A human homolog of the *C. elegans* polarity determinant Par-6 links Rac and Cdc42 to PKCζ signaling and cell transformation

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Background: Rac and Cdc42 are members of the Rho family of small GTPases. They modulate cell growth and polarity, and contribute to oncogenic transformation by Ras. The molecular mechanisms underlying these functions remain elusive, however.

Results: We have identified a novel effector of Rac and Cdc42, hPar-6, which is the human homolog of a cell-polarity determinant in *Caenorhabditis elegans*. hPar-6 contains a PDZ domain and a Cdc42/Rac interactive binding (CRIB) motif, and interacts with Rac1 and Cdc42 in a GTP-dependent manner. hPar-6 also binds directly to an atypical protein kinase C isoform, PKCζ, and forms a stable ternary complex with Rac1 or Cdc42 and PKCζ. This association results in stimulation of PKCζ kinase activity. Moreover, hPar-6 potentiates cell transformation by Rac1/Cdc42 and its interaction with Rac1/Cdc42 is essential for this effect. Cell transformation by hPar-6 involves a PKCζ-dependent pathway distinct from the pathway mediated by Raf.

Conclusions: These findings indicate that Rac/Cdc42 can regulate cell growth through Par-6 and PKCζ, and suggest that deregulation of cell-polarity signaling can lead to cell transformation.

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Background

Rac and Cdc42 are members of the Rho GTPase family, which in turn constitutes a subfamily of the Ras superfamily [1]. Like all other Ras-related proteins, Rac and Cdc42 act as molecular switches, cycling between the active GTP-bound and the inactive GDP-bound states [2]. In the GTP-bound form, they interact with downstream effectors to elicit biological responses, including actin cytoskeletal organization, membrane trafficking and cell adhesion [2–4].

An important theme that has emerged during the past several years is that Rac and Cdc42 also have essential roles in growth control and contribute to oncogenic transformation [5]. They stimulate DNA synthesis, promote cell-cycle progression and mediate survival signaling [6,7]. Several observations indicate that deregulation of Rac1 and Cdc42 signaling can lead to cell transformation. First, overexpression of activated mutants of Rac1 or Cdc42 results in growth transformation in fibroblasts [8–11]. In addition, activated Rac1 and Cdc42 have been reported to synergize with activated Raf in focus assays [8,10,12]. Moreover, Rac and Cdc42 are required for cell transformation induced by oncogenic Ras in fibroblasts [8–10] and epithelial cells [13]. Despite many efforts to identify the downstream targets responsible for Rac- and Cdc42-mediated growth control [5,14], the pathway by which Rac1 and Cdc42 transform cells remains unknown.

It is becoming increasingly evident that Rac and Cdc42 are also important regulators of cell polarity in many different eukaryotic cells [2,15–19]. Two other groups of proteins have also been implicated in the determination of cell polarity. In *Caenorhabditis elegans* six *par* (partitioning defective; *par-1* to *par-6*) genes required for asymmetric segregation of cell-fate determinants have been identified [20] and homologs of some of these *par* genes have been isolated in other metazoans [21–24]. Recent studies have also suggested a role for the atypical PKCs (PKCζ, PKCλ/ι of mammals and PKC-3 of *C. elegans*) in cell polarity [22,24]. In addition to its role in cell polarity, PKCζ also has a critical role in many processes involved in mitogenic signaling and growth control [25–28].

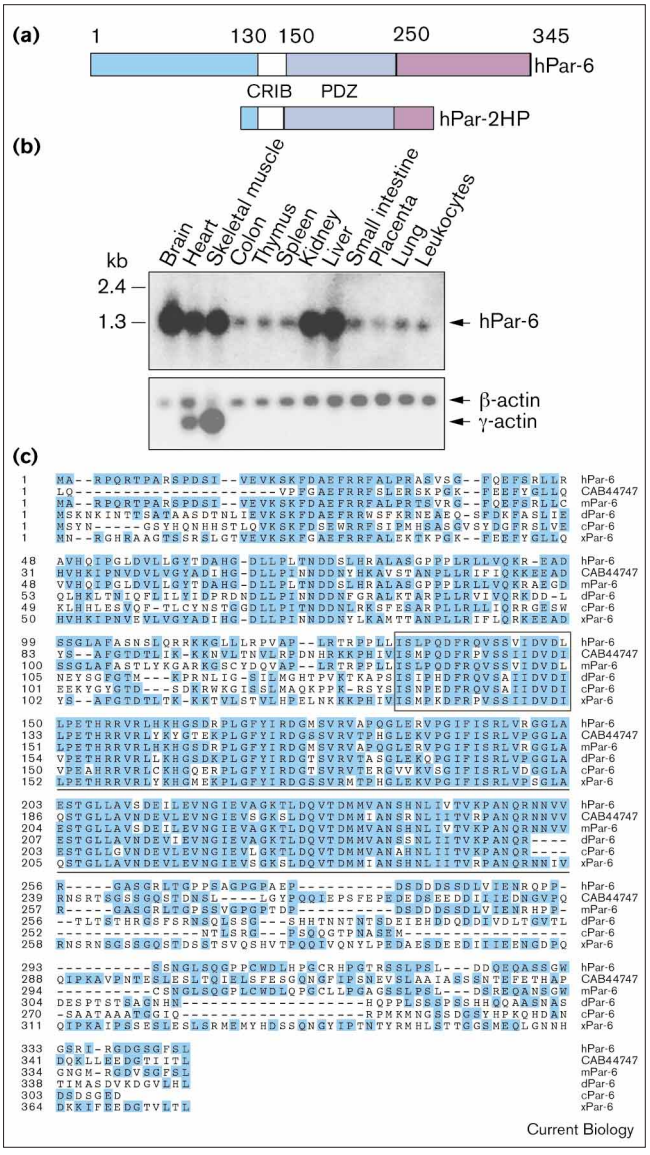
Here we report the cloning and characterization of a human homolog of Par-6 (hPar-6) and present evidence that hPar-6 is a novel effector of Rac1 and Cdc42. We show that hPar-6 physically links Rac1 or Cdc42 to PKCζ and mediates PKCζ-dependent transformation by Rac1 and Cdc42.

Results

Two-hybrid screen identifies hPar-6 as a putative effector of Rac1 and Cdc42

To isolate novel effectors of Cdc42, we performed a yeast two-hybrid screen [29] using activated Cdc42(Q61L) as bait. Of seven positive clones obtained from a human cDNA library prepared from HeLa cells, clone 2HP-4

Figure 1



Par-6 is conserved in evolution. **(a)** Full-length hPar-6 and the 2HP-4 clone (hPar-2HP) are shown schematically. CRIB, Cdc42/Rac interactive binding motif; PDZ, PDZ domain. The residue numbers indicate the approximate limits of each domain. **(b)** Tissue distribution of hPar-6 mRNA. The size markers (in kb) are shown at the left. The blot was also probed with actin cDNA as an RNA loading control (bottom panel). **(c)** Sequence alignments of Par-6 from *C. elegans* (cPar-6), *Drosophila* (dPar-6), *Xenopus* (xPar-6), mouse (mPar-6) and human (hPar-6), and clone CAB44747. The sequences corresponding to the CRIB motif in hPar-6 are boxed and those corresponding to the PDZ domain are underlined.

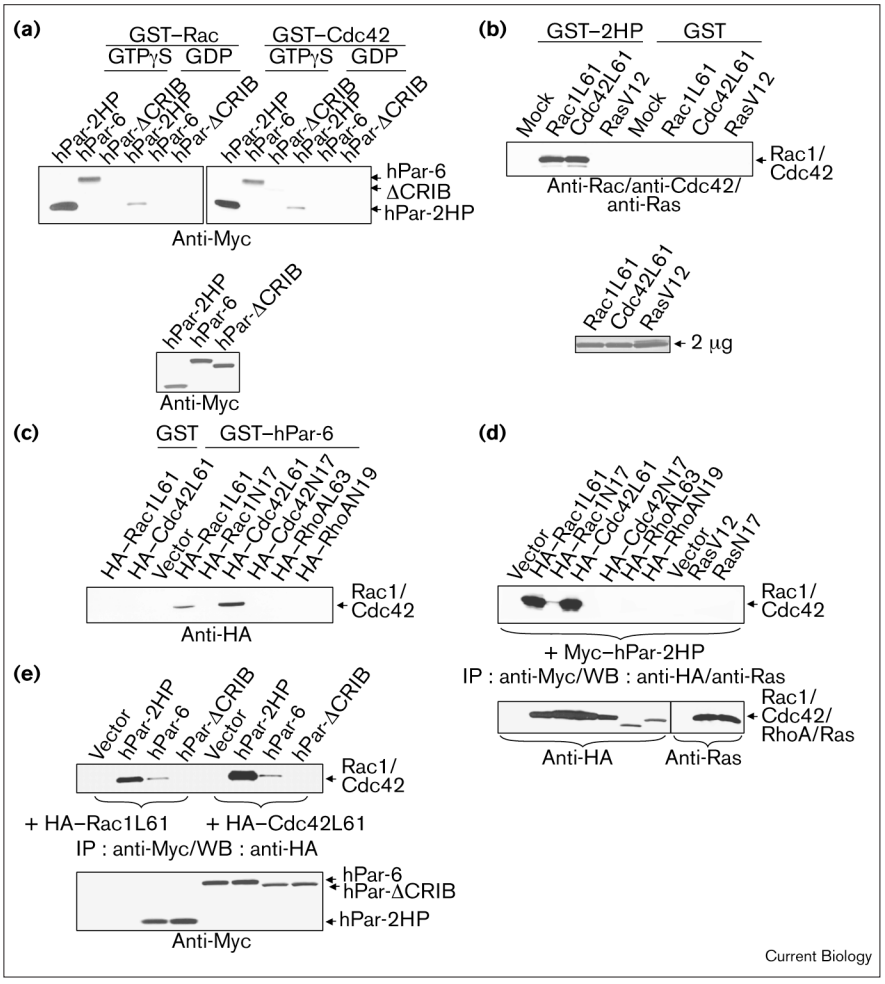
interacted strongly with constitutively active Rac1(Q61L) and Cdc42(Q61L), but not with their dominant-negative counterparts, Rac1(T17N) and Cdc42(T17N), nor with activated RhoA or Ras or the unrelated protein lamin (data not shown; see Supplementary material), suggesting that it is a putative effector of Rac1 and Cdc42. The interaction of 2HP-4 with Cdc42 appears stronger than the interaction between p21-activated kinase, PAK [30], and Cdc42 (see Supplementary material). Sequence analysis revealed that 2HP-4 encodes part of a protein that contains a PDZ domain and a CRIB motif (Figure 1a,c). This protein is a human homolog of *C. elegans* Par-6, the sequence of which was published during the course of this study [22]. We therefore named this novel protein hPar-6. A partial cDNA with identical sequence to 2HP-4 was recently isolated in an unrelated study as a protein (TIP40) that interacts with Tax, the transactivator of human T-cell leukemia virus [31].

To determine the tissue distributions of hPar-6 and the size of the full-length cDNA, a multiple-tissue northern blot was analyzed using the 2HP-4 cDNA insert as a probe. The gene is ubiquitously, but differentially, transcribed as a 1.3 kb mRNA in all tissues examined, with highest levels of expression in brain, heart, skeletal muscle, kidney and liver (Figure 1b).

A cDNA clone of hPar-6 was then obtained from the EST (expressed sequence tag) database and its sequence re-analyzed. It contains a cDNA insert of 1246 bp (data not shown). Three lines of evidence indicate that this EST clone encodes full-length hPar-6. First, the insert size corresponds to that of the mRNA (Figure 1b). Second, the clone contains an open reading frame of 345 amino acids (Figure 1a,c), only one amino acid less than that of its mouse counterpart which has been deposited in the database [22]. Finally, the nucleotides CCGCC immediately upstream of the start codon form a perfect consensus Kozak sequence. Homologs of Par-6 from *Drosophila* and *Xenopus* were also found in the EST database or identified [22,32], indicating that Par-6 has been conserved in evolution. In addition to a high degree of sequence similarity within the PDZ domain, Par-6 proteins show a high degree of sequence similarity in the CRIB-like motif (Figure 1c). Nevertheless, sequence comparison indicates that the CRIB motif of hPar-6 does not retain some of the most conserved residues (for example HXXH, where X is any amino acid) that are present in other CRIB-motif-containing effectors [5], suggesting that these residues are not required for interaction between hPar-6 and Rac1/Cdc42. The hPar-6 cDNA sequence has been deposited in GenBank, accession number AF265565.

We have also identified in the database the partial sequence of a human protein, CAB44747, which shows about 46% sequence identity to hPar-6 (Figure 1c). In addition, we have found other human EST clones (for example IMAGE clones 1540865, 1474395 and 2531285) that share sequence homology with hPar-6. These findings indicate the existence of a family of hPar-6 proteins.

Figure 2

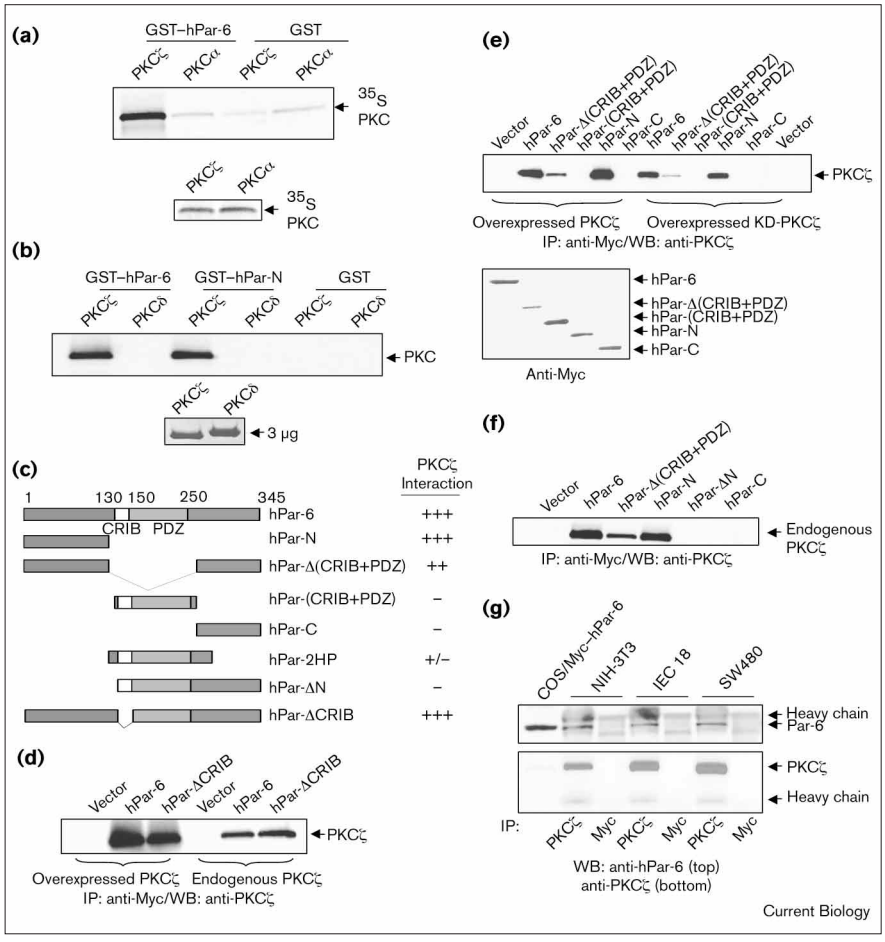


hPar-6 shows GTP-dependent interaction with Rac1/Cdc42 *in vitro* and *in vivo*. **(a)** *In vitro* interaction assays using GST–Rac1 and Cdc42 loaded with either GTPγS or GDP, and COS cell lysates containing Myc-tagged versions of the two-hybrid protein (hPar-2HP), full-length hPar-6, or the CRIB deletion mutant (hPar-ΔCRIB) of hPar-6 (see Figure 3c for descriptions of mutants). The precipitates were analyzed by western blotting with 9E10 anti-Myc monoclonal antibody (upper panels). The lower panel shows the levels of hPar-2HP, hPar-6 and hPar-ΔCRIB in 16% of the cell lysates used in the assay. **(b)** *In vitro* interaction assays showing direct interaction of hPar-2HP with Rac1 and Cdc42. Purified recombinant Rac1(Q61L), Cdc42(Q61L) or Ras(G12V) (2 μg each) were incubated with either GST–hPar-2HP (GST–2HP) or GST control. Precipitated proteins were detected by western blotting with a combination of antibodies against Rac, Cdc42 and Ras. The upper panel shows the results of the precipitations. The lower panel shows a Coomassie blue stain of 2 μg purified Rac1, Cdc42 and Ras. **(c)** *In vitro* interaction assays showing interaction of full-length hPar-6 with Rac1 and Cdc42. COS cell lysates containing the proteins as indicated above each lane were incubated with either GST–hPar-6 or GST control. Precipitated proteins were detected by western blotting with F-7 anti-HA antibody. **(d)** Co-immunoprecipitation (IP) of hPar-6 with Rac1/Cdc42. An expression construct encoding Myc-tagged hPar-2HP was co-transfected into COS cells with either empty vector or expression plasmids encoding the proteins as indicated above each lane. Myc–hPar-2HP was immunoprecipitated from the cell lysates with 9E10 anti-Myc antibody, and the precipitates were analyzed by western blotting (WB) with a combination of F-7 anti-HA and anti-Ras antibodies (upper panel). Similar blots were also probed separately with F-7 or anti-Ras with identical results (data not shown). The lower panel shows the levels of HA-tagged Rac1/Cdc42/RhoA mutants, and Ras mutants in 16% of the input cell lysates used in the assay. Levels of the proteins in the cell lysates were similar in (c) and (d). **(e)** CRIB-dependent interaction *in vivo*. Constructs encoding HA-tagged Rac1(Q61L) or Cdc42(Q61L) were co-transfected into COS cells with either empty vector, or a construct encoding Myc-tagged hPar-2HP, hPar-6 or hPar-ΔCRIB. Myc-tagged proteins were immunoprecipitated from the cell lysates with 9E10 anti-Myc, and the precipitates were analyzed by western blotting with F-7 anti-HA (upper panel). The lower panel shows in duplicate the levels of hPar-2HP, hPar-6 and hPar-ΔCRIB in 20% of the input cell lysates in the assay.

hPar-6 interacts with activated Rac1 and Cdc42 *in vitro* and *in vivo*

To obtain direct evidence that hPar-6 is an effector of Rac1 and Cdc42, we assayed their interaction both *in vitro* and *in vivo* (see Materials and methods). Full-length hPar-6 binds specifically to glutathione-*S*-transferase (GST) fusions of wild-type Rac1 (GST–Rac) or Cdc42 (GST–Cdc42) when these proteins are pre-loaded with GTPγS (a non-hydrolyzable analog of GTP), but not when pre-loaded with GDP (Figure 2a). Conversely, GST–hPar-6 or GST–hPar-2HP (corresponding to 2HP-4) binds activated mutants of Rac1 and Cdc42 (provided as either *in vitro* translated proteins, transfected cell lysates or purified proteins), but does not bind their dominant-negative versions, nor other control proteins such as activated RhoA, activated Ras or E-cadherin (Figure 2b,c; see Supplementary materials). Thus, the interaction between hPar-6 and Rac1/Cdc42 is direct, specific and GTP dependent. Moreover, Rac1(Q61L) and Cdc42(Q61L) are co-immunoprecipitated with hPar-6 or hPar-2HP from cells overexpressing both proteins, indicating that the interaction can occur *in vivo* (Figure 2d,e). Deletion of the CRIB motif of hPar-6 abolishes its interaction with Rac1/Cdc42 both *in vitro* and *in vivo* (Figure 2a,e), suggesting that binding of hPar-6 to Rac1/Cdc42 is mediated by this motif. Taken together, these data indicate that hPar-6 interacts

Figure 3



hPar-6 interacts specifically with atypical PKC. (**a**) *In vitro* interaction of hPar-6 with PKCζ but not PKCα. Equal amounts of ^{35}S-labeled PKCζ and PKCα made by TNT (see Materials and methods) were incubated with either GST–hPar-6 or GST control. The precipitates were detected by autoradiography (upper panel). The lower panel shows 20% of the input TNT PKC signals. **(b)** Direct interaction of hPar-6 with PKCζ but not with PKCδ. Assays were performed as in (a) except that 3 μg of purified recombinant PKCζ or PKCδ (Calbiochem) were used instead of *in vitro* translated proteins. The precipitates were analyzed by western blotting with anti-PKCζ and anti-PKCδ antibodies (Santa Cruz Biotechnology) (upper panel). The lower panel shows the Coomassie blue stain of 3 μg of the purified PKC. **(c)** Schematic representation of different mutants of hPar-6 and summary of their interactions with PKCζ. +++, Strong interaction; +/–, very little interaction; –, no detectable interaction. Data documenting interaction between hPar-2HP and PKCζ are not shown. **(d)** Co-immunoprecipitation of hPar-6 with PKCζ. COS cell lysates containing Myc-tagged vector, hPar-6 or hPar-ΔCRIB with (left three lanes) or without (right three lanes) co-transfected PKCζ were immunoprecipitated (IP) with 9E10 anti-Myc, and the immunoprecipitates were analyzed by western blotting (WB) with anti-PKCζ. The expression level of each hPar-6 construct was detected by western blotting with 9E10 (data not shown). Equivalent amounts of hPar-6 or hPar-ΔCRIB were then used in the experiment. **(e)** Mapping the PKCζ interaction region of hPar-6. Constructs expressing Myc-tagged hPar-6 and different deletion mutants of hPar-6 (see (c)) were co-transfected into COS cells with a construct expressing either PKCζ or kinase-dead (KD) PKCζ (PKCζ(K281W)). The cell lysates were then analyzed by immunoprecipitation with 9E10 anti-Myc followed by western blotting with anti-PKCζ (upper panel). The expression level of each hPar-6 mutant was detected by western blotting with 9E10 (lower panel). Equivalent amounts of hPar-6 or its mutants were then used in the experiment. **(f)** Interaction of overexpressed hPar-6 with endogenous PKCζ. Constructs encoding Myc-tagged hPar-6 or different mutants of hPar-6 were transfected into COS cells and the lysates were analyzed by immunoprecipitation with 9E10 anti-Myc followed by western blotting with anti-PKCζ. Expression of Myc-tagged hPar-6 mutants was confirmed by western blotting with 9E10 antibody (data not shown). Equivalent amounts of hPar-6 or its mutants were then used in the experiment. **(g)** Complex formation between endogenous Par-6 and PKCζ. Endogenous PKCζ in cell lysates containing 1.5 mg (NIH-3T3 and IEC18) or 1 mg (SW480) of total protein were immunoprecipitated with anti-PKCζ or control anti-Myc antibody. The precipitates were then analyzed with anti-hPar-6 antiserum for co-precipitated endogenous Par-6. The first lane was loaded with 10 μg of COS cell lysate containing transfected hPar-6 as a size control.

with Rac1/Cdc42 both *in vitro* and *in vivo*, and further confirm that hPar-6 is an effector of Rac1 and Cdc42.

Full-length hPar-6 interacts with Rac1/Cdc42 less efficiently than does hPar-2HP (Figure 2a,e), suggesting that in the full-length protein the CRIB motif is less accessible or has a lower affinity for Rac1/Cdc42. One possible interpretation is that an intramolecular interaction may occur between the amino and carboxyl termini of hPar-6, masking the CRIB domain and preventing its interaction with Rac1 and Cdc42. Alternatively, some proteins that bind to the amino- and/or carboxy-terminal regions may influence the interaction.

hPar-6 links Rac1/Cdc42 to PKCζ

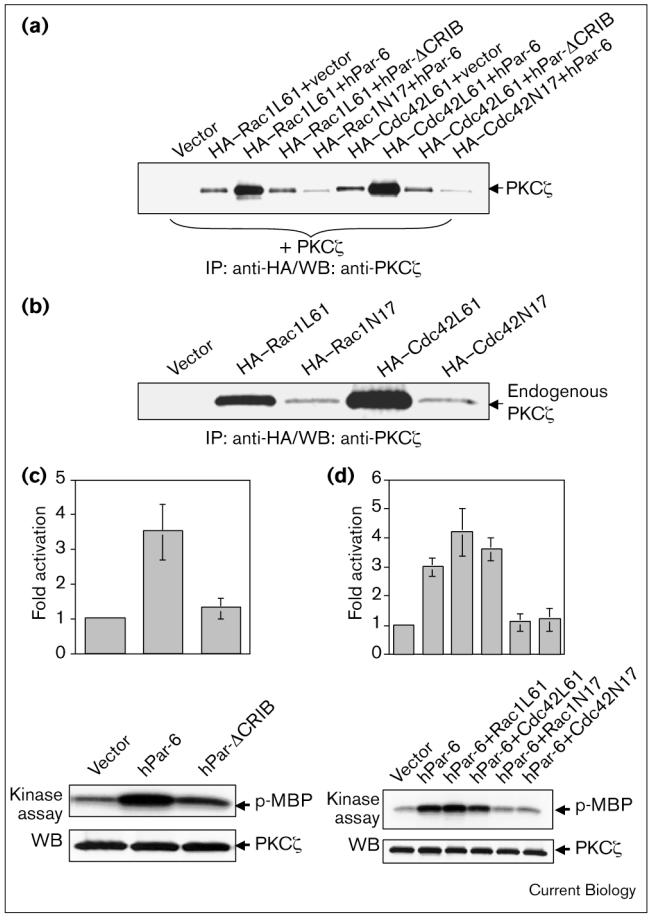
It was demonstrated recently that in the early embryo of *C. elegans*, Par-6 and an atypical PKC homolog, PKC-3, have an overlapping distribution and that stable localization of these two proteins at the cell periphery is dependent on each other's function [22]. This observation suggests that Par-6 and PKC-3 may form a complex. To address the possibility that hPar-6 might associate with a

mammalian atypical PKC, we first examined the ability of GST–hPar-6 to bind to human PKCζ translated *in vitro*. GST–hPar-6 efficiently bound PKCζ whereas the GST control showed little binding (Figure 3a). In contrast, GST–hPar-6 did not bind a conventional PKC, PKCα (Figure 3a) [33]. Furthermore, GST–hPar-6 bound purified recombinant human PKCζ, but not a novel PKC, PKCδ (Figure 3b) [33]. These data indicate that hPar-6 binds directly and specifically to PKCζ.

To define the region in hPar-6 responsible for its interaction with PKCζ, we generated various deletion mutants of hPar-6 (Figure 3c). Constructs encoding Myc-tagged versions of these mutants were co-transfected with a construct encoding PKCζ into COS cells and immunoprecipitation was carried out using the anti-Myc monoclonal antibody, 9E10. PKCζ co-immunoprecipitated with hPar-6, indicating that these two proteins interact *in vivo* (Figure 3d). Deletion of the CRIB motif did not affect the binding of PKCζ (Figure 3d) whereas it abolished the interaction of hPar-6 with Rac1/Cdc42 (Figure 2a,e), suggesting that PKCζ and Rac1/Cdc42 interact with hPar-6 at different sites. The amino-terminal region of hPar-6 was necessary and sufficient for interaction with PKCζ, and the PDZ plus CRIB domains or the carboxyl terminus displayed no detectable binding (Figure 3e,f). Deletion of both CRIB and PDZ domains from hPar-6, however, caused a reduction in PKCζ binding (Figure 3e,f), possibly because accessibility of the amino-terminal domain is compromised. Alternatively, elements in the CRIB and PDZ domains may contribute to PKCζ binding. Similar co-immunoprecipitation experiments demonstrated that overexpressed hPar-6 or hPar-N (see Fig. 3c) interacted with endogenous PKCζ (Figure 3d,f). hPar-6 also interacted with catalytically inactive (kinase-dead) PKCζ (Figure 3e). Furthermore, we showed that endogenous Par-6 and PKCζ co-immunoprecipitated from three different cell lines including murine fibroblasts (NIH-3T3), rat intestinal epithelial cells (IEC18), and human colon carcinoma cells (SW480) (Figure 3g). Taken together, these data indicate that Par-6 associates with PKCζ *in vivo* and that this association is mediated by the amino-terminal region of hPar-6 and is independent of PKCζ kinase activity. The physiological significance of this association is suggested by the formation of a complex between the endogenous proteins (Figure 3g).

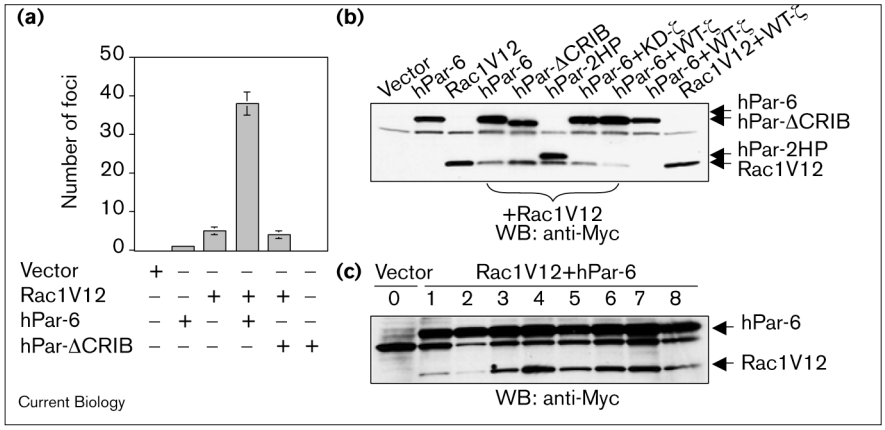
Because hPar-6 interacts with both Rac1/Cdc42 and PKCζ, we investigated whether Rac1 or Cdc42 and PKCζ form ternary complexes with hPar-6 by co-transfection in COS cells. Immunoprecipitation of hemagglutinin (HA)-tagged Rac1(Q61L) or Cdc42(Q61L) recovered considerably more PKCζ in the presence of overexpressed hPar-6 than in its absence (Figure 4a). Rac1(T17N) or Cdc42(T17N) did not precipitate significant amounts of PKCζ even when hPar-6 was overexpressed. Similarly, endogenous PKCζ co-immunoprecipitated with activated Rac1 and Cdc42, but to a much lesser extent with dominant-negative versions of these proteins (Figure 4b). In addition, when the CRIB deletion mutant of hPar-6 (hPar-ΔCRIB) was overexpressed, the amount of PKCζ co-precipitated

Figure 4



hPar-6 physically links Rac1/Cdc42 to PKCζ. **(a)** hPar-6-dependent association between Rac1/Cdc42 and PKCζ. The expression construct indicated above each lane was co-transfected into COS cells with a construct expressing PKCζ. Cell lysates were analyzed by immunoprecipitation (IP) with F-7 anti-HA followed by western blotting (WB) with anti-PKCζ. **(b)** Association of endogenous PKCζ with Rac1/Cdc42. COS cell lysates containing the proteins indicated above each lane were analyzed by immunoprecipitation with F-7 anti-HA followed by western blotting with anti-PKCζ. **(c)** CRIB-dependent activation of PKCζ by hPar-6. Expression constructs encoding Myc-tagged vector, hPar-6 or hPar-ΔCRIB were co-transfected into COS cells with the construct expressing PKCζ. Cell lysates were immunoprecipitated with anti-PKCζ and PKCζ kinase activity assayed (see Materials and methods). The upper panel shows the quantification of the radiolabel incorporated into myelin basic protein (MBP) (relative to the vector control). The middle panel is a representative autoradiogram showing phosphorylated MBP (p-MBP). The lower panel displays the expression of PKCζ in the lysates. **(d)** Effect of Rac1/Cdc42 on PKCζ activation by hPar-6. Assays were carried out and are represented as in (c). The error bars in (c) and (d) represent standard deviation of two to three independent experiments carried out in single set. In (b,c), the order of the bars in the upper panel corresponds to the order of the lanes in the lower panels.

Figure 5



hPar-6 potentiates transformation by Rac1. **(a)** Results of focus assays in NIH-3T3 cells, showing the CRIB-dependent potentiation of Rac1 transformation by hPar-6. Data are from at least four independent experiments performed in duplicate. The amount of DNA used in each transfection was 1 μg for each plasmid. **(b)** Western blotting with 9E10 anti-Myc showing expression levels of Myc-tagged hPar-6, hPar-ΔCRIB, hPar-2HP and Rac1(G12V) in transfected NIH-3T3 cells in a typical focus assay. Cells were transfected with constructs encoding the proteins indicated above each lane. Lanes 4–8 (designated +Rac1V12) were also co-transfected with Myc–Rac1(G12V). KD-ζ, kinase-dead PKCζ; WT-ζ, wild-type PKCζ. **(c)** Western blotting with 9E10 anti-Myc showing expression of both Myc-tagged hPar-6 and Rac1V12 in independent foci isolated from dishes transfected with plasmids encoding Myc–hPar-6 and Rac1(G12V). Lanes 1–8 represent lysates from different foci, and lane 0 represents a lysate from cells transfected with vector alone.

with Rac1 or Cdc42 did not increase (Figure 4a). These results strongly suggest that hPar-6 can bridge activated Rac1/Cdc42 and PKCζ to form a stable complex *in vivo*.

Overexpression of hPar-6 stimulates PKCζ kinase activity

To determine whether complex formation with Rac1 and hPar-6 affects PKCζ kinase activity, immune-complex kinase assays were carried out using myelin basic protein (MBP) as a substrate. Overexpression of hPar-6 in COS cells stimulated PKCζ kinase activity about threefold, whereas deletion of the CRIB motif abrogated this stimulation (Figure 4c). These results indicate that hPar-6 not only interacts with PKCζ, but also stimulates its catalytic activity. They also suggest that the interaction of hPar-6 with Rac1/Cdc42 is required for this stimulation. Consistent with a role for Rac1/Cdc42 in hPar-6-mediated PKCζ activation, co-expression of Rac1(T17N) or Cdc42(T17N) blocked this effect (Figure 4d). Conversely, Rac1(Q61L) and Cdc42(Q61L) further potentiated PKCζ activation by hPar-6 but the increase was modest (Figure 4d), suggesting that endogenous Rac and Cdc42 may be sufficient for activation of PKCζ by overexpressed hPar-6.

Par-6 potentiates cell transformation by Rac1

We and others have shown previously that constitutive activation of Rac1 and Cdc42 leads to cell transformation and that the pathway(s) mediated by Rac1/Cdc42 are critical for Ras transformation [8–11,13]. The downstream pathway(s) responsible for the transforming activity of Rac/Cdc42 remains elusive, however. Although many effectors of Rac1 and Cdc42 have been isolated [2], none of them has been shown to directly mediate Rac1- or Cdc42-induced transformation. The demonstrated roles of PKCζ in mitogenic [25] and survival signaling [28,34] and the biochemical link that we had found between Rac1/Cdc42, hPar-6 and PKCζ led us to test the possibility that Rac1/Cdc42 might elicit cell transformation via hPar-6 and PKCζ.

We first examined interaction of hPar-6 with a panel of Rac1 effector domain mutants that had been characterized with respect to transformation in cooperation with activated Raf [35]. Rac1(G12V;T35S) with no transforming ability showed no binding to hPar-6, whereas Rac1(N26D;Q61L) and Rac1(N43D;Q61L) with reduced transforming potential had reduced affinity toward hPar-6 (data not shown). Thus, the strength of Rac1–hPar-6 association correlates with the potency of Rac1-induced transformation.

To provide direct evidence for a role of hPar-6 and PKCζ in Rac1/Cdc42 transformation, focus assays were carried out in NIH-3T3 cells. Consistent with previous observations [8,10], activated Rac1 alone weakly transformed the cells. hPar-6 or hPar-ΔCRIB alone showed even weaker, if any, transforming capability. In marked contrast, co-transfection of hPar-6 with Rac1(G12V) potentiated focus formation by about tenfold, whereas deletion of the hPar-6 CRIB motif completely abolished this effect (Figure 5a). To rule out the possibility that the increase in focus formation results merely from an increase in expression of Rac1 and/or hPar-6, we examined transiently transfected lysates by western blotting. hPar-6 and hPar-ΔCRIB were expressed at comparable levels, whereas the level of Rac1(G12V) was actually lower in cells co-expressing hPar-6 (Figure 5b), despite the more potent transformation effect observed. This result suggests that hPar-6 causes the transformation effect directly by interacting with Rac1. A number of independent foci from cells transfected with Rac1(G12V) plus hPar-6 were examined for expression of these proteins. All eight foci analyzed contained both Myc–Rac1(G12V) and Myc–hPar-6 (Figure 5c), supporting

the conclusion that the cell transformation observed is promoted by Rac1 and hPar-6 acting in concert. Moreover, the morphology of foci induced by Rac1(G12V) plus hPar-6 appears very similar to that induced by Rac1(G12V) alone, except that the Rac1 foci are usually smaller (see below). On the other hand, we did not observe any effect of PAK1 (a Rac/Cdc42 effector) or POR1 (a Rac-specific effector) on Rac1(G12V) transformation [2] (data not shown), suggesting that the effect of hPar-6 on Rac1 transformation is specific.

PKCζ has an essential role in cell transformation by Par-6

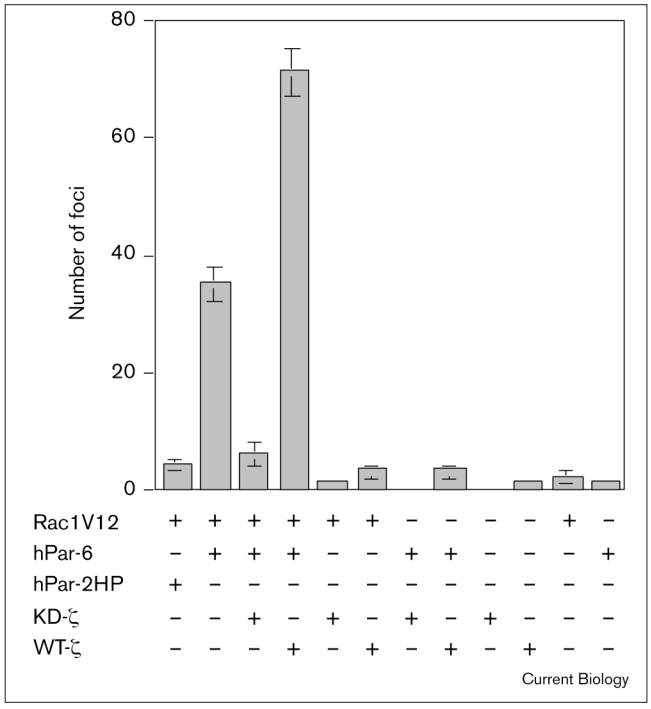
To further elucidate the downstream elements in the Rac1/hPar-6 pathway, we investigated the role of PKCζ in mediating transformation by Rac1 and hPar-6. We made use of a well-characterized, catalytically inactive (kinase-dead), dominant-negative mutant of PKCζ (KD-ζ) [25,36,37] to inhibit endogenous PKCζ activation. Co-expression of KD-ζ strongly reduced the transforming potential of Rac1 plus hPar-6, whereas co-expression of wild-type PKCζ (WT-ζ) further enhanced transformation by Rac1 plus hPar-6 (Figure 6). WT-ζ only slightly increased transformation by Rac1 or hPar-6 alone (Figure 6). Introduction of KD-ζ and WT-ζ did not increase the expression level of Rac1(G12V) or hPar-6 (Figure 5b). These data strongly suggest that PKCζ kinase activity is required for Rac1 and hPar-6 to transform fibroblasts. Consistent with a role for PKCζ in mediating hPar-6 transformation, hPar-2HP and hPar-ΔN (see Figure 3c), which interacted at most very weakly with PKCζ, did not potentiate Rac1 focus formation (Figures 3c,6 and data not shown). Moreover, hPar-C and hPar-(CRIB+PDZ) (Figure 3c), which did not interact with PKCζ, also did not promote cell transformation (data not shown).

To examine whether focus formation induced by Rac1, hPar-6 and PKCζ correlates with other transformation phenotypes such as anchorage-independent growth, we assayed cells from transformed foci for growth in soft agar. Cells grown from four independent foci induced by either Rac1(G12V) and hPar-6, or Rac1(G12V) and hPar-6 plus PKCζ all formed colonies in soft agar, whereas cells transfected by the vector only did not (see Supplementary material). The growth efficiency in soft agar ranges from 1 to 7%. This result suggests that activation of the Rac1/hPar-6/PKCζ pathway can also confer other transformation phenotypes on NIH-3T3 cells.

The Rac1/hPar-6/PKCζ pathway is distinct from the RafCAAX-mediated pathway

It has been demonstrated by us and others that Rac1 and RafCAAX, a membrane-targeted, constitutively active Raf kinase, may control two distinct pathways downstream of Ras that cooperate to promote cell transformation [5,8,10]. To assess whether hPar-6 and PKCζ comprise the pathway downstream of Rac1 that synergizes with RafCAAX, we

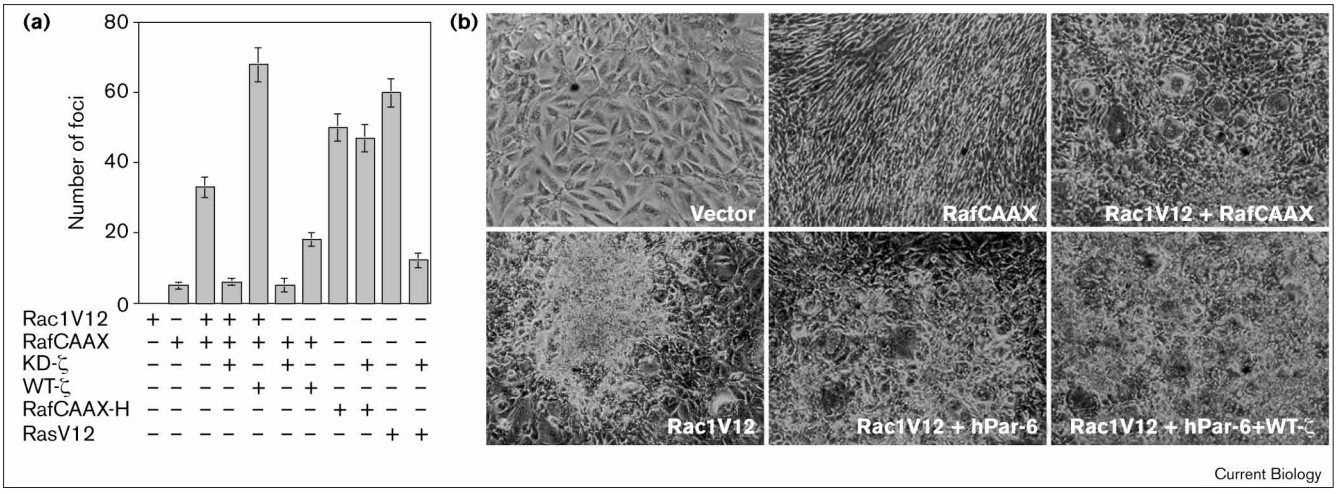
Figure 6



PKCζ mediates transformation by Rac1/hPar-6. Results of focus assays showing the effect of PKCζ on cell transformation induced by Rac1 and hPar-6. Data are from two to four independent experiments performed in duplicate. The amount of DNA in each transfection was: 1 μg for Rac1V12, hPar-6 or hPar-2HP; 2 μg for KD-ζ or WT-ζ.

tested the effect of PKCζ on RafCAAX-induced transformation. The synergy between Rac1 and RafCAAX was inhibited by KD-ζ and enhanced by WT-ζ (Figure 7a). Moreover, WT-ζ modestly stimulated RafCAAX transformation. On the other hand, KD-ζ did not have a significant effect on focus formation induced by RafCAAX alone (Figure 7a) or by RhoA(G14V) (data not shown). In addition, the morphology of transformed foci induced by Rac1 and hPar-6, or by Rac1 and hPar-6 plus PKCζ, is similar to that induced by Rac1 alone, but quite different from that induced by RafCAAX (Figure 7b): foci induced by the Rac1/hPar-6/PKCζ pathway are dense, and contain rounded cells that pile up, including many multinucleated cells, whereas foci induced by RafCAAX are less dense and contain spindle-shaped cells that do not pile up (Figure 7b). Moreover, many foci induced by Rac1 plus RafCAAX look similar to those induced by the Rac1/hPar-6/PKCζ pathway (Figure 7b). These observations indicate that the inhibitory effect of KD-ζ is specific to the Rac1 pathway and support the idea that hPar-6 and PKCζ mediate a signaling pathway downstream of Rac1 that is distinct from the RafCAAX-mediated pathway. Consistent with the proposal that Rac1 mediates a growth control pathway downstream of Ras [8,10], KD-ζ also significantly inhibited transformation induced by oncogenic Ras (Figure 7a). In agreement with

Figure 7



The hPar-6/PKCζ pathway is distinct from the RafCAAX-mediated pathway. **(a)** Results of focus assays showing the effect of PKCζ on the transforming synergy between Rac1 and RafCAAX and on transformation induced by Ras. The amount of DNA used in each transfection was: 100 ng for Rac1(G12V) or RafCAAX; 2 μg for KD-ζ or WT-ζ; 10 ng for Ras(G12V). RafCAAX-H designates the higher amount of DNA (1 μg) that was used to obtain a higher number of foci. Data are from two independent experiments performed in duplicate. **(b)** Morphologies of different foci transformed by the protein(s) indicated in each panel.

these observations, it was recently reported that PKCζ can act in a pathway downstream of Ras and Rac1 that leads to disassembly of actin stress fibers, a phenotype characteristic of transformed cells [38].

hPar-6 and PKCζ mediate cell transformation by Cdc42

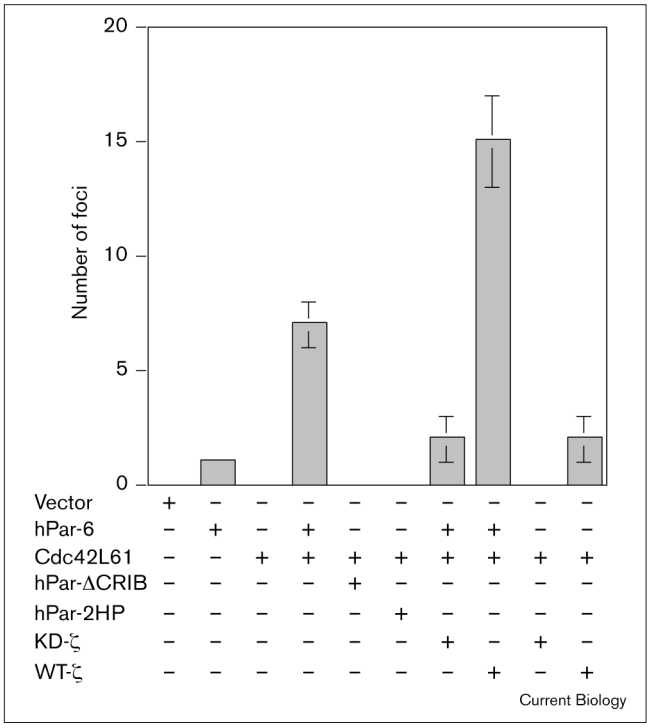
As Cdc42 has a role in cell transformation [5] and can also undergo GTP-dependent interaction with hPar-6, we carried out similar focus assays to determine whether hPar-6 and PKCζ mediate transformation by Cdc42. We found that overexpression of hPar-6 and PKCζ increased focus formation by Cdc42(Q61L) (Figure 8), suggesting that signaling from hPar-6 to PKCζ also promotes transformation by Cdc42. This effect was, however, much weaker than in the case of Rac1, and Cdc42 alone did not induce any foci (Figure 8). In addition, the transformed foci induced by Cdc42 and hPar-6 appeared flatter than those induced by Cdc42 and hPar-6 plus PKCζ (see Supplementary material), and the foci contained fewer multinucleated cells than those induced by the Rac1/hPar-6/ PKCζ pathway (compare Figure 7b with Figure S4 in Supplementary material).

Discussion

It has become evident in the past several years that Rac1 and Cdc42 have important roles in cell growth control and that their signaling contributes to cell transformation by oncogenic Ras. The biochemical mechanisms underlying this effect remain obscure, however. Many direct targets of Rac1 and Cdc42 have been identified [14], but none has been shown to have a direct role in cell transformation by Rac1 and Cdc42. We have identified hPar-6 as a novel effector of Rac1 and Cdc42 that promotes PKCζ-dependent transformation by both GTPases. Although it has been suggested that PAK1 may also contribute to transformation by Rac1 in Rat1 fibroblasts [39], we found that PAK1 does not enhance transformation by activated Raf or activated Rac1 in NIH-3T3 cells (data not shown), and studies using effector domain mutants indicated that interaction of PAK1 with Rac1 does not correlate with cell-cycle progression or transformation [2,5]. Thus, to our knowledge hPar-6 is the first effector shown to directly mediate transformation by Rac1 and Cdc42. The identification of PKCζ as a downstream effector of hPar-6 represents the first elucidation of a signaling pathway linking Rac1/Cdc42 to cell transformation (Figure 9).

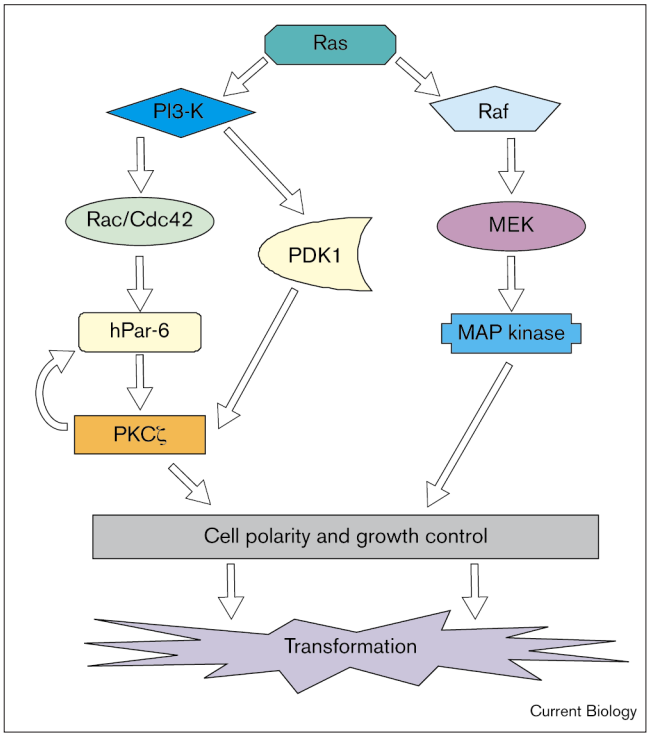
The mechanism by which hPar-6 regulates the kinase activity of PKCζ is currently under investigation. Subcellular targeting by interaction with specific proteins provides an attractive mechanism for PKC isozyme-specific regulation [40]. It is possible that hPar-6 and PKCζ are translocated by Rac1 or Cdc42 to the membrane, where PKCζ could interact with an activator. One candidate activator is the phosphatidylinositol 3-kinase (PI3-kinase) target PDK1, as PDK1 and PKCζ associate *in vivo* via their catalytic domains, and both PI3-kinase and PDK1 stimulate PKCζ activity [41–43]. Consistent with this model (Figure 9), it has been demonstrated that PI3-kinase can act as a link between Ras and Rac in transformation and that membrane-targeted PKCζ is constitutively active [43–45]. The observation that hPar-6 alone exhibits little, if any, transforming activity is also

Figure 8



hPar-6 promotes transformation by Cdc42. Results of focus assays showing CRIB- and PKCζ-dependent transformation by hPar-6 together with Cdc42(Q61L). The amount of DNA used in each transfection was: 1 μg for vector, Cdc42(Q61L), hPar-6, hPar2HP or hPar-ΔCRIB; 2 μg for KD-ζ or WT-ζ. Data are from two independent experiments performed in duplicate.

Figure 9



Schematic of the model depicting two separate pathways downstream of Ras that lead to cell polarity and growth control, and which can contribute to cell transformation. One comprises Rac/Cdc42, hPar-6 and PKCζ, and the other is mediated by Raf, MEK and MAP kinase.

consistent with the membrane-targeting model. It should also be noted that although overexpression of hPar-6 alone (i.e., in the absence of Rac1[G12V]) is sufficient to activate PKCζ kinase activity (Figure 4c), overexpression of hPar-6 and PKCζ only marginally promotes focus formation (Figure 6), suggesting that activated Rac1 is necessary to target PKCζ to substrates involved in transformation. However, we cannot rule out the possibility that Rac1 activates some other pathway that is also necessary for transformation. In addition to being activated by hPar-6, PKCζ might in turn phosphorylate hPar-6 (Figure 9). In this regard, it should be noted that there is a putative PKCζ-phosphorylation site [46] in mammalian Par-6.

The mechanism underlying transformation by hPar-6 and PKCζ is not yet clear. Stimulation of cell proliferation and inhibition of apoptosis are, however, important characteristics of cell transformation [47]. In this regard, it has been shown that Rac1 and Cdc42 induce cyclin D1 transcription and accumulation, phosphorylation and inactivation of the tumor suppressor protein Rb, and activation of the transcription factor E2F [5,48]. Inactivation of Rb may be necessary for Rac1/Cdc42 stimulation of cell proliferation, and it is possible that hPar-6 and PKCζ have a role in this pathway. In addition, Ras, Rac1, Cdc42 and PKCζ are all able to activate the transcription factor NF-κB [2,5,27,37]. NF-κB activation is associated with mitogenesis, anti-apoptotic activity and cell transformation [7,49,50]. Thus, the hPar-6–PKCζ pathway might mediate NF-κB activation, and thereby contribute to cell transformation by Rac1 and Ras. Another possibility is that the hPar-6–PKCζ pathway may mediate growth control by Rac1/Cdc42 by inducing downregulation of the pro-apoptotic protein Par-4 (prostate apoptosis response-4; unrelated to the *C. elegans* Par gene product) [28]. Par-4 interacts with PKCζ and overexpression of PKCζ downregulates Par-4, an event that appears important for Ras transformation and tumor progression [28]. Thus, cyclin D1, Rb, E2F, NF-κB and Par-4 all warrant further investigation as possible downstream targets of the hPar-6–PKCζ pathway.

Polarity is a fundamental feature of all eukaryotic cells [51]. Rac, Cdc42, Par-6 and atypical PKCs appear to be conserved in diverse metazoans, including *Drosophila*, *C. elegans*, *Xenopus*, mouse and humans [22,32,52]. The CRIB motif of Par-6 is also conserved, suggesting that it interacts with Rac and/or Cdc42 in these different species. In *C. elegans*, inhibition of Cdc42 function by

RNA-mediated gene interference (RNAi) [53] produced defects in cell polarity similar to those observed in *par* and *pkc-3* mutants (A. Kay and C.P. Hunter, personal communication; A. Romano and M. Glotzer, personal communication), while in mammalian cells, Par-6 is localized to tight junctions, together with atypical PKC and ASIP, the mammalian homolog of Par-3 (T. Yamanaka and S. Ohno, personal communication). Moreover in *C. elegans*, Par-6 interacts with Par-3, and in *Drosophila* the Par-3 homolog has an important role in the asymmetric cleavage of epithelial cells and neuroblasts [21,54,55]. Taken together, these observations suggest that Rac or Cdc42, Par-6, atypical PKC, and perhaps Par-3, constitute a conserved pathway that regulates cell polarity. As we have shown that hPar-6 and PKCζ mediate cell transformation by Rac1 and Cdc42, there may be a link between cell-polarity signaling and growth control: aberrant cell-polarity signaling could lead to oncogenic transformation (Figure 9). In the light of the important roles of Rac1/Cdc42 in Ras-induced transformation, hPar-6 and PKCζ could represent potential targets for anti-cancer therapeutics.

Conclusions

Our findings indicate that hPar-6, the human homolog of a *C. elegans* cell-polarity determinant, is a novel effector of Rac1 and Cdc42. hPar-6 provides a link between Rac or Cdc42 and PKCζ in a pathway that is involved in cell transformation. To our knowledge, hPar-6 is the first effector shown to directly mediate Rac1- or Cdc42-induced transformation, and the identification of PKCζ as a downstream element in this pathway represents the first elucidation of a molecular mechanism by which Rac and Cdc42 can control cell growth. This pathway seems to be distinct from the Raf-mediated pathway, and both pathways are essential for cell transformation by Ras. These findings suggest a link between cell-polarity signaling and cell transformation.

Materials and methods

Northern blot analysis

A poly(A)$^+$ multiple-tissue Northern blot (Clontech) was probed with 2HP-4 cDNA labeled with [^{32}P]dCTP using the Megaprime DNA Labelling System (Amersham).

Transient transfections

All transfections were carried out in COS cells using either Superfect (Qiagen) according to the protocol provided by the manufacturer, or electroporation at 250 V and 125 μF. Cell lysates were prepared 48 h post-transfection using mammalian cell lysis buffer containing 40 mM HEPES pH 7.4, 100 mM NaCl, 1% TritonX-100, 5 mM $MgCl_2$, 1 mM EDTA and protease and phosphatase inhibitors.

SDS–PAGE and western blot analyses

All the SDS–PAGE and western blot analyses were carried out as previously described [9].

In vitro *interaction assays (GST fusion affinity precipitation)*

GST fusions (10–20 μg) were incubated for 2 h at 4°C with cell lysates containing proteins of interest, then washed three times with bacterial lysis buffer containing 40 mM Tris pH 8.0, 100 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 1 mM EDTA and protease inhibitors. When *in vitro* translated products were used, the proteins were generated using a coupled transcription and translation (TNT) kit (Promega) and labeled with [^{35}S]methionine; the precipitated proteins were visualized by autoradiography. When purified recombinant proteins were used, 2–3 μg of each recombinant protein was mixed with 20 μg of BSA in 200 μl bacterial lysis buffer. Proteins precipitated from lysates of transiently transfected cells or from purified preparations were visualized by western blot analysis.

In vivo *interaction assays (co-immunoprecipitation)*

Constructs encoding proteins of interest were transfected into COS cells. Cell lysates containing 200–500 μg of total proteins were incubated at 4°C for 2 h with either 9E10 anti-Myc monoclonal antibody to precipitate Myc-tagged hPar-6-derived proteins, F-7 anti-HA monoclonal antibody (Santa Cruz Biotechnology) to precipitate HA-tagged Rac1/Cdc42/RhoA mutants, or anti-PKCζ polyclonal antibody (Santa Cruz Biotechnology) to precipitate endogenous and overexpressed PKCζ. The immunoprecipitates were washed three times with mammalian cell lysis buffer and the co-precipitated proteins were detected by western blot analysis with specific antibodies.

Immune-complex PKCζ kinase assays

Total protein from COS cell lysates (500 μg in each assay) overexpressing PKCζ and proteins of interest were immunoprecipitated with 1–2 μg anti-PKCζ polyclonal antibody or 1 μg anti-V5 epitope tag monoclonal antibody (Invitrogen). The PKCζ construct contained the V5 epitope tag at the carboxyl terminus of PKCζ. The immunoprecipitates were then incubated with MBP and [γ-^{32}P]ATP [56]. The phosphorylated MBP was visualized by autoradiography and quantified.

Focus assays

The assays were carried out in NIH-3T3 cells essentially as described previously [8]. For the experiment shown in Figure 5b, transfection was done in triplicate. Two were maintained for focus formation, the other one was used to make cell lysate 2 days post-transfection for western blot analysis.

Soft agar assays

Individual transformed foci were isolated and cultured. Cells from these foci were then assayed for growth in soft agar essentially as described previously [8].

Supplementary material

Supplementary material including a description of the cloning and subcloning of hPar-6 cDNA and additional figures is available at http://current-biology.com/supmat/supmatin.htm.

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