Human homologues of the *Caenorhabditis elegans* cell polarity protein PAR6 as an adaptor that links the small GTPases Rac and Cdc42 to atypical protein kinase C

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Abstract

***Background*: Asymmetric cell division in the *Caenorhabditis elegans* embryos requires products of *par* (*par*titioning defective) genes 1–6 and atypical protein kinase C (aPKC), whereas Cdc42 and Rac, members of the Rho family GTPases, play an essential role in cell polarity establishment in yeast and mammalian cells. However, little is known about a link between PAR proteins and the GTPases in cell polarization.**

***Results*: Here we have cloned cDNAs for three human homologues of PAR6, designated PAR6α, β and γ, comprising 345, 372 and 376 amino acids, respectively. The PAR6 proteins harbour a PDZ domain and a CRIB-like motif, and directly interact with GTP-bound Rac and Cdc42 via this motif and with the aPKC isoforms PKCι/λ and PKCζ via the N-terminal head-to–head association. These interactions are not mutually exclusive, thereby allowing the PAR6 proteins to form a ternary complex with the GTPases and aPKC, both *in vitro* and *in vivo*. When PAR6 and aPKC are expressed with a constitutively active form of Rac in HeLa or COS-7 cells, these proteins co-localize to membrane ruffles, which are known to occur at the leading edge of polarized cells during cell movement.**

***Conclusion*: Human PAR6 homologues most likely play an important role in the cell polarization of mammalian cells, by functioning as an adaptor protein that links activated Rac and Cdc42 to aPKC signalling.**

Introduction

Cell polarization is crucial for a variety of biological processes such as asymmetrical cell division, directional cell migration and the establishment and maintenance of apical-basal polarity in epithelial cells (Drubin & Nelson 1996). Generation of asymmetry in the one-cell embryo of *Caenorhabditis elegans* is necessary for the establishment of the anterior-posterior axis and to ensure the proper identity of early blastomeres. It requires six *par* (*par*titioning defective) genes, mutations of which lead to cleavage pattern defects and alterations in the fates of the founder cells (Bowerman & Shelton 1999; Kemphues 2000). The PAR proteins are asymmetrically distributed in dividing cells, e.g. the PDZ (PSD95/Dlg/ZO-1) domain-containing proteins PAR-3 and PAR-6 become enriched at the anterior periphery (Watts *et al.* 1996; Hung & Kemphues 1999). Another protein, PKC-3, an atypical protein kinase C (aPKC) in the worm, co-localizes with PAR-3 and PAR-6 and exhibits a similar loss-of-function phenotype (Tabuse *et al.* 1998). The peripheral localizations of the three proteins are interdependent, suggesting that they might act together in a complex (Hung & Kemphues 1999). The molecular mechanisms of their functions, however, are largely unknown at present. A mammalian homologue of PAR-3, designated ASIP, directly associates and co-localizes with aPKC at tight junctions of epithelial cells (Izumi *et al.* 1998). In *Drosophila*, asymmetric division of neuroblasts is governed by the apical-basal polarity, in which the fruitfly PAR-3 homologue called Bazooka functions

Communicated by: Takao Shimizu
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by recruiting Inscuteable (a key organizing molecule) to the apical cortex (Jan & Jan 2000). Although one *Drosophila*, one *Xenopus*, and one mouse homologue of PAR6 have recently been identified (Hung & Kemphues 1999; Choi *et al.* 2000), their roles in these animals have remained elusive.

The Rho family GTPases, including Rac and Cdc42, serve as a molecular switch which cycles between an active GTP-bound and inactive GDP-bound states, and function by controlling the organization and dynamics of the actin cytoskeleton (Van Aelst & D'Souza-Schorey 1997; Hall 1998). In the active state, Rac and Cdc42 bind to a variety of effector proteins via interactions with target motifs, such as *C*dc42/*R*ac *i*nteractive *b*inding (CRIB) motif (Burbelo *et al.* 1995), to elicit cellular responses. Cell polarity in the budding yeast *Saccharomyces cerevisiae*, which is indispensable for budding and mating, appears ultimately to be determined by regulation of the activity of Cdc42 (Chant 1999; O'Shea & Herskowitz 2000). In mammals, this GTPase also seems to participate in the polarization of migrating macrophages (Allen *et al.* 1998) and of helper T lymphocytes towards antigen-presenting cells in the mammalian immune system (Stowers *et al.* 1995), although its downstream effector functioning in polarization remains unknown. Activated Rac induces membrane ruffles that are enriched with actin filaments around the perimeter of cells, and it is necessary for the formation of lamellipodia and cell movement (Van Aelst & D'Souza-Schorey 1997; Hall 1998). During chemotaxis (directed cell movement toward a chemoattractant) cells exhibit an elongated, polarized shape with a wide flat lamella that terminates in a ruffling lamellipodium at the leading edge, facing the direction of migration (Van Aelst & D'Souza-Schorey 1997; Hall 1998). It has recently been shown that *Dictyostellium* cells expressing a dominant negative form of Rac fail to polarize and migrate; Rac most likely regulates cellular polarity during chemotaxis by interacting with an unidentified effector protein (Chumg *et al.* 2000).

In the present study we have cloned cDNAs for three human PAR6 homologues, proteins of which are all capable of interacting directly with GTP-bound Rac and Cdc42 and simultaneously with aPKC, thereby forming a ternary complex both *in vitro* and *in vivo*. The novel complex occurs at membrane ruffles induced by activated Rac, which is suggestive of a function of PAR6 as an adaptor protein that links Rac to aPKC in cell polarization.

Results

Human homologues of PAR6

Using RT-PCR with primers synthesized based on sequences of EST clones showing similarities to the *C. elegans* PAR-6 (for detail, see Experimental procedures), we have cloned cDNAs for three human PAR6 homologues, tentatively designated PAR6α (GenBank accession no. AB041642), PAR6β (AB044555), and PAR6γ (AB044556), which are presumably encoded by distinct genes (Fig. 1). The predicted proteins PAR6α, β and γ of 345, 372 and 376 amino acids, respectively, harbour a PDZ domain, which is most highly conserved among PAR6 proteins from *C. elegans*, *Drosophila*, *Xenopus*, mouse and human (Fig. 1A). The N-terminus of the PAR6 proteins is also conserved, but to a lesser extent, and contains a stretch with high identity, which is similar to the CRIB motif (Burbelo *et al.* 1995) (amino acids 132–149, 133–150 and 134–151 of PAR6α, β and γ, respectively). On the other hand, the regions C-terminal to the PDZ domain are much less conserved.

To build a phylogenetic tree, we aligned the full lengths, N-terminal regions, or PDZ domains of PAR6 proteins from a variety of species using the Clustal W program (Thompson *et al.* 1994). The trees of the individual regions were constructed by using the neighbour-joining method with the program Treeview (Page 1996). Since we found that the three trees were essentially the same, only that which was constructed from the full-length proteins is shown in Fig. 1B. Among the human PAR6 proteins, PAR6α appears to be most comparable to the worm and fruitfly PAR6s (Hung & Kemphues 1999), suggestive of its identity as the most widespread and founding member of this family. The phylogenetic tree also indicates that the mouse and frog PAR6 proteins which had previously been identified (Hung & Kemphues 1999; Choi *et al.* 2000) are the α and β isoforms, respectively.

Expression of mRNAs for human PAR6 homologues

We next investigated the tissue distribution of human PAR6 mRNA. Northern blot analysis revealed that the PAR6α gene is expressed as an mRNA of 1.4 kb in a variety of human tissues, with preference in the brain, skeletal muscle and kidney, followed by the heart and liver (Fig. 2, upper panel). Among foetal tissues tested, the brain also contained the greatest concentration of

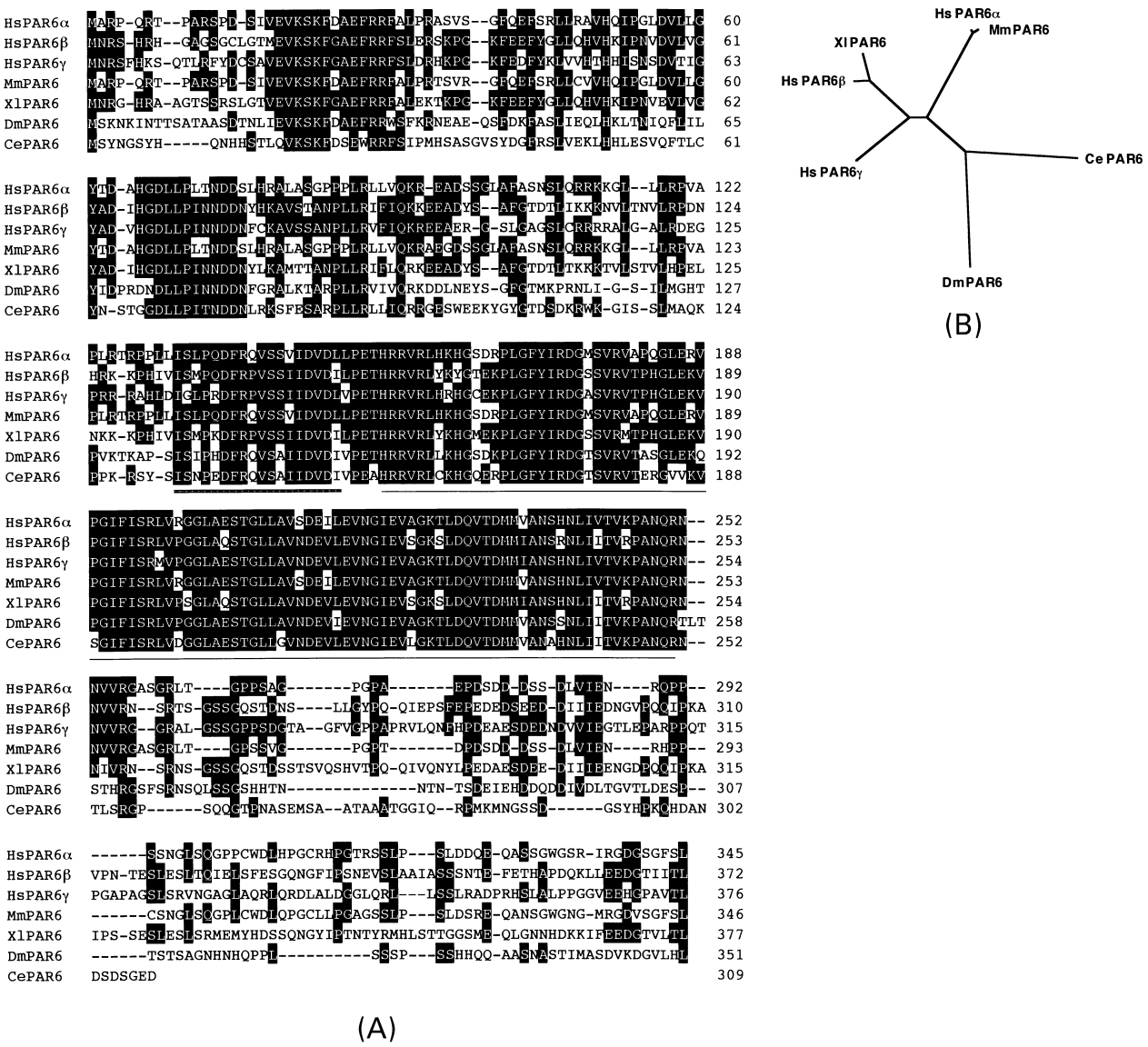

Figure 1 Deduced amino acid sequences of human PAR6α, PAR6β and PAR6γ, and a comparison with those of the worm, fruitfly, frog and mouse PAR6. (A) Alignment of the amino acid sequences of PAR6 from human (HsPAR6α, β, and γ), mouse PAR6 (MmPAR6) (Hung & Kemphues 1999), *Xenopus laevis* PAR6 (XlPAR6) (Choi *et al*. 2000), *Drosophila melanogaster* (DmPAR6) (Hung & Kemphues 1999), and *Caenorhabditis elegans* (CePAR6) (Hung & Kemphues 1999). Residues identical among at least two of the human PAR6 proteins are boxed in black. The PDZ domain and the CRIB-like motif are underlined and doubly underlined, respectively. (B) Branches of the PAR6 family tree. To build a phylogenetic tree, the full-lengths of PAR6 were aligned using the CLUSTAL W program (Thompson *et al*. 1994). The unrooted tree was constructed by using the neighbour-joining method with the program TREEVIEW (Page 1996).

the message of PAR6α. The approximately 4.8 kb-long PAR6β mRNA was predominantly detected in both adult and foetal kidneys, while much weaker but significant signals were observed in the placenta, lung and liver (Fig. 2, middle panel). Among the three PAR6 homologues, the PAR6γ mRNA of 4.0 kb was most similarly expressed in human tissues tested, with the strongest signal in the kidney of both adult and foetus (Fig. 2, lower panel). Thus the three PAR6 homologues are expressed in various tissues, but in a distinct manner, suggesting that they may function differently.

Interaction of human PAR6 homologues with atypical PKC

In *C. elegans*, PKC-3, an aPKC, has a loss-of-function phenotype that is similar to that of PAR-6 (Tabuse *et al.* 1998), and the peripheral localizations of PKC-3 and PAR-6 in embryos are interdependent (Hung & Kemphues 1999). These observations suggest a physical interaction between the proteins, although it has not been demonstrated. To test whether human PAR6 proteins bind to human aPKC, we prepared various constructs of these proteins (Fig. 3A) and used them in the yeast two-hybrid system. As shown in Fig. 3B, the full-length PAR6α, β and γ were all capable of associating with the full-length PKCι/λ. In addition, both N-terminal regions of PAR6 and aPKC were required and sufficient for this interaction (Fig. 3B). Thus PAR6 proteins most likely bind to aPKC via the N-terminal head-to-head interaction.

To address the question of whether or not the interaction between PAR6 and aPKC is direct, we expressed and purified the full-length PAR6β and the N-terminal noncatalytic domain of aPKC as His- and glutathione *S*-transferase (GST)-tagged proteins, respectively, and tested their ability to bind to each other *in vitro*. His–PAR6β was efficiently precipitated with GST–PKCι/λ (1–107) or GST–PKCζ (1–106), but not with GST alone (Fig. 3C). Similarly, His–PAR6α and His–PAR6γ were capable of binding to GST–PKCι/λ and GST–PKCζ under the same conditions (data not shown). These findings indicate that the PAR6 homologues directly interact with the members of aPKC.

We next expressed FLAG-tagged PAR6β in HeLa cells to test whether the protein interacts with aPKC. As shown in Fig. 3D, PAR6β, as well as aPKC, was precipitated with an anti-aPKC antibody, indicating that the association between PAR6 and aPKC occurs in mammalian cells. The same results were obtained when PAR6α or PAR6γ was expressed instead of PAR6β (data not shown). Taken together, PAR6 proteins most likely form a complex with aPKC in cells via the direct N-terminal head-to-head interaction.

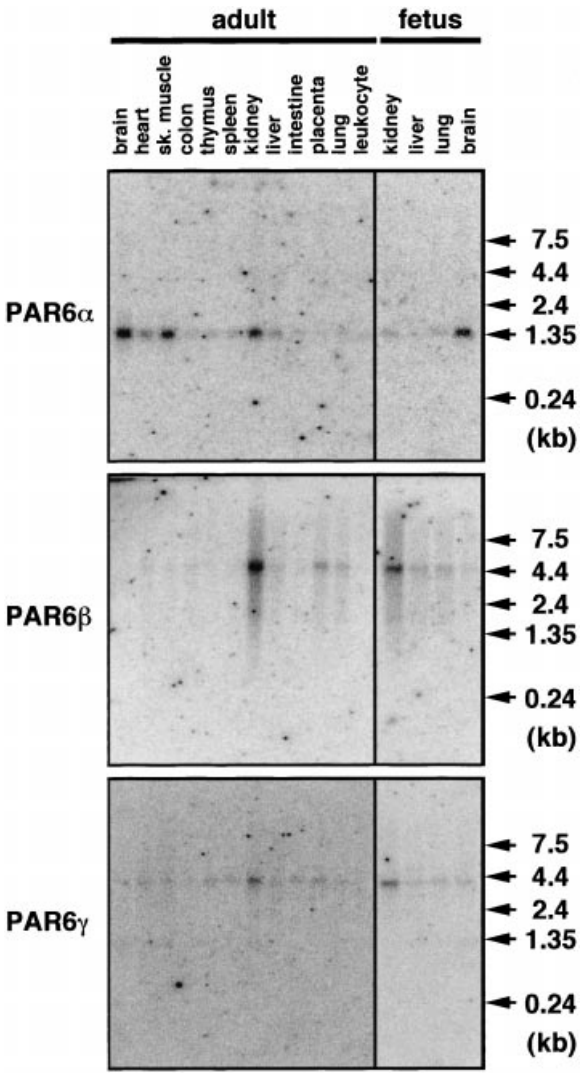

Figure 2 Expression of mRNAs of human PAR6 homologues. Human Multiple Tissue Northern blots (Clontech) were hybridized with ^{32}P-labelled cDNA fragments of human PAR6α (upper panel), PAR6β (middle panel), and PAR6γ (lower panel) (corresponding to regions encoding amino acids 19–253, 65–372 and 1–258, respectively) under high-stringency conditions using ExpressHyb (Clontech). The results are representative of two independent experiments. Positions of RNA molecular size markers are shown in kilo-bases.

Interaction of Rac and Cdc42 with human PAR6 homologues

A recent report has demonstrated that aPKC can be precipitated with GTP-bound Rac or Cdc42 (Coghlan *et al.* 2000), indicative of the occurrence of a Rac/Cdc42–aPKC interaction. However neither Rac nor Cdc42, even in their GTP-bound active form, could bind to PKCι/λ in the yeast two-hybrid system (Fig. 4A) as well as in a pull-down assay using purified proteins (data not shown), suggesting that the reported interaction of Rac/Cdc42 with aPKC is mediated by a third-party protein. One of the candidates would be PAR6, since it binds directly to aPKC as described above, and contains a remnant of the CRIB motif (Fig. 1A), a target of Rac and Cdc42 (Burbelo *et al.* 1995). As expected, all the three PAR6 proteins exhibited

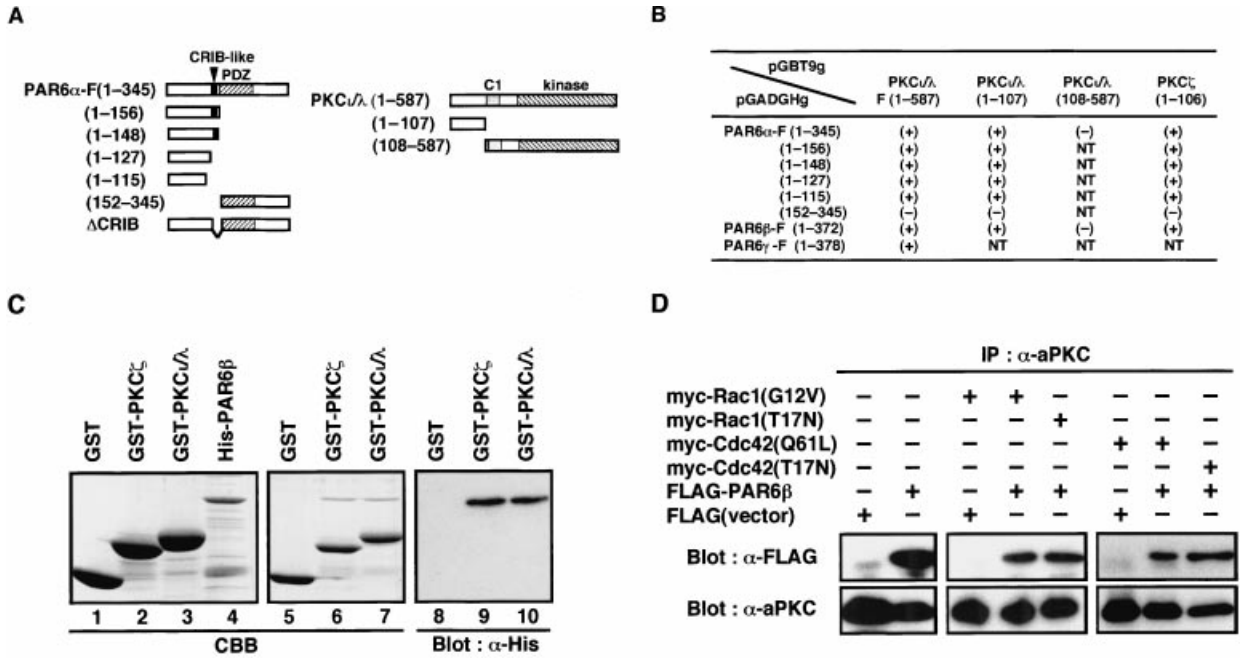

Figure 3 Interaction of human PAR6 homologues with PKCι/λ and PKCζ. (A) Schematic representation of constructs of human PAR6α and PKCι/λ. PAR6α contains a CRIB-like motif and a PDZ domain. PKCι/λ comprises the C-terminal kinase domain and the N-terminal noncatalytic domain, the latter of which contains a remnant of the cysteine-rich C1 domain. (B) Two–hybrid interactions of human PAR6 homologues with PKCι/λ and PKCζ. Histidine-independent growth was tested using yeast Y190 cells that were co-transformed by pairs of pGADGHg encoding the PAR6 proteins and pGBT9g encoding the indicated form of PKCι/λ or PKCζ, as described under 'Experimental procedures.' NT, not tested. (C) Direct interaction of human PAR6β with aPKC. Twenty μg of recombinant GST alone (lane 1), GST-tagged PKCζ (lane 2), and GST-tagged PKCι/λ (lane 3), and 2 μg of recombinant His-tagged PAR6β (lane 4) were applied to SDS-PAGE and stained with Coomassie Brilliant Blue. His–PAR6β (20 μg) was incubated with 10 μg of GST alone (lanes 5 and 8), GST–PKCζ (lanes 6 and 9), or GST–PKCι/λ (lanes 7 and 10), and proteins were pulled down with glutathione-Sepharose-4B beads. The precipitated proteins were subjected to SDS-PAGE, and stained with Coomassie Brilliant Blue (lanes 5–7) or analysed by Western blot with an anti-$(His)_5$ antibody (lanes 8–10). (D) Physical interaction of human PAR6β with aPKC in HeLa cells. The expression constructs indicated above each lane were co-transfected into HeLa cells. Cell lysates (equivalent to 6×10^5 cells) were analysed by immunoprecipitation (IP) with an anti-aPKC antibody followed by Western blot (Blot) with an anti-FLAG (upper panels) or anti-aPKC (lower panels) antibody.

yeast two–hybrid interactions with activated Rac1, Rac2 and Cdc42, but not with RhoA (Fig. 4A, and data not shown). The interactions appear to require the CRIB-like motif, since truncation of the motif resulted in defective binding to Cdc42 and Rac1 (Fig. 4A).

To clarify whether Rac1 and Cdc42 bind directly to the PAR6 homologues, we performed a pull-down binding assay using purified proteins. As shown in Fig. 4B, GTP-bound Rac1, interacted with PAR6α, β and γ, whereas GDP-bound one did not. Similarly, Cdc42 in the GTP-bound state associated with the three isoforms of PAR6 (Fig. 4B). From these experiments, we roughly estimated the K_d values of Rac/Cdc42 for the PAR6 proteins at approximately 3–15 μM, which are higher than those for other targets of the GTPases (Van Aelst & D'Souza-Schorey 1997; Thompson *et al.* 1998; Hall 1998). To compare the affinities of Rac/Cdc42 for the PAR6 proteins with that for the protein kinase PAK2, a typical high-affinity effector of the GTPases (Thompson *et al.* 1998; Zhang *et al.* 1998; Akasaki *et al.* 1999), we expressed and purified the Rac/Cdc42-binding region of human PAK2, and used the domain for the pull-down assay. PAK2 bound to GTP-loaded Rac/Cdc42 with about two orders of magnitude higher affinity ($K_d = 0.1–0.2$ μM) under the same experimental conditions (data not shown).

Formation of a ternary complex containing Rac/Cdc42, PAR6 and aPKC, both *in vitro* and *in vivo*

The present findings show that PAR6 interacts directly with Rac/Cdc42 and aPKC via its distinct moieties,

A

pGADGHg \ pGBT9g	Rac1 G12V	Rac1 T17N	Cdc42 Q61L	Cdc42 T17N	RhoA Q63L	RhoA T19N
PAR6α-F (1–345)	(+)	(–)	(+)	(–)	(–)	(–)
(1–156)	(+)	(–)	(+)	(–)	NT	NT
(1–148)	(–)	(–)	(–)	(–)	NT	NT
(1–127)	(–)	(–)	(–)	(–)	NT	NT
(1–115)	(–)	(–)	(–)	(–)	NT	NT
(152–345)	(–)	(–)	(–)	(–)	NT	NT
ΔCRIB	(–)	(–)	(–)	(–)	NT	NT
PAR6β-F (1–372)	(+)	(–)	(+)	(±)	(–)	(–)
ΔCRIB	(–)	(–)	(–)	(–)	NT	NT
PAR6γ-F (1–378)	(+)	(–)	(+)	(–)	(–)	(–)
ΔCRIB	(–)	(–)	(–)	(–)	NT	NT
PKCι/λ-F (1–587)	(–)	(–)	(–)	(–)	NT	NT

B

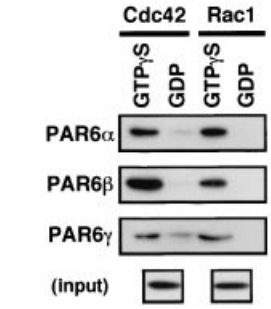

Figure 4 Interaction of human PAR6 homologues with the small GTPases Rac and Cdc42. (A) Two–hybrid interaction of human PAR6 homologues with the small GTPases Rac and Cdc42. Histidine-independent growth was tested using yeast Y190 cells that were co-transformed by pairs of pGADGHg encoding the PAR6 proteins or PKCι/λ, and pGBT9g encoding the indicated form of Rac1, Cdc42, or RhoA, as described under 'Experimental procedures.' NT, not tested. (B) Direct interaction of human PAR6 homologues with the small GTPases Rac and Cdc42. Recombinant His–Cdc42 or His–Rac1 (20 μg), preloaded with GTPγS or GDP, was incubated with 100 μg of GST–PAR6α (the most upper panel), GST–PAR6β (the second panel), or GST–PAR6γ (the third panel) in 500 μL of PBS containing 1% Triton X-100. Proteins were pulled down with glutathione-Sepharose-4B, and analysed by Western blot with an anti-$(His)_5$ antibody as described in 'Experimental procedures.' The results are representative of three independent experiments. (input): Recombinant GTPases used, His–Rac1 (0.5 μg) and His–Cdc42 (0.5 μg), were also analysed directly by Western blot with an anti-$(His)_5$ antibody (the lowest panels).

the CRIB-like motif and the N-terminal domain, respectively, suggesting that PAR6 can bind simultaneously to both partners. To test this possibility, we expressed and purified His–Rac1, His–PAR6β, and GST–PKCι/λ (1–107) in order to use them in a pull-down assay. In the presence of PAR6β, PKCι/λ precipitated GTPγS-loaded Rac1 (Fig. 5A, lane 5), while the kinase by itself failed to interact with Rac (Fig. 5A, lane 3). Similarly, PAR6β tethered GTPγS-loaded Cdc42 to PKCι/λ (Fig. 5B). These findings clearly demonstrate that PAR6β is capable of directly linking GTP-bound Rac/Cdc42 to PKCι/λ as an adaptor protein. The same results were obtained when PAR6α or PAR6γ was used instead of PAR6β (data not shown).

To test whether activated Rac/Cdc42 exists in a complex containing PAR6 and aPKC *in vivo*, we expressed myc–Rac1(G12V), a constitutively active form of the GTPase, and FLAG–PAR6β in HeLa cells. As shown in Fig. 5C, aPKC was co-immunoprecipitated with Rac1(G12V) in a manner that was dependent on the existence of PAR6β. On the other hand, Rac1(T17N), a dominant negative mutant, was not complexed with aPKC. Similarly, in the presence of PAR6β, a GTP-bound form of Cdc42(Q61L), but not a GDP-bound form of Cdc42(T17N), associated with aPKC in HeLa cells (Fig. 5D). In both cases, PAR6β interacted with aPKC independently of the state of Rac1 or Cdc42 (Fig. 3D), indicating that this interaction is constitutive. Thus Rac and Cdc42, in their active state, most likely form a complex with aPKC via the adaptor protein PAR6 in cells.

Co-localization of PAR6β and PKCι/λ with an active form of Cdc42 or Rac1 in cells

We finally investigated the intracellular localization of PAR6 and aPKC in mammalian cells expressing activated Cdc42 or Rac1. When PKCι/λ was over-expressed as a green fluorescent protein (GFP)-fusion protein in HeLa cells (Fig. 6A), the kinase primarily localized to the nucleus, as was previously reported (Akimoto *et al*. 1996; Coghlan *et al*. 2000). In cells transfected with FLAG-tagged Cdc42(Q61L), a constitutively active mutant, PKCι/λ translocated to the cytoplasm and cell periphery, where the active Cdc42 existed (Fig. 6B,C). This is consistent with a recent observation that expression of activated Cdc42 in NIH 3T3 cells results in the translocation of PKCι/λ from the nucleus into the cytosol, and Cdc42 and PKCι/λ co-localize at the plasma membrane and cytosol (Coghlan *et al*. 2000). Intriguingly, GFP–PAR6α also co-localized with Cdc42(Q61L) to the cell periphery, when it was co-expressed in HeLa cells (Fig. 6D,E). The translocation of PAR6α to the cell periphery was not observed in cells transfected with Cdc42(T17N), a dominant negative form of the GTPase (Fig. 6F,G). The same results were obtained when COS-7 cells were used instead of HeLa cells (data not shown).

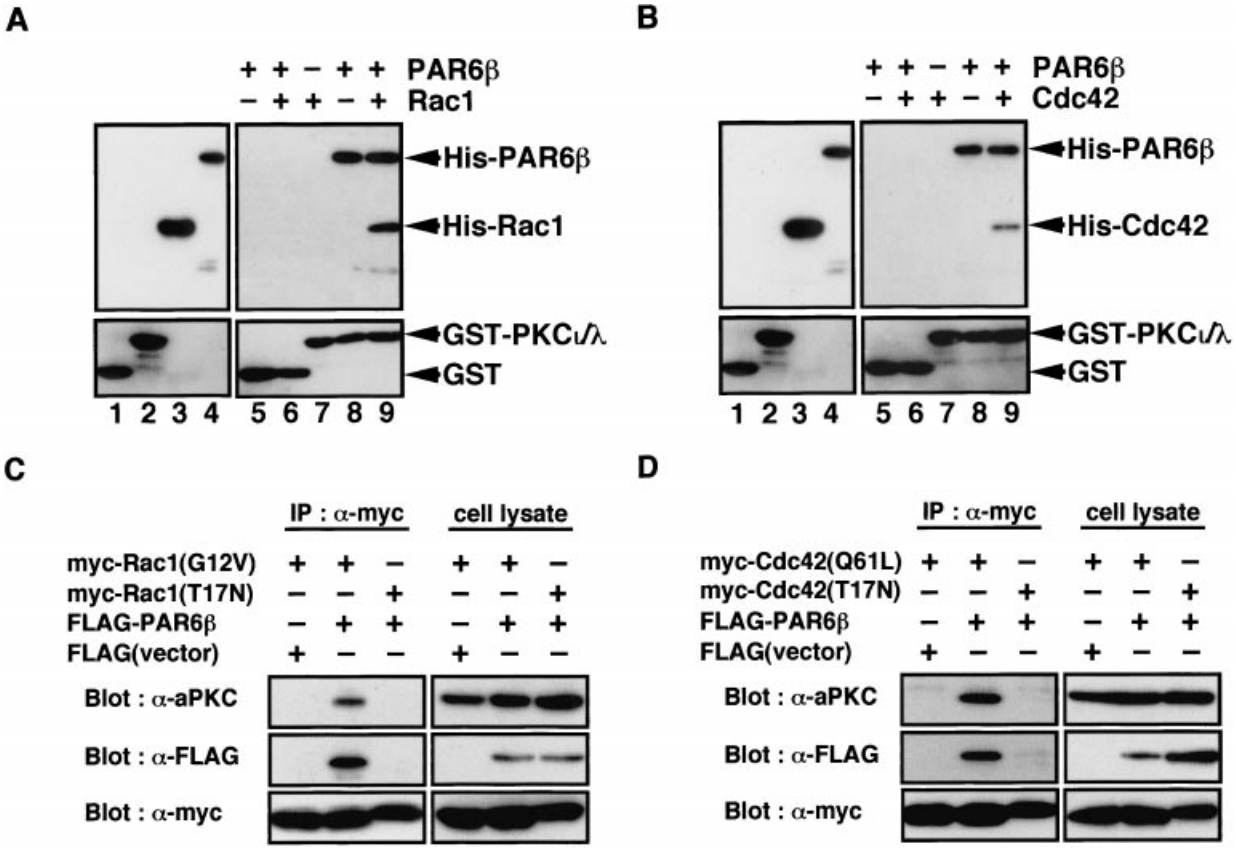

Figure 5 Formation of a ternary complex containing activated Rac1/Cdc42, PAR6β and aPKCι/λ. (A) *In vitro* formation of a complex containing purified Rac1, PAR6β, and aPKCι/λ. Twenty μg of GST alone (lanes 5 and 6) or GST–PKCι/λ (1–107) (lanes 7–9) was incubated with or without 40 μg of His–PAR6β in the presence or absence of 40 μg of GTPγS-loaded His–Rac1, and pulled down with glutathione-Sepharose-4B. The precipitated proteins were analysed by Western blotting with an anti-$(His)_5$ (right upper panel) or anti-GST (right lower panel) antibody as described in 'Experimental procedures.' Recombinant proteins used: GST alone (5 μg; lane 1), GST–PKCι/λ (1–107) (5 μg; lane 2), His–Rac1 (1 μg; lane 3), and His–PAR6β (0.5 μg; lane 4), were also analysed directly by Western blot with an anti-$(His)_5$ (left upper panel) or anti-GST (left lower panel) antibody. (B) *In vitro* formation of a complex containing purified Cdc42, PAR6β and aPKCι/λ. Twenty μg of GST alone (lanes 5 and 6) or GST–PKCι/λ (1–107) (lanes 7–9) was incubated with or without 40 μg of His–PAR6β in the presence or absence of 40 μg of GTPγS-loaded His–Cdc42, and pulled down with glutathione-Sepharose-4B. The precipitated proteins were analysed by Western blot with an anti-$(His)_5$ (upper panel) or anti-GST (lower panel) antibody as described in 'Experimental procedures.' Recombinant proteins used, GST alone (5 μg; lane 1), GST–PKCι/λ (1–107) (5 μg; lane 2), His–Cdc42 (1 μg; lane 3), and His–PAR6β (0.5 μg; lane 4), were also analysed directly by Western blot with an anti-$(His)_5$ (upper panel) or anti-GST (lower panel) antibody. (C) Formation of a complex containing activated Rac1, PAR6β, and aPKCι/λ in HeLa cells. The expression constructs indicated above each lane were co-transfected into HeLa cells. Cell lysates (equivalent to 6×10^5 cells) were analysed by immunoprecipitation (IP) with an anti-myc antibody followed by Western blot (Blot) with an anti-aPKC (upper panels), anti-FLAG (middle panels) or anti-myc (lower panels) antibody. Proteins in cell lysates (equivalent to 2×10^4 cells) were also analysed directly by Western blot. (D) Formation of a complex containing activated Cdc42, PAR6β, and aPKCι/λ in HeLa cells. The expression constructs indicated above each lane were co-transfected into HeLa cells. Cell lysates (equivalent to 6×10^5 cells) were analysed by immunoprecipitation (IP) with an anti-myc antibody followed by Western blot (Blot) with an anti-aPKC (upper panels), anti-FLAG (middle panels) or anti-myc (lower panels) antibody. Proteins in cell lysates (equivalent to 2×10^4 cells) were also analysed directly by Western blot. The results of this figure are representative of three independent experiments.

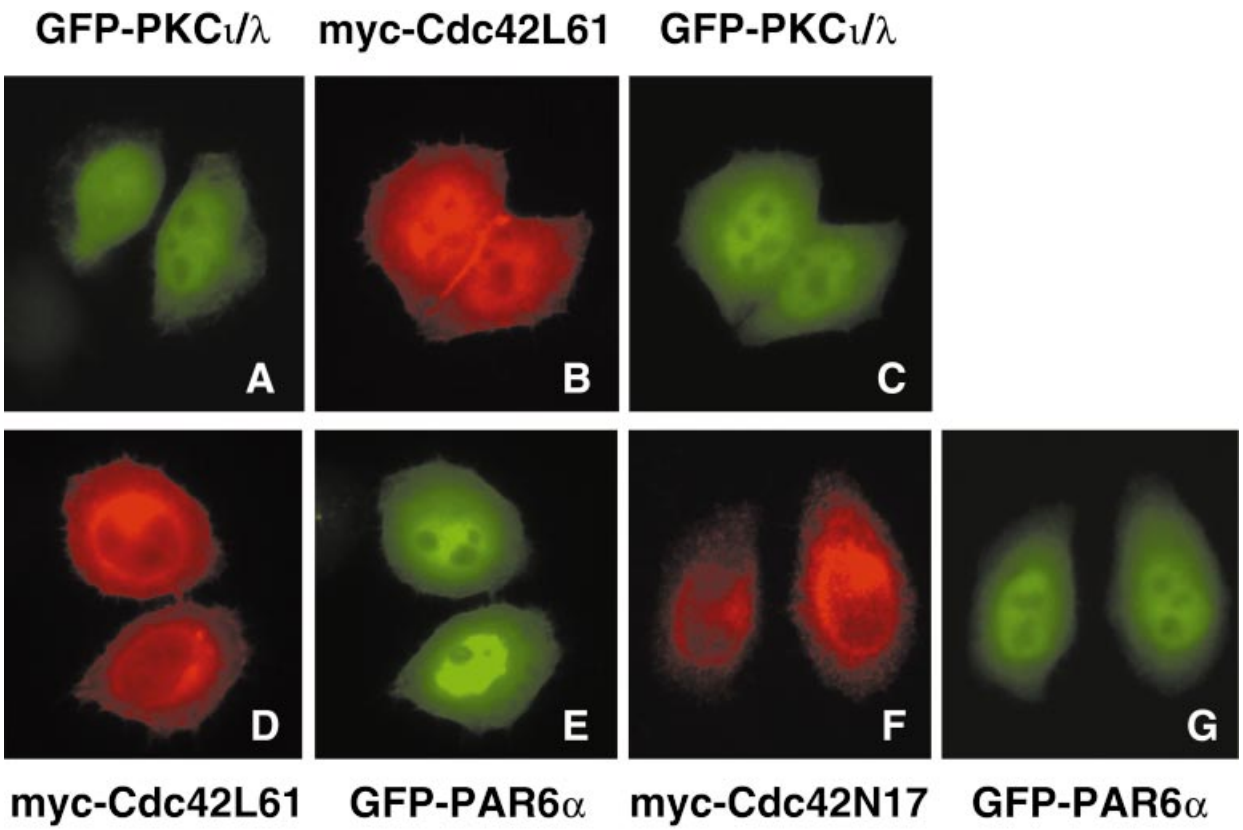

Figure 6 Co-localization of human PAR6α and PKCι/λ with activated Cdc42 at the cell periphery. Vector encoding GFP–PKCι/λ (panels A–C) or GFP–PAR6α (panels D–G) was co-transfected in HeLa cells with pEF-BOS vector (panel A), or the vector for myc-tagged Cdc42 (Q61L) (panels B–E) or myc-tagged Cdc42 (T17N) (panels F and G). Cells were fixed and stained for myc–Cdc42 with an anti-myc antibody (panels B, D and F). Expressed GFP-fusion proteins are shown in green (panels A, C, E and G), while myc-tagged proteins expressed are presented in red (panels B, D and F).

The finding that GTP-bound Rac1 is capable of forming a complex with PAR6 and aPKC (Fig. 5) raised the possibility that PAR6 and aPKC may localize to membrane ruffles which are induced by activated Rac (Van Aelst & D'Souza-Schorey 1997; Hall 1998). To test this, we examined the localization of PAR6α and PKCι/λ in HeLa cells transfected with a constitutively active, GTP-bound form of Rac1, Rac1(G12V). When PAR6α (Fig. 7A,B) or PKCι/λ (Fig. 7E,F) was expressed as a GFP-fusion protein, both proteins co-localized with myc-tagged Rac1(G12V) to membrane ruffles, which were also stained with phalloidin (data not shown). In addition, when both FLAG-tagged PAR6α and GFP–PKCι/λ were simultaneously expressed in HeLa cells, transfection of Rac1(G12V) led to a co-localization of PAR6α and PKCι/λ to membrane ruffles (Fig. 7G,H). On the other hand, a dominant negative, GDP-bound form of Rac1, Rac1(T17N), did not cause a membrane localization of PAR6α (Fig. 7C,D) or PKCι/λ (data not shown). The same results were obtained when COS-7 cells were used instead of HeLa cells, or when HeLa cells were transfected with PAR6β or PAR6γ instead of PAR6α (data not shown). These observations suggest that a ternary complex containing activated Rac, PAR6 and aPKC occurs at the membrane ruffles induced by activated Rac. Thus, PAR6 proteins appear to function as adaptors that link activated Rac/Cdc42 to aPKC at the cell level.

Discussion

In the present study we have cloned three human cDNA, each encoding a distinct homologue of the *C. elegans* polarity protein PAR6, designated PAR6α, β and γ. Among the three PAR6 proteins, those which are conserved are the N-terminal domain, the CRIB-like motif and the PDZ domain (Fig. 1). Through the

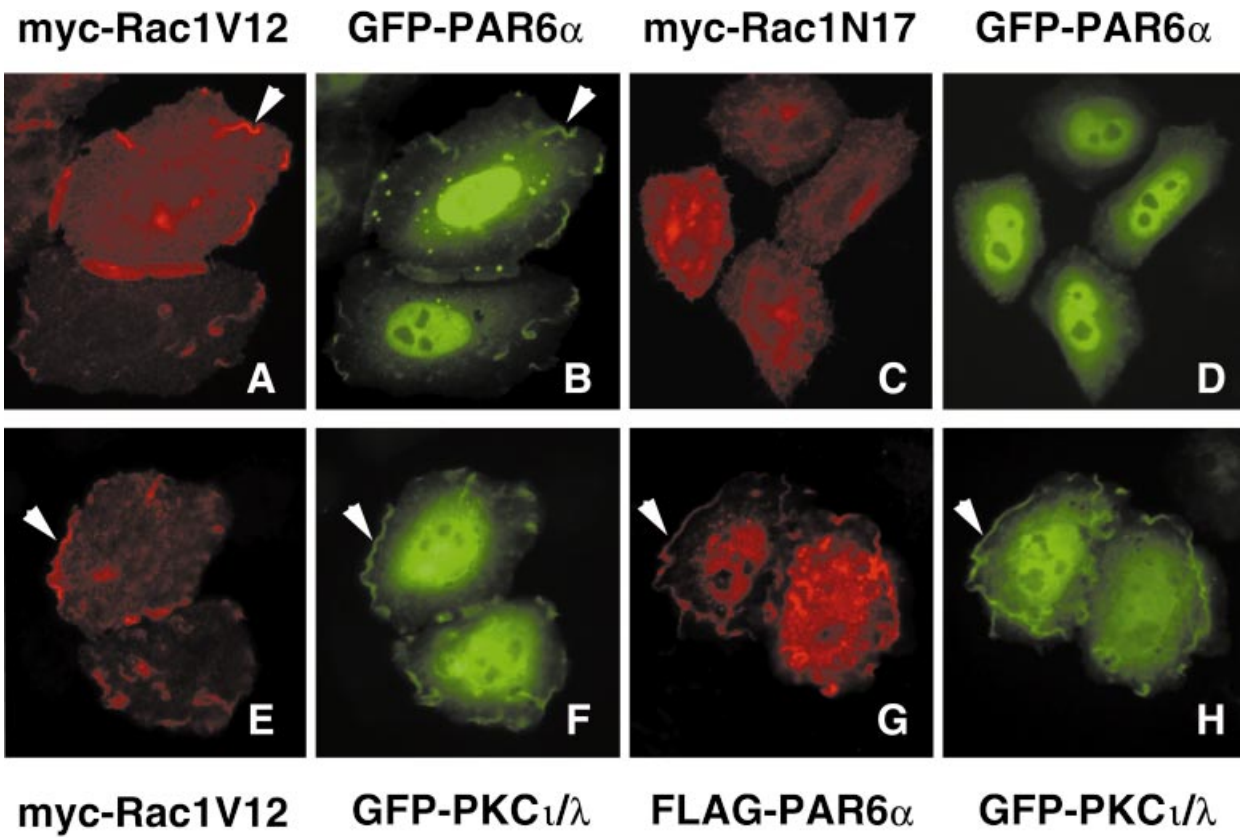

Figure 7 Co-localization of human PAR6α with Rac1 and PKCι/λ to membrane ruffles. Vector encoding myc-tagged Rac1 (G12V) (panels A, B and E–H) or myc-tagged Rac1 (T17N) (panels C and D) was co-transfected in HeLa cells with vector for GFP–PAR6α (panels A–D), that for GFP–PKCι/λ (panels E and F), or both those for FLAG-tagged PAR6α and GFP–PKCι/λ (panels G and H). Cells were fixed and stained for myc–Rac (panels A, C and E) or FLAG–PAR6α (panel G). The myc- or FLAG-tagged proteins expressed are shown in red (panels A, C, E and G), while expressed GFP-fusion proteins in green (panels B, D, F and H). Arrows indicate membrane ruffles.

N-terminal domain, the human PAR6 proteins directly associate with the two isoforms of human aPKC, PKCι/λ and PKCζ (Fig. 3), a physical interaction which appears to constitutively occur in human cells (Fig. 3). The N-terminal noncatalytic region of aPKC is required and sufficient for the association with the PAR6 proteins (Fig. 3), indicating that PAR6 binds to aPKC via the N-terminal head-to-head interaction. We also present evidence that the PAR6 proteins interact directly—via the CRIB-like motif—with the Rho family GTPases Rac and Cdc42 in their active states (Fig. 4). Thus the human PAR6 proteins most likely function as an adaptor for a novel link between activated Rac/Cdc42 and aPKC (Figs 5, 6 and 7).

Among effector proteins of Rac and Cdc42, PAR6 displays a low affinity for the GTPases: the K_d is about one-to-two orders of magnitude higher than that for other typical targets (Van Aelst & D'Souza-Schorey 1997; Hall 1998; Thompson *et al.* 1998; Zhang *et al.* 1998; Akasaki *et al.* 1999). Rac is also known to bind, with a low affinity, to $p67^{phox}$, an indispensable activator of the microbicidal NADPH oxidase complex in phagocytes; this interaction is absolutely required for the oxidase activation (Koga *et al.* 1999). In both cases, their affinities are estimated in a solution, while these *in vivo* bindings occur at the membrane, where another interaction via other components of the complex might stabilize a Rac/Cdc42–effector dimer. What is the role for the incorporation of GTP-bound Rac/Cdc42 into the PAR6–aPKC complex? The binding leads to recruitment of aPKC to the plasma membrane (Figs 6 and 7), where the activated GTPases exist. Intriguingly, it has recently been shown that the kinase activity of aPKC is positively regulated by the Cdc42–PAR6 complex (Yamanaka & Ohno, unpublished results). Thus, the formation of the Rac/Cdc42–PAR6–aPKC complex likely elicits two events, i.e. recruitment of aPKC and activation of the kinase. Phosphorylation of

an unidentified substrate by activated aPKC at the membrane may play important roles in biological processes such as cell polarization.

As is shown in the present study, the human PAR6 proteins can be co-localized with activated Rac and aPKC to membrane ruffles and lamellipodia (Fig. 7), which are known to occur at the leading edge of polarized cells during cell movement (Van Aelst & D'Souza-Schorey 1997; Hall 1998). Thus PAR6 homologues are likely to play an important role in polarization in mammalian cells, as in *C. elegans*. It is, however, unclear at present whether the Rac–PAR6–aPKC complex is involved in membrane ruffle formation. The N-terminus of PAR6α (amino acids 1–115), lacking the CRIB-like motif, is capable of binding to aPKC but not to Rac1(G12V) (Figs 3 and 4), and is therefore expected to block the ternary complex formation as a dominant negative inhibitor. Our preliminary experiments show that the expression of PAR6α (1–115) fused to GFP does not appear to suppress Rac1(G12V)-induced membrane ruffling in HeLa cells (Noda & Sumimoto, unpublished observation), suggesting that the Rac–PAR6–aPKC complex may not be required for membrane ruffle formation *per se*.

At the molecular level, the present study may fill a gap between two ideas about cell polarization, which have thus far independent. The one is based on genetic studies of *C. elegans*, and has established the importance of the PAR proteins and aPKC (Drubin & Nelson 1996; Kemphues 2000). The other has its origin in a series of genetic and molecular biological studies on the budding yeast, leading to identification of Cdc42 as an essential factor for the establishment of polarity (Chant 1999; O'Shea & Herskowitz 2000). A currently increasing body of evidence suggests that the Rho family GTPases Cdc42 and Rac also participate in the polarization of mammalian cells (Nobes & Hall 1999; Waterman-Storer *et al.* 1999). It has also been demonstrated that the mutation of a putative exchange factor for the Rho family GTPases leads to cell polarization defects in *Drosophila* embryogenesis (Häcker & Perrimon 1998), and that CED-10, a *C. elegans* homologue of Rac, is involved in the control of the polarized extension of cell surfaces (Reddien & Horvitz 2000). In addition, actin rearrangement, which is regulated by the Rho family GTPases (Van Aelst & D'Souza-Schorey 1997; Hall 1998), plays a crucial role in cell polarity establishment of the budding yeast and mammalian epithelial cells (Drubin & Nelson 1996) as well as in the asymmetric cell division of *C. elegans* embryos (Hill & Strome 1990; Hird & White 1993; Rose *et al.* 1995). Thus the Rac/Cdc42 signalling pathway appears to regulate cell polarity in a wide variety of organisms. The present finding of a PAR6-mediated link between Rac/Cdc42 and aPKC suggest that Rac and Cdc42 function along with PAR6 and aPKC in cell polarization.

In *C. elegans* embryos, PAR-6 co-localizes with PAR-3 to the anterior periphery (Hung & Kemphues 1999). Human PAR6 proteins interact directly with the N-terminal region of aPKC, while the mammalian PAR3 homologue ASIP binds to the C-terminal catalytic domain of aPKC (Izumi *et al.* 1998). It is therefore likely that aPKC can simultaneously bind to both PAR6 and ASIP, and thus ASIP is involved in the Rac/Cdc42–PAR6–aPKC complex. The theory of this involvement may be supported by the fact that ASIP co-localizes with aPKC at tight junctions (Izumi *et al.* 1998), whose structure and function seem to be regulated by Rac in mammalian epithelial cells (Jou *et al.* 1998).

The molecules that act upstream or downstream of the Rac/Cdc42–PAR6–aPKC complex in cell polarization are presently unknown. Phosphatidylinositol 3-kinase may function upstream, since its products can activate guanine nucleotide exchange factors for Rac/Cdc42 (Van Aelst & D'Souza-Schorey 1997; Hall 1998; Akasaki *et al.* 1999), which seems to be consistent with the previous observation that the lipid kinase likely mediates EGF- or PDGF-induced activation of aPKC (Akimoto *et al.* 1996). The PAR6-centred complex may contain more proteins, because PAR6 harbours a PDZ domain, a module involved in protein–protein interactions (Ponting *et al.* 1997). Identification of molecules that interact with the PDZ domain or the C-terminal less conserved region of PAR6 will help our understanding of the role of this evolutionarily conserved adaptor protein in cell polarization. A recent report has shown that PKCζ and a human homologue of PAR6, identical to the PAR6α of the present study, are involved in the cell transformation that is induced by activated Rac/Cdc42 (Qiu *et al.* 2000), suggestive of pleiotropic function of the PAR6-centred complex. During the completion of the present study, another two papers have reported that mammalian PAR6 links Cdc42/Rac to PAR3 and aPKC (Joberty *et al.* 2000; Lin *et al.* 2000).

In summary, we have cloned cDNAs for three human PAR6 homologues, designated PAR6α, β and γ (Fig. 1), which are expressed in various tissues, but in a distinct manner (Fig. 2). The PAR6 proteins are all capable of interacting directly with GTP-bound Rac and Cdc42 via the CRIB-like motifs (Fig. 4) and

simultaneously with aPKC via the N-terminal head-to-head interaction (Fig. 3), thereby forming a ternary complex both *in vitro* and *in vivo* (Figs 5–7). The novel complex occurs at membrane ruffles induced by activated Rac (Fig. 7). These findings suggest that PAR6 proteins play an important role in cell polarization by functioning as an adaptor that links Rac to aPKC.

Experimental procedures

Cloning of human homologues of PAR6

A search of EST databases with the *C. elegans* PAR-6 yielded three groups of human cDNA clones (GENBANK accession numbers AA 975142, AI 950015 and AI 991628, which are representatives of the three groups). We thus synthesized three pairs of unique oligonucleotide primers: 5′-GCAGGATCCATGGCCCGGCCGCAGAGGAC-3′ and 5′-GATGAATTCAGAGGCTGAAGCCACTACC-3′; 5′-GCAGGATCCATGAACCGCAGCCACCG-3′ and 5′-GATGAATTCATAATGTTATGATTGTTCCATCT-3′; and 5′-GCTGGATCCATGAACCGAAGTTTTCACAAG-3′ and 5′-GATGTCGACTCAGAGCGTGACCGCGGG-3′. Here sequences from the EST clones are underlined. The cDNA for the first group of PAR6 homologues (PAR6α) was amplified by RT-PCR using RNA prepared from the human neuroblastoma SH-SY5Y cells, the cDNA for the second group (PAR6β) from the human renal cell carcinoma KPK1 cells (Naito *et al.* 1982), and the cDNA for the third group (PAR6γ) from human renal cell carcinoma KPK1 and SN12C cells (Naito *et al.* 1988). The products were cloned into the modified two-hybrid vector pGBT9g (Ago *et al.* 1999; Koga *et al.* 1999). All clones thus obtained were sequenced in both directions.

Northern blot analysis

Human Multiple Tissue Northern blots (Clontech) were hybridized with ^{32}P-labelled cDNA fragments of PAR6α, PAR6β and PAR6γ (corresponding to the regions encoding amino acids 19–253, 65–372 and 1–258, respectively) under highly stringents condition using ExpressHyb (Clontech).

Plasmid construction

The constructs of human Rac1, Rac2, Cdc42 and RhoA were prepared as previously described (19–21) except for pEF-BOS-myc-Rac1(G12V) (Komuro *et al.* 1996), which was a generous gift from Dr Yoshimi Takai (Osaka University). The cDNA for human PKCι/λ and PKCζ were obtained by RT-PCR using RNA from SH-SY5Y cells. The DNA fragments encoding PAR6α (1–156), PAR6α (1–148), PAR6α (1–127), PAR6α (1–115), and PAR6α (152–345) were amplified from the human PAR6α cDNA described above by PCR using specific primers. The cDNAs encoding CRIB-like motif-truncated PAR6 proteins, PAR6αΔCRIB (lacking amino acids 131–149), PAR6βΔCRIB (lacking amino acids 132–150), and PAR6γΔCRIB (lacking amino acids 133–151), were obtained by PCR-mediated site-directed mutagenesis. The PCR products were ligated to pGBT9g (Akasaki *et al.* 1999; Koga *et al.* 1999), pGADGHg (Akasaki *et al.* 1999; Koga *et al.* 1999), pGEX-2T or -4T (both from Amersham Pharmacia Biotech), pProEX-HTb (Life Tech), pEGFP-C1 (Clontech), or pEF-BOS (Mizushima & Nagata 1990), which was a generous gift form Dr Shigekazu Nagata (Osaka University). All the constructs were sequenced to confirm their identities.

Two-hybrid experiments

Various combinations between the pGBT9g and pGADGHg plasmids were co-transformed into competent yeast Y190 cells containing *HIS3* and *lacZ* reporter genes, as previously described (Ago *et al.* 1999; Koga *et al.* 1999). Following selection for the Trp^+ and Leu^+ phenotype, the transformants were tested for their ability to grow on plates lacking histidine supplemented with 25 mM 3-aminotriazole to suppress background growth.

An *in vitro* pull-down binding assay using purified proteins

GST- or His-tagged proteins were expressed in *Escherichia coli* strain BL21 and purified by glutathione-Sepharose-4B (Amersham Pharmacia Biotech) or His-bind resin (Novagen), respectively, as previously described (Ago *et al.* 1999; Akasaki *et al.* 1999; Shiose & Sumimoto 2000). Recombinant His-Rac1 or His-Cdc42 was loaded with GTPγS or GDP, as previously described (Akasaki *et al.* 1999). Pull-down binding assays were performed as previously described (Ago *et al.* 1999; Shiose & Sumimoto 2000). Briefly, a pair of a GST-fusion and a His-tagged protein were incubated in 500 μL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4) containing 1% Triton X-100, and precipitated by glutathione-Sepharose-4B. After washing six times with the same buffer containing 1% Triton X-100, bound proteins were eluted with 10 mM glutathione. The eluates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with anti-GST (Amersham Pharmacia Biotech) or anti-$(His)_5$ (Qiagen) monoclonal antibody. The blots were developed using ECL-plus (Amersham Pharmacia Biotech) to visualize the antibodies.

Transfection and immunofluorescence

COS-7 and HeLa cells, a generous gift from Dr Shuh Narumiya (Kyoto University), were cultured in DMEM supplemented with 10% FCS. Cells were transfected with plasmids with or without the cDNA using LipofectAMINE (Life Tech), and cultured for 3 h. After adding DMEM containing 10% FCS, the cells were cultured for another 13 h. Following three washes with PBS, cells were fixed for 15 min in 3.7% formaldehyde.

Cells were permeabilized for 4 min in PBS containing 0.1% Triton X-100, washed three times with PBS, and blocked with PBS containing 3% bovine serum albumin for 60 min. The samples were first incubated with either anti-Myc (9E10) antibody (Roche) or anti-FLAG (M2) antibody (Sigma-Aldrich). Alexa Flour™ 594-labelled goat anti-mouse IgG antibody (Molecular Probes) was used as a secondary antibody. For F-actin staining, Texas Red-X phalloidin (Molecular Probes) was added after incubation with the second antibody. Images were visualized with a Nikon Eclipse TE300 microscope and captured on an ORCA digital camera (Hamamatsu Photonics).

Immunoprecipitation

HeLa cells (4×10^6 cells) were transfected with pEF-BOS-myc-Rac1(G12V) or pEF-BOS-myc-Rac1(T17N), and simultaneously with pEF-BOS-FLAG-PAR6β using LipofectAMINE (Life Tech), and cultured for 36 h in DMEM supplemented with 10% FCS. The cells were lysed with 1 mL of a lysis buffer (100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and 40 mM Hepes, pH 7.4) containing 1% Triton X-100. The lysate was precipitated with anti-Myc monoclonal antibody (9E10, Roche) or anti-aPKC rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.), and protein G-Sepharose. After washing three times with the lysis buffer, the precipitant was applied to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was probed with anti-Myc (9E10), anti-FLAG (M2) monoclonal antibody, or anti-aPKC rabbit polyclonal antibody.

Acknowledgements

We are grateful to Drs Shuh Narumiya and Toshimasa Ishizaki (Kyoto University) for helpful advice and for providing the HeLa cells, Dr Shigekazu Nagata (Osaka University) for providing the plasmid pEF-BOS, Dr Yoshimi Takai (Osaka University) for the plasmid pEF-BOS-myc-Rac1(G12V), and Ms Yohko Kage (Kyushu University) for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and from CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST), and partly carried out in Kyushu University Station for Collaborative Research.

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Received: 1 September 2000
Accepted: 31 October 2000