The mammalian homologue of the *Caenorhabditis elegans* polarity protein PAR-6 is a binding partner for the Rho GTPases Cdc42 and Rac1

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SUMMARY

A mammalian homologue of the PDZ domain containing *Caenorhabditis elegans* protein PAR-6 was found in a yeast two-hybrid system screen as binding to the Rho family member Cdc42. PAR-6 contains a PDZ domain and in *C. elegans* it has been shown to be crucial for the asymmetric cleavage and establishment of cell polarity during the first cell divisions in the growing embryo. Mammalian PAR-6 interacted with Cdc42 and Rac1 both in the yeast two-hybrid system and in in vitro binding assays. Co-immunoprecipitation experiments, employing transiently transfected Cos-1 cells, further confirmed that Cdc42 and Rac1 are physiological binding partners for PAR-6. We found that, in epithelial Madin-Darby canine kidney cells (MDCK), endogenous PAR-6 was present in the tight junctions, as judged from its co-localisation with the tight junction protein ZO-1, however, PAR-6 was also detected in the cell nucleus. Stimulation of MDCK cells with scatter factor/hepatocyte growth factor induced a loss of PAR-6 from the areas of cell-cell contacts in conformity with their progressive breakdown. In *C. elegans* PAR-6 co-localises with PAR-3 and has been suggested to form a direct complex. In agreement with earlier studies, mammalian PAR-3 was found to be present in tight junctions of MDCK cells but, in contrast to PAR-6, the protein could not be detected in the nucleus. Furthermore, co-immunoprecipitation experiments, employing Cos-1 cells, demonstrated that mammalian PAR-6 and PAR-3 formed a direct complex. These findings, together with the reported roles of PAR-6 and PAR-3 in *C. elegans*, suggest that Cdc42 and Rac1 and PAR-6/PAR-3 are involved in the establishment of cell polarity in epithelial cells.

Key words: Rho GTPase, par gene, Cell polarity, Tight junction, Epithelial cell

INTRODUCTION

The Rho GTPases are important regulators of the actin cytoskeleton and thereby of the morphology and motile behaviour of eukaryotic cells (Van Aelst and D’Souza-Schorey, 1997; Hall, 1998; Kjoller and Hall, 1999). This growing family of GTP-binding proteins share a simple enzymatic property; they bind and hydrolyse GTP and, in this process, they cycle between inactive, GDP-bound, and active, GTP-bound, conformations. Currently, 15 distinct Rho GTPases have been distinguished, which can be further divided into six subgroups; Rac (Rac1-3, Rhog), Cdc42 (Cdc42, Tc10, Chp), Rho (RhoAC), Rnd (Rnd1-2, RhoE), RhoD and RhoH (Aspenström, 1999a). The enzyme activity of the Rho GTPases is regulated by proteins that facilitate the exchange of GDP for GTP (guanine nucleotide exchange factors or GEFs), proteins that increase the GTP hydrolysis activity (GTPase activating proteins or GAPs) and proteins that inhibit the dissociation of the nucleotide from the GTPase (GDP dissociation inhibitors or GDIs). A large number of GEFs and GAPs have been characterised during the last couples of years and currently each group comprises at least 20-30 distinct members (Van Aelst and D’Souza-Schorey, 1997; Kjoller and Hall, 1999).

Furthermore, the number of known target proteins, so called effectors, for the Rho GTPases has increased substantially (Van Aelst and D’Souza-Schorey, 1997; Aspenström, 1999b). The Rho GTPases participate in a multitude of vital cellular processes in addition to regulating the actin cytoskeleton. For instance, they are involved in transcriptional regulation via signalling pathways controlled by the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), p38 mitogen activated protein (p38 MAP) kinase and serum response factor (SRF). In addition, the Rho GTPases participate in induction of apoptosis, control of cell cycle progression and maintenance of the transformed phenotype of Ras-transformed cells (Van Aelst and D’Souza-Schorey, 1997; Hall, 1998; Kjoller and Hall, 1999; Aspenström, 1999a,b).

A vast majority of the work on Rho GTPases has been performed using RhoA, Rac1 and Cdc42 and they have been found to regulate the assembly of distinct actin filament-containing structures. In fibroblasts, Rho controls the assembly of focal adhesion complexes and stress fibres, Rac controls the formation of peripheral membrane lamellae in response to growth factors, whereas Cdc42 controls the formation of finger-like protrusions, so called filopodia, at the cell periphery (Van Aelst and D’Souza-Schorey, 1997; Hall, 1998; Kjoller...
These findings, together with the roles of the PAR-6 and PAR-3 are integral components of tight junctions also in mammalian cells (Izumi et al., 1998). In the present tight junctions in epithelial cells, indicating that mammalian specific interacting protein), has been shown to be present in the human homologue of PAR-3, called ASIP (atypical PKC isotype-specific interacting protein), has been shown to be present in the human homologue of PAR-3, called ASIP (atypical PKC isotype-

Additionally, studies of migrating mouse macrophages demonstrated that Cdc42 was needed in order to give the cells a directionality in locomotion toward a gradient of colony stimulating factor 1 (CSF-1) (Allen et al., 1998). A similar observation was made from studies on primary rat embryonic fibroblast (REF) cells employing a wound healing assay to induce unidirectional motility of cells in monolayers. Under these conditions, Cdc42 was required to establish polarity during migration, contrary to Rac, which was necessary for the forward movement of the cells (Nobes and Hall, 1999).

The yeast two-hybrid system was used in an effort to isolate novel Cdc42-binding proteins and we have previously reported the characterisation of Wiskott Aldrich syndrome protein (WASP) and Cdc42-interacting protein 4 (CIP4) as Cdc42 effectors (Aspenström et al., 1996; Aspenström, 1997). An additional set of potential Cdc42-binding clones encoded the human homologue of the Caenorhabditis elegans genes in C. elegans has demonstrated that PAR-6 is involved in the process of asymmetric cleavage in the early worm embryo (Guo and Kephues, 1996; Watts et al., 1996). During this process PAR-6 co-localises with the PAR-3 gene and an atypical PKC isoform, PKC-3 (Tabuse et al., 1998), and a correct localisation of each of these components depend on the presence of the other, suggesting that they are forming a direct complex (Hun and Kephues, 1999). A mammalian homologue of PAR-3, called ASIP (atypical PKC isoform-specific interacting protein), has been shown to be present in tight junctions in epithelial cells, indicating that mammalian par genes could have roles in the establishment of cell polarity also in mammalian cells (Izumi et al., 1998). In the present study we show that PAR-6 binds to Cdc42 and Rac1 in a GTP-dependent manner. The data also indicated that mammalian PAR-6 and PAR-3 are integral components of tight junctions and that they are forming a direct complex with each other. These findings, together with the roles of the C. elegans PAR-6 and PAR-3, indicate that Cdc42 and Rac1 and PAR-6/PAR-3 are involved in the establishment of cell polarity.

**MATERIALS AND METHODS**

**DNA work and yeast two-hybrid system screen**

The Saccharomyces cerevisae strain Y190 (genotype: MATa, gal4-542, gal80-538, his3, trp1-901, ade2-101, ura3-52, leu2-3,112, URA3::GAL1-LacZ, Lys2::GAL1-HIS3cyh) was transformed with a cDNA encoding L61Cdc42 (G25K isoform) fused to the GAL4 DNA-binding domain (GAL4DB) in the pYTH6 vector. This GAL4DB-L61Cdc42-expressing yeast strain was used to screen a cDNA library from EBV-transformed human B-cells fused to the GAL4 activation domain (GAL4AD) in the pACT vector as described before (Aspenström et al., 1996; Aspenström, 1997).

The C-terminal 230-439 amino-acids encoding the GAP domain of RhoGAP was subcloned into pACTII, L61Cdc42, N17Cdc42, L61Rac1, N17Rac1, L63RhoA, N19RhoA and L87R-Ras were introduced into pYTH6 and transformed into Y190. The ability of the GAL4DB-fusion small GTPases to bind to GAL4AD-PAR-6:125-346 was analysed by transforming pACT-PAR-6:125-346 into Y190 cells expressing the various GALDB-GTTPase constructs. The cells were grown on medium lacking histidine and supplemented with 25 mM 3-aminotriazole, as described previously (Aspenström et al., 1996, Aspenström, 1997).

A cDNA encoding a full-length murine homologue of the C. elegans PAR-6 protein was obtained from the American Type Culture Collection (clone mg89602.r1, acc. no. AA016558). Accession number for the human PAR-6 is CABB5490. For expression in mammalian cells the mouse PAR-6 was subcloned into the pi3H vector or the pR5myc vector. The cDNAs for the murine PAR-3 as well as the splice variants (100 kDa and 150 kDa) in the pcDNA3 vector were generous gifts from Dan Lin (Toronto, Canada), pR5Kmycl61Cdc42F37A and pR5KmycL61Cdc42F40C were generous gifts from Alan Hall (London, UK). The DNA work followed standard procedures (Sambrook et al., 1989). The DNA sequencing was performed on a Perkin Elmer Genetic Analyzer 310.

**Northern blot analysis**

A hybridisation-ready northern blot (Human Multiple Tissue Northern Blot) was purchased from Clonetech. The blot contained mRNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. A probe representing the C-terminal half of human PAR-6, starting from codon 125, was labelled with [35P]CTP employing the T-cell hybridoma. This work showed that the GST-fusion proteins were purified essentially following the protocol provided by the manufacturer (Amersham Pharmacia Biotech) 250-346 and 125-183 were generated by PCR and subcloned into pGEX-KG. Fragments of PAR-6 corresponding to amino acids 58-346 and 158-256 were subcloned into pGEX-2T. These vectors, as well as the pGEX-2T-RhoGAP-230-439, pGEX-KG-WASP-201-321 (Aspenström et al., 1996) and pGEX-KG-p67hox:1-199 (a gift from D. Diekmann, London), were transformed into the BL21 E. coli strain and the GST-fusion proteins were purified essentially following the protocol provided by the manufacturer (Amersham Pharmacia Biotech), eluted by 5 mM reduced glutathione after which the glutathione was removed by passing the eluted proteins over PD10 pre-packed chromatography columns (Amersham Pharmacia Biotech) equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2 and 1 mM dithiothreitol (DTT). Small GTPases were isolated from GST-fusion proteins by thrombin cleavage (Self and Hall, 1995).

**Dot-blot assay and GTP-dependence assay**

GST fusion proteins were spotted onto nitrocellulose filters and incubated in blocking buffer (5% dried milk, 5% foetal calf serum (FCS), 1% glycine and 1% ovalbumin) for one hour at room temperature. The filters were washed twice in buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA). The filters were then incubated on ice for 10 minutes in 5 ml buffer A containing 0.1 mM GTP, 1 mg/ml BSA and 0.5 μg of GTase preloaded with 10...
μCi of [γ-32P]GTP (Amersham Pharmacia Biotech, 5000 Ci/mmole). The filters were washed three times with 0.1 M Tris-HCl, pH 8, 0.15 M NaCl, 0.1% Tween-20 wrapped in plastic foil and visualised using the FUJIX BAS2000 phosphoimaging system.

For GTP-dependence analysis, a total amount of 0.5 μg of L61Cdc42 and L61Rac1 was preloaded with 2 μCi of [3H]GTP or [3H]GDP (8.3 Ci/mmmol and 11.9 Ci/mmol, respectively, Amersham Pharmacia Biotech) and incubated with GST-PAR-6:58-346 or GST-PAR-6:256-346 (40 μg of each) for 10 minutes on ice (input counts of the GTP/GDP loaded GTPases were 100,000 cpm in all cases). Thereafter, 40 μl of glutathione-Sepharose (Amersham Pharmacia Biotech) beads were added and the mixture incubated end over end for 30 minutes in the cold-room. The beads were washed 3 times with buffer A containing 0.1% Triton X-100 and the beads were collected on nitrocellulose filters and subjected to scintillation counting.

Cell cultivation, transfection and immunoprecipitation
MDCK-II cells were a gift from Björn Öbrink (Stockholm, Sweden). SF/HGF was from RD Systems. Cos-1 and MDCKII cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS and penicillin/streptomycin and incubated at 37°C in an atmosphere of 5% CO2. MDCKII cells were transfected by Lipofectamine (Life Technology) employing the protocol provided by the manufacturer. Cos-1 cells were transfected by the DEAE-dextran method (Kriegler, 1990). 48 hours after transfection, the Cos-1 cells were washed once in ice-cold PBS and lysed in buffer containing (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM PMSF, 1% aprotinin (Trasylo, Bayer). For co-immunoprecipitations of PAR-6 and PAR-3, Cos-1 cells were instead lysed in a RIPA-buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Brij 97, 0.5% deoxycholic acid, 0.1% SDS, 1 mM PMSF, 1% aprotinin) but otherwise handled as the lysed cells above. After incubation on ice, the cells were scraped off the dishes and the lysates were clarified by centrifugation at 13,000 rpm for 15 minutes. The supernatants were incubated together with agarose-conjugated anti-myc antibodies (9E10, mouse monoclonal, Santa Cruz) for 2 hours at 4°C. The beads were thereafter washed three times with lysis buffer after which SDS-PAGE sample buffer was added to each sample. The immunoprecipitates as well as control cell lysates were subjected to SDS-PAGE and the proteins were then transferred to nitrocellulose filters (Hybond C, Amersham). Western blots were detected by the BM chemiluminescence blotting substrate (Boehringer Mannheim).

Antibodies and immunohistochemistry
An antiserum was raised against a peptide representing amino acid residues 2-16 of mouse PAR-6. The IgG fraction of the PAR-6 antiserum was accumulated on a Protein A-Sepharose (Immunosorb, Medicagco) by passing the anti sera over the column. The adsorbed IgG fraction was washed with 0.01 M sodium phosphate, pH 7.5, 0.15 M NaCl, and then eluted by addition of 50 mM glycine, pH 2.7, 10% glycerol; thereafter, the eluted material was dialysed against PBS.

Rabbit polyclonal antiserum recognising PAR-3 was a generous gift from Dan Lin. Commercial antibodies were obtained as follows: rat anti-ZO-1 (Chemicon International, Inc.), mouse anti-E-cadherin and mouse anti-β-catenin (Transduction laboratories), mouse anti-myosin (9E10, Santa Cruz), mouse anti-hemagglutinin (HA) (12C5A, Boehringer Mannheim), goat TRITC-conjugated anti-rat (Jackson ImmunoResearch Laboratories), FITC-or TRITC-conjugated anti-rabbit, and FITC-conjugated anti-mouse antibodies (DAKO). FITC-conjugated phallolidin (Sigma) was used to visualise filamentous actin.

Cells grown on coverslips were fixed in 2% paraformaldehyde in PBS for 20 minutes, washed with PBS and then permeabilised in 0.2% Triton X-100 in PBS for 5 minutes. The cells were washed again and incubated in the presence of 10 mM glycine in PBS for 1 hour. Primary as well as secondary antibodies were diluted in PBS containing 5% FCS. Cells were incubated with primary antibodies followed by secondary antibodies for intervals of 1 hour with a washing step in between. The coverslips were mounted on object slides by the use of Fluoromount-G (Southern Biotechnology Associates, Inc.). Cells were photographed by the QED Imaging System software using a Zeiss Axioplan2 microscope and a Hamamatsu ORCA CCD digital camera.

RESULTS
Isolation of PAR-6 as a Cdc42 binding protein
The yeast two-hybrid system was used to isolate cDNAs from EBV-transformed human B-cells encoding proteins able to bind the activated L61Cdc42 mutant protein which is constitutively in a GTP-bound conformation. We have already reported the identification of WASP, 14-3-3β, MLK3 and CIP4 as Cdc42-binding proteins employing this system (Aspenström et al., 1996; Aspenström, 1997). In addition, some of the L61Cdc42-interacting clones encoded the human homologue of the C. elegans PAR-6 gene-product (Hung and Kemphues, 1999). Since none of these clones encoded a full length human PAR-6, we instead obtained an EST clone encoding a full length mouse PAR-6. The protein contained a central PDZ domain (Fig. 1A), a domain structure originally characterised in the postsynaptic density protein PSD/SAP90, the Drosophila tumour suppressor DlgA and in ZO-1, hence the acronym PDZ (Fanning and Anderson, 1999). This type of domain has been demonstrated to participate in protein:protein interactions, either by binding to the C-termini of proteins or by inter PDZ-domain interactions (Fanning and Anderson, 1999). Database searches demonstrated the presence of EST clones, contained in the UniGene database, encoding two additional genes for human PAR-6 isoforms (Hs.164219 and Hs.225994), suggesting that the PAR-6 family consists of at least three members (Fig. 1B). Northern blot analysis using the human PAR-6 as a probe demonstrated that a transcript of 1.4 kb was present in pancreas, skeletal muscle, brain and heart. Low level expression was also noticed in kidney and placenta (Fig. 1C).

In vitro binding assays reveal that PAR-6 selectively interacts with the active, GTP-bound, Cdc42 and Rac1
The binding of PAR-6 to Rho GTPases was analysed by transforming GAL4 activation domain (GAL4AD)-PAR-6:125-346 in the pACT vector into yeast cells expressing GAL4 DNA-binding domain (GAL4DB)-fusions of Rho GTPases and spreading the cells on medium lacking histidine. The transformants were then patched onto new plates with medium lacking histidine, under which conditions the cells grew only when an interaction between the two fusion proteins had restored a functional GAL4 protein and thereby induced transcription of the reporter genes (Fig. 2A). It was found that GAL4AD-PAR-6:125-346 interacted with GAL4DB-L61Cdc42 and GAL4DB-L61Rac1, which are constitutively GTP-bound, but not with the mutant GAL4DB-N17Cdc42 or GAL4DB-N17Rac1 which are folded in GDP-bound conformations. This indicated that PAR-6 is only recognised by the active conformation of Cdc42 or Rac1 (Fig. 2A). PAR-6 was furthermore tested for binding to the Rho GTPases in vitro. Fragments of PAR-6 were produced as GST-
fusion proteins in E. coli. The GST-fusion proteins were spotted onto nitro-cellulose filters and overlaid with Rho GTPases preloaded with \[^{32}P\]GTP. The GST-PAR-6:58-346 fragment bound to L61Cdc42, V12Cdc42 and to L61Rac1 with the same apparent affinity (Fig. 2B). This PAR-6 fragment also bound wtCdc42 and wtRac1 but not at all L63RhoA (Fig. 2B). We were only able to detect robust interactions between the fragment encompassing amino acid residues 58-346 of PAR-6 and Cdc42/Rac1 GTPases, and none of the truncated versions of PAR-6 bound detectably to the GTPases. The reason for this is not clear but it either suggested that the other GST-fusion proteins produced were unable to fold into a Cdc42/Rac1 interacting domain or, alternatively, the major part of the protein is needed to stabilise high affinity binding to the GTPases. However, the Cdc42/Rac1 binding-site must reside between amino acid residues 125 and 346, since this fragment strongly interacted in the yeast two-hybrid system.

GTP-dependence for the interaction between Cdc42/Rac1 and PAR-6 was tested by incubating GST-PAR-6:58-346 or the GST-PAR-6:256-346 in the presence of L61Cdc42 or L61Rac1 preloaded with \[^{3}H\]GTP or \[^{3}H\]GDP. Glutathione-Sepharose beads were then added to the samples and the radioactivity...
PAR-6 is a target for Cdc42

remaining bound to the beads after washing was determined by collecting the beads on nitro-cellulose filters followed by scintillation counting. This confirmed that PAR-6 only bound to GTP-bound L61Cdc42 and L61Rac1 and not to the GDP-bound proteins (Fig. 2C).

**Co-immunoprecipitation experiments in transfected Cos-1 cells confirmed that PAR-6 interacted with Cdc42 and Rac1**

In order to assay the interaction between PAR-6 and Rho GTPases under in vivo conditions Cos-1 cells were transiently transfected with plasmids expressing HA epitope-tagged PAR-6 together with plasmids expressing Myc epitope-tagged Rho GTPases. Myc-GTPases were immunoprecipitated from cell lysates and the presence of PAR-6 was analysed by western blotting using HA specific antibodies under which conditions PAR-6 was detected as a protein with a molecular mass of 42 kDa (Fig. 3). PAR-6 bound to L61Cdc42 as well as to L61Rac1 with equal affinity but not to N17Cdc42 or N17Rac1, confirming the in vitro binding data. No interaction between L63RhoA and PAR-6 was detected. A similar result was obtained in three independent experiments. This strongly suggested that PAR-6 interacts with the activated Cdc42 and Rac1 in living cells and thereby can function as an effector for these GTPases.

We furthermore tested the affinity of PAR-6 for L61Cdc42 proteins harbouring effector loop mutations (F37A and Y40C). These Cdc42 mutant proteins have been implicated in triggering distinct signalling pathways downstream of the GTPase: F37AL61Cdc42 specifically activates the JNK/SAPK signalling cascade and Y40CL61Cdc42 activates actin cytoskeleton reorganisation (Lamarche et al., 1996). In this case, it was found that PAR-6 interacted with equal apparent affinity with both L61Cdc42 mutant proteins, suggesting that PAR-6 does not mediate specific signals to the actin cytoskeleton or to the JNK/SAPK signalling pathway (Fig. 3). This is in agreement with experiments where PAR-6 was transiently transfected into NIH-3T3 and Cos-1 under which conditions no apparent effect could be detected, neither on actin rearrangement nor activation of the JNK/SAPK signalling pathway (data not shown).

**Subcellular localisation of PAR-6 in epithelial cells**

An antiserum was raised against a peptide derived from the amino terminus of mouse PAR-6, and used to stain MDCK cells. Endogenous PAR-6 was detected in the cell nucleus, as well as in cell-cell contacts (Fig. 4). A similar localisation of PAR-6 was detected also in other cells of epithelial origin, such as normal rat intestinal epithelial (IEC-6) cells (data not shown). The nuclear translocation of PAR-6 might be achieved by the putative nuclear localisation signal present in the N-terminal part of PAR-6. The staining pattern of PAR-6 in MDCK cells was indicative for a localisation to the tight junctions since the protein exhibited a high degree of co-localisation with the tight junction protein ZO-1 (Fig. 4A-C). The proteins showed little or no co-localisation with β-catenin and E-cadherin, which are localised in the basolateral cell-cell contacts of the MDCK cells (Fig. 4G-L). To confirm the nuclear localisation of PAR-6, MDCK cells were transiently transfected with HA epitope-tagged PAR-6 and stained with HA-specific antibodies. As expected, the staining pattern corroborated the staining pattern for endogenous PAR-6. HA-PAR-6 was seen in cell-cell contacts as well as in the nucleus (Fig. 5), however, the cytoplasmic staining was more elaborate presumably due to the overexpression of the exogenous protein.

Activation of MDCK cells with scatter factor/hepatocyte growth factor (SF/HGF) have been shown to induce cell spreading and disruption of cell-cell contacts, and this effect has been shown to require the activity of the Rho GTPases (Ridley et al., 1995; Imamura et al., 1998). Consequently, treatment of the MDCK cells resulted in a loss of cell-cell contacts and displacement of tight junctions (Fig. 6). In accordance with earlier studies (Imamura et al., 1998), an initial increase in the stress fibre content was noticed 4 hours after addition of SF/HGF (Fig. 6D-F) followed by a loss of stress fibres and an increased accumulation of polymerised actin at the cell periphery after 16 hours of SF/HGF stimulation (Fig. 6J-L). The tight junctions started to loosen after 4 hours of HGF treatment as revealed by the ZO-1 staining (Fig. 6A-C). Loss of PAR-6 from the area of tight junctions was concomitant with their progressive breakdown, which was
Further manifested after 16 hours of HGF-treatment where most of the ZO-1 and PAR-6 were dislocated from the area of cell-cell contacts (Fig. 6G-I). Under these conditions the nuclear localisation of PAR-6 remained unaffected.

PAR-6 bind directly to PAR-3
Previous studies found the mammalian PAR-3 homologue localised to tight junctions in epithelial cells (Izumi et al., 1998). We were unable to perform double staining for PAR-6 and PAR-3 in the same cell, since the antisera recognising the respective proteins were both of rabbit polyclonal origin. However, localisation of mammalian PAR-3 to the tight junctions in the MDCK cells was confirmed and the staining pattern closely resembled the one for PAR-6 (Fig. 7B) but, in contrast to PAR-6, no nuclear staining for PAR-3 could be

![Fig. 4. Immunolocalisation of PAR-6 in MDCK cells. PAR-6 (A,D,G,J), ZO-1 (B), merge PAR-6/ZO-1 (C), F-actin (E), merge PAR-6/F-actin (F), β-catenin (H), merge PAR-6/β-catenin (I), E-cadherin (K), merge PAR-6/E-cadherin (L). Bar, 20 μm.](image)

![Fig. 5. Immunolocalisation of HA-PAR-6 in transiently transfected MDCK cells. (A) Short exposure time. (B) long exposure time to visualise localisation in nucleus and cell-cell contacts, respectively. Bar, 20 μm.](image)
PAR-6 is a target for Cdc42 detected. We further tested the interaction between the mammalian PAR-6 and PAR-3 by cotransfecting plasmids expressing PAR-6 and the different splice variants of PAR-3. It was found that PAR-6 interacted with all three splice variants of 180, 150 and 100 kDa respectively, as judged by their presence in PAR-6 immunoprecipitates (Fig. 7A). This strongly suggests that PAR-6 and PAR-3 participate in regulating cell asymmetry also in mammalian cells.

**DISCUSSION**

Studies on organisms like yeast and *C. elegans*, involving both genetic screening procedures and biochemical analyses, have been extremely rewarding in delineating the factors required for the establishment of cell asymmetry (Drubin and Nelson, 1996; Guo and Kemphues, 1996). However, the molecular machinery behind this complex process has only partially been characterised. Several lines of evidence implicate Rho GTPases, in particular Cdc42, as important regulators of cell polarity in eukaryotic cells, roles which have been conserved throughout the phylogenetic tree (Johnson, 1999). The finding that Cdc42 directly bind to the polarity protein PAR-6 suggests that essential parts of the molecular machinery for the establishment of cell polarity has been conserved from *C. elegans* to human.

The establishment of cell polarity is of vital importance already during the first cell divisions and studies on early mouse embryos have indicated a role for Cdc42, as well as for Rho in this process. Microinjection of a constitutively active V12Cdc42 into four-cell blastomeres induced nuclear displacement and aberrations in the actin organisation.
indicative of a defect in polarisation (Clayton et al., 1999). In the adult body, cell polarisation has been extensively studied in epithelial cells (Drubin and Nelson, 1996). In these cells the establishment of cell polarity originates from the adhesion of cells to the substratum or extracellular matrix. This initiates an asymmetrical distribution of cytoskeletal components and subsequently the formation of a polarised gradient from the basolateral to the apical part of the cells. This polarisation is maintained both by the formation of tight junctions at the apical cell-cell contacts and by a continuous sorting of proteins and membrane components to the apical and basolateral cellular compartments (Drubin and Nelson, 1996; Balda and Matter, 1998; Tsukita et al., 1999). The Rho GT-Pases participate during all of these stages of the establishment of cell polarity. Rho has been found to be involved in regulating the assembly of tight junctions in T84 human intestinal epithelial cells. Inactivation of Rho with exotoxin lead to a displacement of the perijunctional actin filaments as well as the tight junction component ZO-1 (Nusrat, 1995). Studies of MDCK cells, furthermore, implicate that Rho affects the assembly of both tight junctions and the basolateral cell-cell contacts involving the activity of β-catenin and E-cadherin in contrast to Rac, which only had a role in the β-catenin/E-cadherin-regulated cell-cell interactions (Takaishi et al., 1997; Gopalakrishnan et al., 1998; Jou and Nelson, 1998; Jou et al., 1998; Imamura et al., 1998). Cdc42 have also been implicated in regulating cadherin-based cell-cell adhesion in a manner independent of Rac (Kodama et al., 1999). Another mechanism for Cdc42-regulated cell polarity was implicated by the finding that Cdc42 regulate endocytic trafficking from the apical to the basolateral part of MDCK cells, a process that is required for the maintenance of a polar gradient in these cells (Kroshewski et al., 1999). All these Rho protein-regulated processes are likely to coordinate signals for the establishment of cell polarity and it is likely that several effector proteins are involved. For instance, the Cdc42 and Rac effector IQGAP1 has been shown to directly bind β-catenin and participate in the regulation of cadherin-based cell-cell adherence (Van Aelst and D’Souza-Schorey, 1997; Fukata et al., 1999a,b). Future work will elucidate how PAR-6/PAR-3 and components like IQGAP1/IQGAP2 work in concert with other effectors for Rho proteins in order to regulate cell adherence and polarity.

In addition to Cdc42 and Rac1, PAR-6 also binds to the mammalian PAR-3. An interaction between PAR-6 and PAR-3 was suggested, but not directly proven, from their co-localisation in the early C. elegans embryo (Hung and Kemphues, 1999). Mutations in the par-6 and par-3 genes result in loss of symmetry during the first cell division and an aberrant distribution of cytoskeletal components associated with loss of cell polarity (Watts et al., 1996; Guo and Kemphues, 1996; Hung and Kemphues, 1999). The molecular mechanisms underlying PAR-6/PAR-3 induced cell asymmetry is currently unclear, but our findings implicate that Cdc42 and Rac1 might participate in this process. A mammalian PAR-3, called ASIP, was recently identified as a protein binding to the atypical PKCβ, and these two proteins co-localised to cell-cell junctions both in fibroblasts and MDCK cells (Izumi et al., 1998). In the latter cells PAR-3 and PKCβ was found in tight junctions and they were suggested to have a role in epithelial cell polarity (Izumi et al., 1998). Interestingly, the mammalian PAR-3 has furthermore been found to bind to members of the B-type ephrins, which are ligands for class B Eph receptors, suggesting a role for this molecule in ephrin signalling (Lin et al., 1999). If Cdc42, Rac1 and PAR-6 can participate in this type of signalling remains to be studied.

A strong nuclear localisation of PAR-6 was detected presumably guided by the potential nuclear localisation signal present in the N-terminal part of PAR-6. Under the conditions for the experiment, PAR-6 was constitutively localised to the nucleus in addition to its tight junctional localisation, in contrast to PAR-3, which only localised to tight junctions. Treatment of cells with SF/HGF resulted in loss of cell-cell contacts as visualised by the gradual displacement of ZO-1 and PAR-6 from the areas of tight junctions. Some integral membrane components like symplekin and cingulin have been reported to be present in the nucleus indicating nuclear localisation under certain conditions; ZO-1 has been found in the nucleus of subconfluent MDCK cells (Gottardi et al., 1999). The tight junction proteins symplekin and cingulin have also been reported to be present in the nucleus indicating the role of these molecules in the establishment of cell polarity.

### Fig. 7

(A) Immunoprecipitation of myc-epitope tagged PAR-6 with the 100, 150 and 180 kDa splice variants of PAR-3 from transiently transfected Cos-1 cell lysates. (B) Immunolocalisation of PAR-3 in MDCK cells. Bar, 20 μm.
that tight junction components could have additional cellular roles (Keon et al., 1996; Cordenosi et al., 1999). Intriguingly, in the case of PAR-6, one such role could be related to the activity of the HTLV-1 oncoprotein Tax. A yeast two-hybrid search identified PAR-6 as a Tax-binding protein (Roussel et al., 1998). The Tax protein acts as a transactivator in activation of gene expression via the ATF/CREB and NF-κB pathways leading to transformation of T-lymphocytes (Bex and Gaynor, 1998). What roles, if any, PAR-6 has in Tax transactivation remains to be studied.

In conclusion, we have reported that PAR-6 is a binding partner for Cdc42 and Rac1 as well as for PAR-3. The colocalisation of PAR-6 with PAR-3 and ZO-1 furthermore suggest that both PAR-6 and PAR-3 are present in tight junctions in MDCK cells. These findings, together with the reported roles of PAR-6 and PAR-3 in *Caenorhabditis elegans*, suggest that Cdc42 an Rac1 and PAR-6/PAR-3 are involved in the establishment of cell polarity.

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