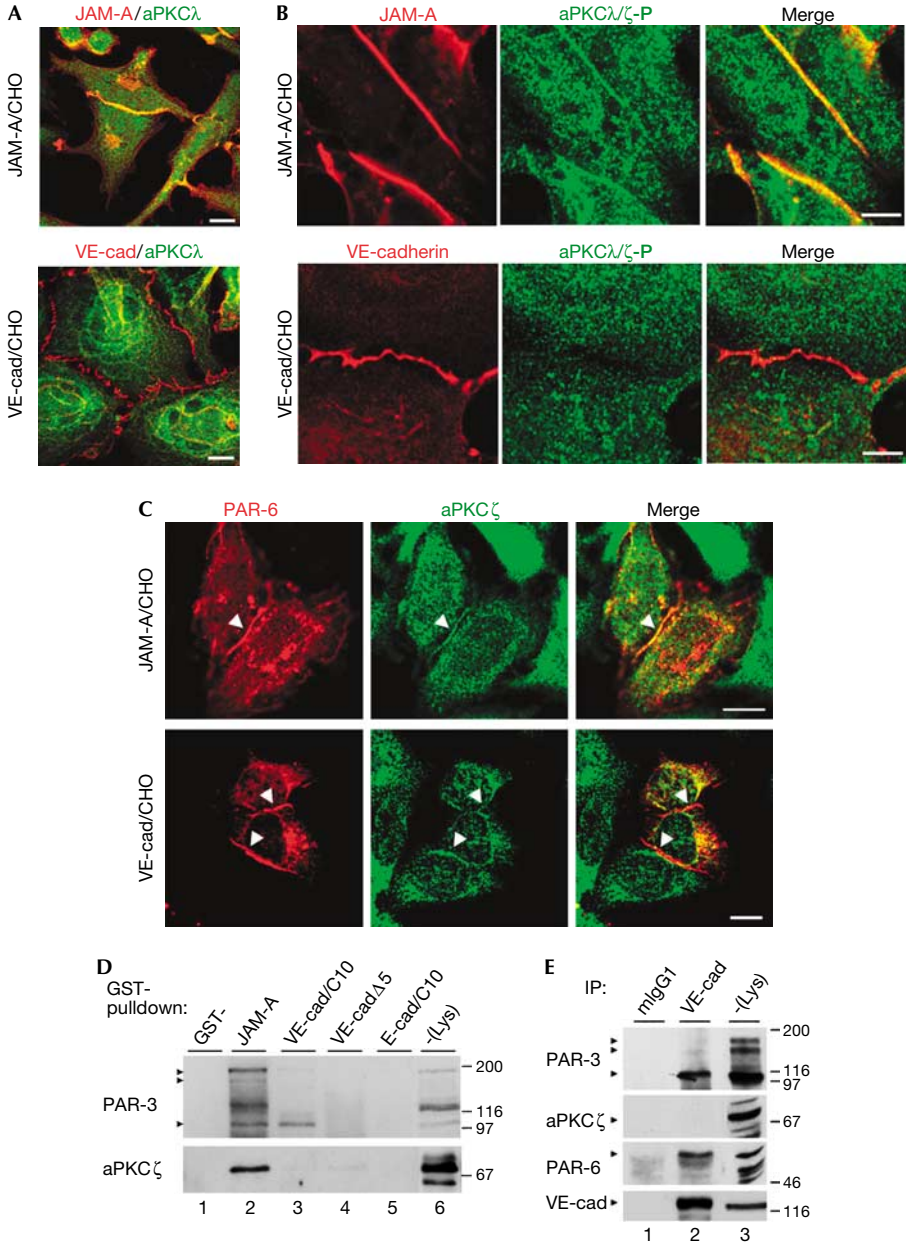

Fig 4 | The VE-cadherin-associated PAR complex lacks atypical protein kinase C. (**A**,**B**) Stable junctional adhesion molecule A (JAM-A)-transfected Chinese hamster ovary cells (JAM-A/CHO) and VE-cadherin/CHO (VE-cad/CHO) were stained for JAM-A or VE-cadherin together with antibodies against atypical protein kinase Cλ (aPKCλ; **A**) or against catalytically active aPKCζ/λ (**B**). aPKC is detectable at JAM-A-based cell–cell contacts but not at VE-cadherin-based cell–cell contacts. Scale bars, 10 μm (**A**), 5 μm (**B**). (**C**) JAM-A/CHO cells and VE-cad/CHO cells were transiently transfected with PAR-6γ and stained for ectopically expressed PAR-6 and endogenous aPKCζ. aPKCζ is not present at VE-cadherin-based cell contacts despite the presence of PAR-6. Scale bars, 10 μm. (**D**) Glutathione *S*-transferase (GST)-fusion proteins (cytoplasmic tail of JAM-A, lane 2; C-terminal ten amino acids of VE-cadherin, lane 3; cytoplasmic tail of VE-cadherin lacking the PDZ motif, lane 4; C-terminal ten amino acids of E-cadherin, lane 5) were used to precipitate PAR-3 from myEnd lysates, and precipitates were blotted with antibodies against PAR-3 and aPKCζ. In lane 6, postnuclear supernatant (1% of input) was loaded. aPKC is associated with PAR-3 precipitated with JAM-A but not with PAR-3 precipitated with VE-cadherin. (**E**) Immunoprecipitations were carried out from confluent human umbilical vein endothelial cells with antibodies indicated at the top, and precipitates were probed with antibodies indicated at the left. In lane 3, postnuclear supernatant (1% of input) was loaded. VE-cadherin immunoprecipitates contain PAR-3 and PAR-6 but not aPKC. Arrowheads in the top panel indicate the three PAR-3 isoforms.

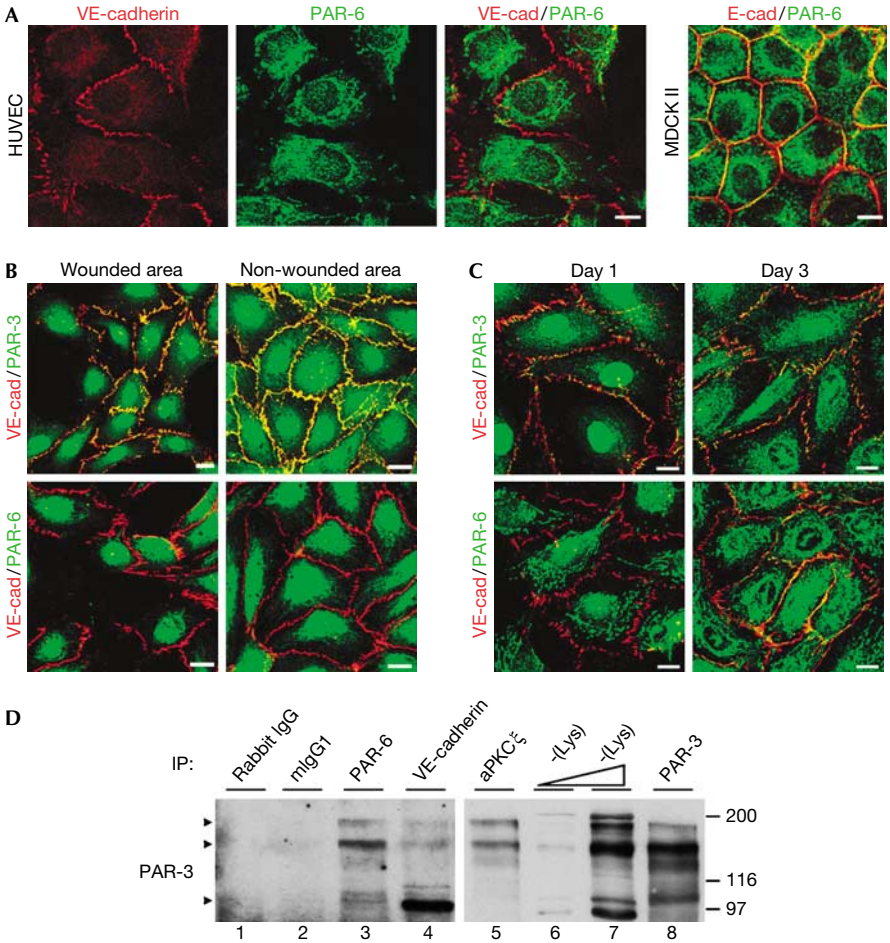

Fig 5 | PAR-3 and PAR-6 are recruited independently to cell–cell contacts in endothelial cells. (**A**) Cultured human umbilical vein endothelial cell (HUVECs) were stained for VE-cadherin and PAR-6α. PAR-6α localized to a cytoplasmic compartment and was absent from VE-cadherin-based cell–cell contacts. In epithelial cells (Mabin–Darby canine kidney (MDCK) II), PAR-6α was readily detectable at E-cadherin-based cell–cell contacts (right panel). Scale bars, 10 μm. (**B**) PAR-3 and PAR-6 do not colocalize during junction formation. HUVEC monolayers were manually wounded, further incubated for 9 h, then fixed and stained. By contrast to PAR-3, PAR-6α is not present at cell–cell contacts in areas of wounding that reflect sites of contact formation (left panels) or in non-wounded areas that reflect more mature cell–cell contacts (right panels). Scale bars, 20 μm. (**C**) PAR-6α is recruited late to endothelial cell–cell contacts during junction maturation. HUVECs were seeded at 5×10^4 cells/cm^2, incubated for 1 or 3 days, then co-stained for VE-cadherin and PAR-3 (top panel) or VE-cadherin and PAR-6α (bottom panel). PAR-6α appeared at cell–cell contacts only at day 3 after seeding, whereas PAR-3-positive cell–cell contacts were detectable already at day 1. Scale bars, 10 μm. (**D**) Two PAR protein complexes exist in endothelial cells. PAR-6 and VE-cadherin were immunoprecipitated from HUVECs and immunoprecipitates were blotted with antibodies against PAR-3. In lanes 6 and 7, postnuclear supernatant (0.2% and 2% of input, respectively) was loaded. PAR-6 is associated predominantly with the 150 and 180 kDa isoforms of PAR-3 (lane 3), whereas VE-cadherin is associated predominantly with the 100 kDa PAR-3 isoform (lane 4). VE-cad, VE-cadherin.

in the same complex (Fig 5D). Interestingly, PAR-6 immunoprecipitates contained predominantly the 180 and 150 kDa isoforms of PAR-3 (Fig 5D, lane 3), whereas VE-cadherin immunoprecipitates contained predominantly the 100 kDa isoform (Fig 5D, lane 4; see also Fig 4D,E), suggesting the existence of two PAR protein complexes: (i) the VE-cadherin-associated PAR complex devoid of aPKC, and (ii) the previously described PAR complex (Macara, 2004) consisting of PAR-3, aPKC and PAR-6 (Fig 5D, lanes 3,5; Fig 3E, lane 3).

VE-cadherin disturbs tight junctions in MDCK cells

The physical interaction of both PAR-3 and PAR-6 with VE-cadherin suggested that overexpressed VE-cadherin might affect tight junction formation, probably by sequestering PAR-3 and PAR-6. In fact, when we ectopically expressed VE-cadherin in Mabin–Darby canine kidney (MDCK) II cells, the staining of the tight-junction-associated proteins zonula occludens 1 (ZO-1) and occludin disappeared in VE-cadherin-transfected cells but not in PECAM-1-transfected cells (Fig 6). These findings suggest that VE-cadherin interacts functionally with PAR-3 and PAR-6 and thus can influence the formation of tight junctions and cell polarity.

The VE-cadherin-associated PAR complex differs from the previously described PAR complex in epithelial cells in several aspects. First, PAR-3 is associated with VE-cadherin through its third PDZ domain. The association of PAR-3 with all thus far identified integral membrane proteins that bind to PAR-3, that is,

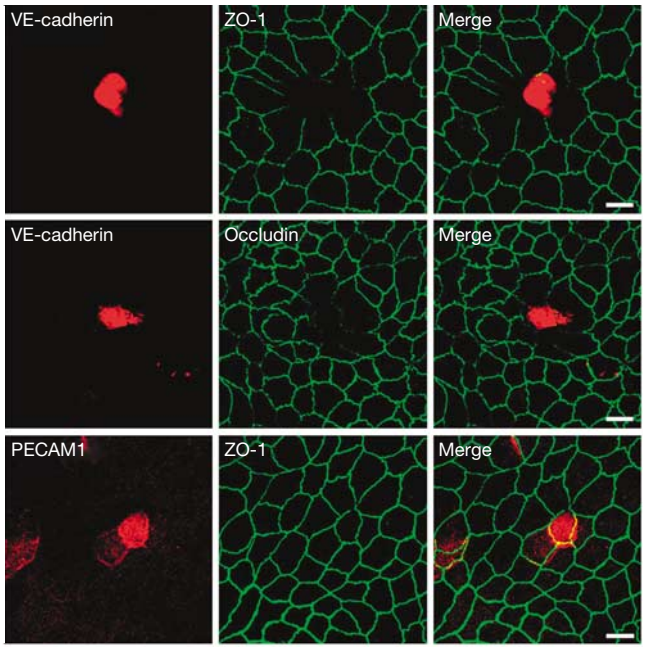

Fig 6 | Expression of VE-cadherin in epithelial cells disturbs tight junction formation. Mabin–Darby canine kidney II cells were transiently transfected with VE-cadherin or platelet–endothelial cell adhesion molecule 1 (PECAM1). Transfected cells were stained for ectopically expressed proteins and endogenous ZO-1 or occludin as indicated. ZO-1 and occludin specifically disappear from cell contacts in VE-cadherin-transfected cells. Scale bars, 10 μm.

JAM-A, JAM-B, JAM-C, nectin 1 and nectin 3, is mediated through the PDZ domain 1 of PAR-3 (Ebnet *et al*, 2003; Takekuni *et al*, 2003). Second, PAR-6 is also directly associated with VE-cadherin. This is in contrast to the tight-junction-associated PAR complex described in epithelial cells, in which PAR-6 is associated with the complex, through binding to either aPKC or PAR-3 (Macara, 2004). To our knowledge, this is the first example of a direct and independent association of both PAR-3 and PAR-6 with the same integral membrane protein. Third, PAR-3 and PAR-6 are recruited independently of each other to cell–cell contacts in endothelial cells. Fourth, the VE-cadherin-associated PAR protein complex lacks aPKC, suggesting that this PAR complex does not promote apico-basal polarity by way of aPKC.

Speculation

During the process of polarization of epithelial cells, proteins of the PAR-3–aPKC–PAR-6 complex translocate to cell–cell junctions after the establishment of spot-like adherens junctions (Suzuki *et al*, 2002). The activation of aPKC through the activity of small GTPases initiates the formation of distinct membrane domains harbouring tight junctions and adherens junctions. During the polarization process, the PAR-3–aPKC–PAR-6 complex co-segregates with other tight junction proteins from the adherens junctions and localizes exclusively to tight junctions in fully polarized epithelial cells. Our data indicate that, in endothelial cells, a distinct PAR complex that does not include aPKC is sequestered at adherens junctions. This might have consequences on endothelial cell polarity. Unlike in polarized epithelial cells, in endothelial cells, tight junctions are distributed along the entire lateral membrane domain and are frequently intermingled with communicating gap junctions and adherens junctions (Simionescu *et al*, 1975). Immunogold electron microscopy and biochemical studies indicate that proteins, which in polarized epithelial cells are separated from each other and localize exclusively to tight junctions or to adherens junctions, are scattered along the entire intercellular cleft and are in close spatial proximity (Ruffer *et al*, 2004; Vorbrodt & Dobrogowska, 2004). We speculate that the localization of a PAR complex devoid of aPKC activity at the adherens junctions of endothelial cells might be inhibitory for the polarization process, which in epithelial cells regulates the apical localization of tight junctions and thus might lead to the intermixing of membrane subdomains harbouring tight junctions and adherens junctions in endothelial cells.

METHODS

Cell culture, transfection, immunoprecipitation and immunofluorescence analyses. HEK293T cells, HeLa cells and MDCK II cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (DMEM standard medium). MyEnd and bEnd.3 cells were cultured in DMEM standard medium supplemented with 1 mM sodium pyruvate. JAM-A- and VE-cadherin-transfected CHO cells and the HUVEC culture have been described elsewhere (Ebnet *et al*, 2001, 2003; Baumeister *et al*, 2005). Cells were transiently transfected using either FuGene6 (Roche, Mannheim, Germany) or Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. GST-pulldown and immunoprecipitation experiments and immunofluorescence analyses were carried out as described previously (Ebnet *et al*, 2001).

Details on plasmid constructs and antibodies used are available as supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank R. Adams, C. Brakebusch and V. Gerke for helpful discussions, E. Cline for the exchange of PAR-6 antibodies, and M. Meyer zu Brickwedde and S. Führer for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (EB 160/2-3, SPP1111; DV, SFB629), the Max-Planck-Society and the Ministry of Education, Culture, Sports, Science and Technology of Japan (to A.S. and S.O.).

REFERENCES

Baumeister U, Funke R, Ebnet K, Vorschmitt H, Koch S, Vestweber D (2005) Association of Csk to VE-cadherin and inhibition of cell proliferation. *EMBO J* **24:** 1686–1695

Bilder D, Schober M, Perrimon N (2003) Integrated activity of PDZ protein complexes regulates epithelial polarity. *Nat Cell Biol* **5:** 53–58

Chen X, Macara IG (2005) Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nat Cell Biol* **7:** 262–269

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

Ebnet K, Aurrand-Lions M, Kuhn A, Kiefer F, Butz S, Zander K, Meyer zu Brickwedde MK, Suzuki A, Imhof BA, Vestweber D (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

Hurd TW, Gao L, Roh MH, Macara IG, Margolis B (2003) Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nat Cell Biol* **5:** 137–142

Itoh M, Sasaki H, Furuse M, Ozaki H, Kita T, Tsukita S (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:** 491–498

Lemmers C, Michel D, Lane-Guermonprez L, Delgrossi MH, Medina E, Arsanto JP, Le Bivic A (2004) CRB3 binds directly to Par6 and regulates the morphogenesis of the tight junctions in mammalian epithelial cells. *Mol Biol Cell* **15:** 1324–1333

Macara IG (2004) Parsing the polarity code. *Nat Rev Mol Cell Biol* **5:** 220–231

Mertens AE, Rygiel TP, Olivo C, van der Kammen R, Collard JG (2005) The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. *J Cell Biol* **170:** 1029–1037

Plant PJ, Fawcett JP, Lin DC, Holdorf AD, Binns K, Kulkarni S, Pawson T (2003) A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. *Nat Cell Biol* **5:** 301–308

Ruffer C, Strey A, Janning A, Kim KS, Gerke V (2004) Cell–cell junctions of dermal microvascular endothelial cells contain tight and adherens junction proteins in spatial proximity. *Biochemistry* **43:** 5360–5369

Simionescu M, Simionescu N, Palade GE (1975) Segmental differentiations of cell junctions in the vascular endothelium. The microvasculature. *J Cell Biol* **67:** 863–885

Suzuki A, Ohno S (2006) The PAR–aPKC system: lessons in polarity. *J Cell Sci* **119:** 979–987

Suzuki A, Ishiyama C, Hashiba K, Shimizu M, Ebnet K, Ohno S (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

Takekuni K, Ikeda W, Fujito T, Morimoto K, Takeuchi M, Monden M, Takai Y (2003) Direct binding of cell polarity protein PAR-3 to cell–cell adhesion molecule nectin at neuroepithelial cells of developing mouse. *J Biol Chem* **278:** 5497–5500

Tanentzapf G, Tepass U (2003) Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. *Nat Cell Biol* **5:** 46–52

Vorbrodt AW, Dobrogowska DH (2004) Molecular anatomy of interendothelial junctions in human blood–brain barrier microvessels. *Folia Histochem Cytobiol* **42:** 67–75

Yamanaka T, Horikoshi Y, Sugiyama Y, Ishiyama C, Suzuki A, Hirose T, Iwamatsu A, Shinohara A, Ohno S (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