# A Cdc42 target protein with homology to the non-kinase domain of FER has a potential role in regulating the actin cytoskeleton

Pontus Aspenström

**Background:** Members of the Rho family of small GTPases have been shown to have a diverse role in cell signalling events. They were originally identified as proteins that, by regulating the assembly of the actin cytoskeleton, are important determinants of cell morphology, and have recently been shown to be involved in transcriptional activation by the JNK/SAPK signalling pathway. In order to understand the mechanisms underlying the effects of Rho GTPases on these processes, the yeast two-hybrid system has been used to identify proteins that bind to an activated mutant of Cdc42, a Rho-family member.

**Results:** A cDNA encoding a previously unidentified Cdc42 target protein, CIP4, which is 545 amino-acids long and contains an SH3 domain at its carboxyl terminus, was cloned from a human B-cell library. The amino terminus of CIP4 bears resemblance to the non-kinase domain of the FER and Fes/Fps family of tyrosine kinases. In addition, similarities to a number of proteins with roles in regulating the actin cytoskeleton were noticed. CIP4 binds to activated Cdc42 *in vitro* and *in vivo* and overexpression of CIP4 in Swiss 3T3 fibroblasts reduces the amount of stress fibres in these cells. Moreover, coexpression of activated Cdc42 and CIP4 leads to clustering of CIP4 to a large number of foci at the dorsal side of the cells.

**Conclusions:** CIP4 is a downstream target of activated GTP-bound Cdc42, and is similar in sequence to proteins involved in signalling and cytoskeletal control. Together, these findings suggest that CIP4 may act as a link between Cdc42 signalling and regulation of the actin cytoskeleton.

Address: Ludwig Institute for Cancer Research, Biomedical Center, Box 595, S-751 24 Uppsala, Sweden. E-mail: pontus.aspenstrom@LICR.uu.se

Received: **12 March 1997**
Revised: **28 April 1997**
Accepted: **19 May 1997**

Published: **13 June 1997**

**Current Biology** 1997, 7:479–487
http://biomednet.com/elecref/0960982200700479



## Background

The actin filament system is an important determinant for the morphological heterogeneity and motile behaviour of eukaryotic cells [1,2]. There is a close correlation between transmembrane signalling events and the mobilisation of the actin filament system. This correlation can be visualised by treating cells in tissue culture with growth factors, such as platelet-derived growth factor (PDGF) or epidermal growth factor: the immediate response is an accumulation of polymerised actin at the cell periphery, a phenomenon known as membrane ruffling [3,4]. The highly dynamic organisation of the actin filament system is indispensable for the capability of cells to respond to changes in the external environment. Members of the Rho family of small GTPases have recently emerged as potent regulators of the actin filament system, linking ligand–receptor interactions to the assembly of actin-containing subcellular compartments [5]. There are at least six distinct members of the Rho GTPases: Rho (isoforms A, B and C), Rac (isoforms 1 and 2), Cdc42 (Cdc42Hs and G25K isoforms), RhoG, RhoE and TC10 [5,6]. They are closely related to the proto-oncogene product Ras, with which they share the ability to bind and hydrolyse GTP. These small GTPases are activated when in the GTP-bound conformation and the hydrolysis of the bound GTP inactivates the proteins. The cycling between active and inactive conformations enables the Rho GTPases to function as binary switches. Furthermore, this cycling is governed by a large number of proteins that either activate the Rho proteins by facilitating GDP/GTP exchange — the so-called guanine nucleotide exchange factors (GEFs) [7] — or inactivate them by increasing their rate of intrinsic GTP hydrolysis [8]. This latter group of proteins are called GTPase activating proteins, or GAPs.

Most of the initial information about the regulatory role of the Rho GTPases on the actin filament system came from studies on Swiss 3T3 fibroblasts. It was shown that Rac regulates the membrane ruffling activity that occurs at the cell edge [9], whereas Rho appears to have a specific role in controlling the assembly of focal contacts [10]. Cdc42, on the other hand, was originally found in the budding yeast *Saccharomyces cerevisiae*, in which it was characterised as a gene involved in the cell division cycle [11]. In *S. cerevisiae*, the activity of Cdc42 is needed for correct assembly of protein components, including actin, at the site of the bud.

In fibroblasts, microinjection of activated Cdc42 triggers formation of filopodia or microspikes at the cell periphery [12,13]. Moreover, recent studies have demonstrated that the Rho GTPases also participate in transcriptional regulation. Cdc42 and Rac are able to induce a cascade of sequential phosphorylations that eventually leads to activation of the Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) [14–17]. Cdc42 and Rac also have a role in cell-cycle control, as they are needed for progression through G1 [17]. In contrast to Ras, the Rho GTPases show very weak transforming activity; however, Rac and Rho appear to be important for maintenance of the transformed phenotype of cells transformed with Ras or Raf [18–20].

A lot of effort has been put into the search for proteins that bind to activated Rac and Cdc42 in order to understand the mechanisms underlying their effects on actin assembly and JNK/SAPK activation. Several proteins that are recognised by the activated, GTP-bound forms have been found: these include the Ser/Thr protein kinases PAK [21] and the SH3-domain-containing proline-rich protein kinase (SPRK), which is also known as mixed lineage kinase 3 (MLK3) [22,23], the non-receptor tyrosine kinase $p120^{ACK}$ [24] and the Wiskott–Aldrich syndrome protein (WASP) [25–27]. Database analysis has shown that these proteins contain a common Cdc42/Rac-binding motif, the so-called CRIB motif, which is present in a number of other proteins from different organisms [28]. Cdc42 and Rac also bind to the RasGAP-related protein IQGAP1 [29], and they have also been implicated in the activation of the $p70^{S6}$ kinase, which leads to transcriptional activation in a pathway distinct from the JNK/SAPK signalling pathway [30].

It is not clear how Cdc42 and Rac can induce such apparently distinct cellular responses as reorganising the actin cytoskeleton and activating JNK/SAPK-controlled transcription. A clue has recently come, however, from studies using Cdc42 and Rac harbouring mutations in their effector loops. This domain of the protein is involved in the interaction of the GTPase with its effector proteins, and the binding can be modulated by the introduction of point mutations. Cdc42 and Rac harbouring Tyr40→Cys amino-acid substitutions no longer bind PAK or WASP, and the mutant proteins are unable to activate the JNK/SAPK signalling pathway; however, they are still able to induce cytoskeletal reorganisation [31,32]. Rac containing a Phe37→Ala or Phe37→Leu mutation is not able to induce lamellipodia, whereas binding to PAK and activation of JNK/SAPK are unaffected [31,32]. These findings suggest that Cdc42 and Rac control the JNK/SAPK pathway and the assembly of the actin cytoskeleton through distinct downstream targets.

I have used the yeast two-hybrid system to isolate proteins that bind specifically to the active GTP-bound conformation of Cdc42. In this screen, using an Epstein–Barr virus (EBV)-transformed B-cell library, five potential Cdc42-interacting proteins (designated CIP1–CIP5) were isolated. We have previously identified one of these as WASP [25]. Here, the isolation of another one, CIP4, which encodes a 545 amino-acid residue protein with a possible role in the regulation of the actin cytoskeleton, is described.

## Results

### Isolation of CIP4

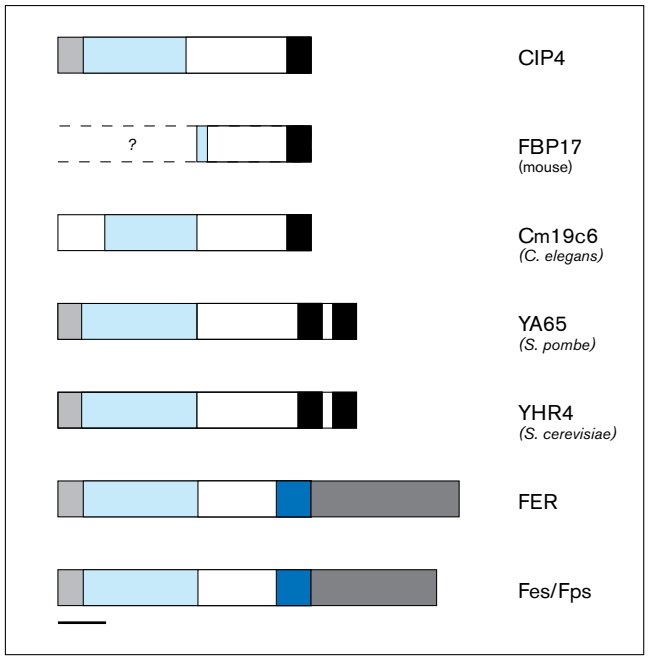
The yeast two-hybrid system was used to isolate cDNAs encoding proteins that bind to a constitutively activated mutant of Cdc42 (L61Cdc42), containing a Gln61→Leu substitution. Using this screen, 19 clones were identified that retained the positive signal when reintroduced into yeast cells expressing a fusion protein of the GAL4 DNA-binding domain (GAL4DB) and L61Cdc42. These cDNAs encoded five different proteins, which were designated Cdc42-interacting protein (CIP) 1–5. Database searches showed that CIP1 was identical to WASP, an observation which has been described elsewhere [25]. CIP2 turned out to be identical to 14-3-3β, a protein implicated in the activation of the c-Raf protein kinase and in protein kinase C (PKC) signalling [33]. However, an interaction between Cdc42 and 14-3-3β could not be detected in *in vitro* binding assays (P.A., unpublished observations), thus the biological significance of this interaction remains to be validated. The single copy of the clone encoding CIP3 was a partial cDNA of the *SPRK/MLK3* gene [22,23]. CIP4 and CIP5 are previously unidentified proteins, although a human cDNA of 620 basepairs (bp) that exactly matched nucleotides 1370–1952 of the *CIP4* gene was found in the database. This partial clone contained an additional 37 bp at the 5′ end that did not match *CIP4* but represented mitochondrial DNA [34].

The longest *CIP4* clone isolated was 2 kb and sequence analysis strongly suggested that it encodes the full-length CIP4 protein as the putative initiator codon is preceded by the hexamer sequence AGCAGC, which is in agreement with the consensus for initiation of transcription [35]. The *CIP4* open reading frame encodes a protein of 545 amino acids with a calculated molecular mass of 63 kDa and an estimated pI of 5.2 (see Supplementary material).

### Domain structure of CIP4

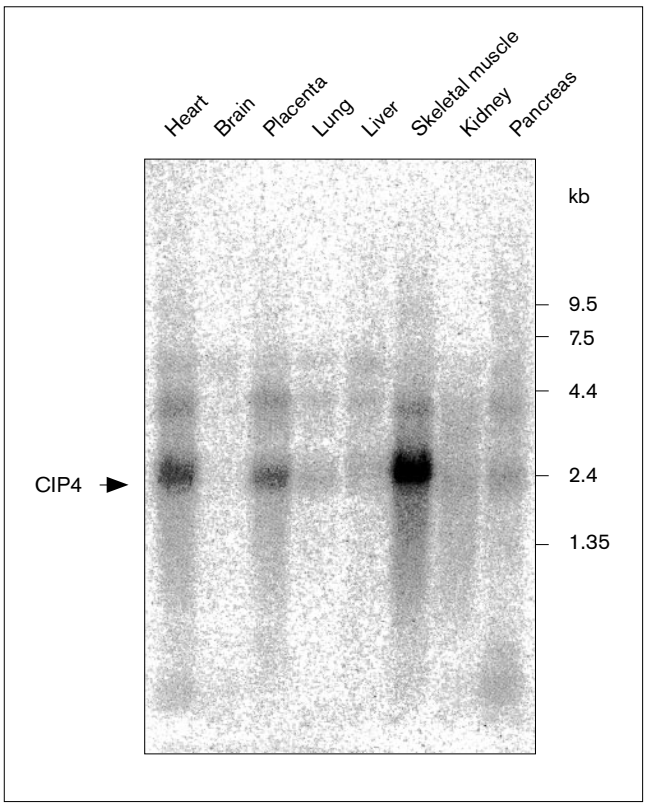
Analysis of the primary structure of CIP4 showed that it contained an SH3 domain at the extreme carboxyl terminus, and database searches revealed the presence of related proteins in several organisms: YHR4 in *S. cerevisiae*, YA65 in *Schizosaccharomyces pombe* and Cm19c6 in *Caenorhabditis elegans* (Figure 1). In addition, a partial, carboxy-terminal sequence of a mouse protein, FBP17, has about 50% identity to the corresponding region of CIP4. FBP17 has been shown to bind to a proline-rich peptide derived from formin, which is a protein implicated in the development of limbs [36]. Like CIP4, FBP17 and

**Figure 1**




Proteins with homology to CIP4 are shown, with the Fer–CIP4 homology (FCH) domains (light grey), FER-homologous domains (light blue), tyrosine kinase domains (dark grey), SH2 domains (dark blue) and SH3 domains (black) indicated.

**Figure 2**




Tissue distribution of CIP4. Northern blot analysis of mRNA from the human tissues indicated.

Cm19c6 contain carboxy-terminal SH3 domains, whereas YHR4 and YA65 each contains two such domains in tandem. The SH3 domain of CIP4 most closely resembles the one in FBP17 (67% identity) and to a lesser extent the one in Cm19c6 (52% identity) and the first of the two in YA65 and YHR4 (42% and 33% identity, respectively); its identity to the SH3 domains of Abl2, Src and Fyn is approximately 30% (see Supplementary material). Furthermore, the first 300 amino acids of CIP4 show similarity to the non-catalytic amino termini of the non-receptor tyrosine kinase FER and the Fujinami Sarcoma virus Fes/Fps family of proto-oncogene products [37,38]. The part of CIP4 with the highest degree of similarity to other protein domains is the region between amino acids 132 and 294 (see supplementary material).

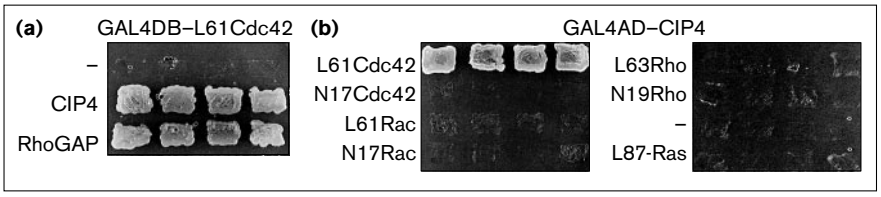
Further computer analysis suggested the presence of previously unidentified domain structure at the extreme amino terminus of CIP4, amino acids 1–56 (see supplementary material). This motif, which we have chosen to call FER–CIP4 homology (FCH) domain, is present in FER and CIP4 as well as in a number of proteins throughout the phylogenetic tree, most notably the RhoGAP protein p115 [39], the actin-organising protein Cdc15 from *S. pombe* [40], the mouse proteins h74 and growth-arrest-specific gene product. In addition, this domain is present in two gene products from *C. elegans* (F09E10.9 and F45E1.7) and one from *S. cerevisiae* (YM9973.05). Nothing is known about the function of these latter proteins.

In contrast to the other CIP4-like proteins shown in Figure 1, CIP4 shows some similarity to moesin and to other members of the ezrin/radixin/moesin (ERM) family of actin-binding proteins [41]. The overall identity between CIP4 and moesin is relatively low, around 17%, but the degree of homology is more pronounced, around 30% identity, between a fragment encompassing amino acids 329–410 of CIP4 and an analogous domain of moesin and the other ERM proteins (see Supplementary material). This domain overlaps with the Cdc42-binding domain of CIP4, amino acids 293–481.

### Tissue distribution of CIP4

Northern blot analysis demonstrated that a major CIP4 transcript of 2.2 kb was abundant in skeletal muscle, heart and placenta (Figure 2). Lower levels of transcript were present in pancreas, lung, liver and kidney, and there was almost none at all in brain. The transcript size of 2.2 kb correlates well with the isolated 2 kb *CIP4* cDNA and further supports the notion that the isolated clone is close to full length. In addition, weak bands of 3.5 and 5 kb

**Figure 3**




Interaction of Cdc42 with CIP4 in the yeast two-hybrid system. Four independent colonies were patched onto selective medium as described in Materials and methods and analysed for growth. **(a)** The interaction between GAL4DB–L61Cdc42 and the indicated GAL4AD fusion proteins. **(b)** The ability of GAL4AD–CIP4 to interact with the indicated GAL4DB fusion proteins.

were visible, which most probably represent alternatively spliced variants of CIP4.

### Binding of GTPases to CIP4 in the yeast two-hybrid system

The binding of full length CIP4 ($CIP4_{1–545}$) to L61Cdc42 was analysed by reintroducing a fusion of $CIP4_{1–545}$ and the GAL4 activation domain (GAL4AD) in the pACT vector into yeast cells expressing GAL4DB–L61Cdc42, after which the cells were spread on medium lacking histidine, as described in Materials and methods. The transformants were then patched onto new plates with medium lacking histidine. Under these conditions, cells grow only in those cases where an interaction between the two fusion proteins restores a functional GAL4 protein and thereby induces transcription of the reporter genes. Figure 3a shows that GAL4AD–$CIP4_{1–545}$ interacts with GAL4DB–L61Cdc42 but not with GAL4DB fused to a dominant-negative Cdc42 mutant that is constitutively in the GDP-bound form (N17Cdc42). GAL4AD–RhoGAP, which is known to bind to GAL4DB fused to Rho and Rac activated mutants containing leucine at positions 63 and 61, respectively (L63Rho and L61Rac) as well as to GAL4DB–L61Cdc42 in this system [42], was included as a control (Figure 3a). GAL4AD–$CIP4_{1–545}$ did not bind to any other GAL4DB–GTPase tested (Figure 3b). In addition, the plates were analysed for lacZ activity using a membrane transfer assay and X-gal as a substrate [42] (data not shown).

**Table 1**

**Interaction between GAL4BD–L61Cdc42 and GAL4AD fusion proteins in the yeast two-hybrid system.**

| GAL4DB fusion | GAL4AD fusion | β-galactosidase activity (U) |
|---|---|---|
| L61Cdc42 | RhoGAP | 4.9 (± 0.6) |
| L61Cdc42 | CIP4 | 59.2 (± 5.1) |
| – | CIP4 | 0.2 (± 0.1) |

Vectors encoding CIP4 and RhoGAP GAL4AD fusion proteins were transformed into Y190 cells expressing GAL4DB–L61Cdc42 or GAL4–DB alone. The transformed yeast cells were plated onto selective media lacking histidine and supplemented with 25 mM 3-aminotriazole. Three colonies from each transformation were isolated and cultivated overnight in selective medium. The cells were collected and permeabilised, after which the β-galactosidase activity was measured using ONPG as a substrate. The results represent the means of duplicate samples from each of the three independent transformations.

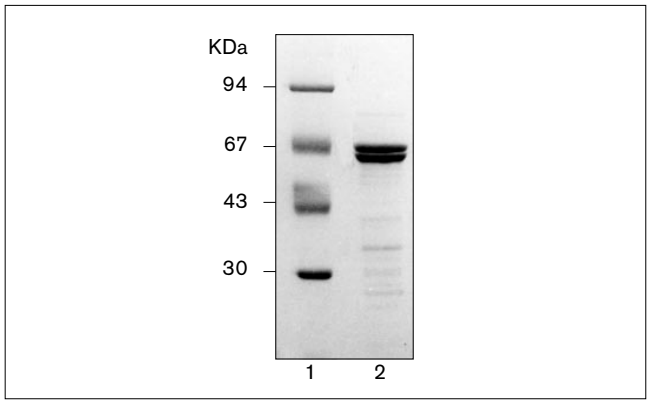
The strength of the interaction between GAL4AD–$CIP4_{1–545}$ and GAL4DB–L61Cdc42 was determined using a solution assay in which cells were lysed and the β-galactosidase activity determined spectrophotometrically by using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate. The β-galactosidase activity in yeast transformed with both CIP4 and L61Cdc42 fusion proteins was 59 units, which was more than 10 times the activity estimated for yeast transformed with both RhoGAP and L61Cdc42 fusion proteins [Table 1].

### *In vitro* binding analysis

In order to analyse the binding of CIP4 to Cdc42 *in vitro*, amino acids 293–481 of CIP were produced as a glutathione-S transferase (GST) fusion protein (GST–$CIP4_{293–481}$) in *Escherichia coli*. This fragment was selected because CIP4 lacking residues 1–292 was still able to bind to Cdc42 in the yeast two-hybrid system (data not shown) and to exclude the SH3 domain, which is unlikely to bind directly to Cdc42. The expression level of GST–$CIP4_{293–481}$ was relatively high; however, the protein appeared as a doublet when analysed by gel electrophoresis, presumably due to partial degradation (Figure 4). The interaction between CIP4 and Rho GTPases was analysed using a dot-blot assay, in which GST–$CIP4_{293–481}$ protein was spotted onto nitrocellulose filters and overlaid with Rho GTPases preloaded with [$^{32}$P]GTP. GST–$CIP4_{293–481}$ bound equally well to L61Cdc42 and to another mutant of Cdc42 (V12Cdc42; which contains a valine at position 12), and with a slightly weaker apparent affinity to wild-type Cdc42 (Figure 5a). CIP4 did not bind at all to L61Rac, whereas the interaction with L63Rho was of the same apparent strength as the one with L61Cdc42 (Figure 5a). After washing the filters with buffer containing 0.5 M NaCl, however, CIP4 no longer bound to L63Rho, whereas its interaction with L61Cdc42 remained strong (Figure 5b).

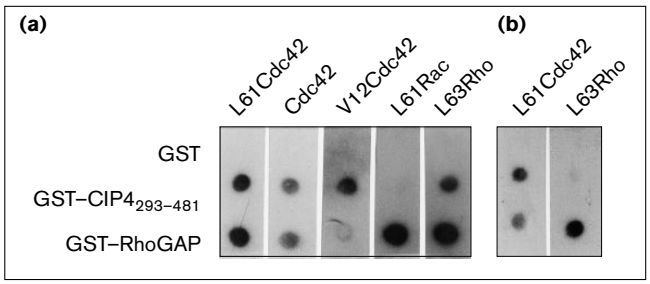
The GTP dependence of the interaction between Cdc42 and CIP4 was analysed using a bead assay in which GST–$CIP4_{293–481}$ was incubated with L61Cdc42 preloaded with either [$^{3}$H]GTP or [$^{3}$H]GDP. Glutathione–Sepharose

**Figure 4**




Gel electrophoretic analysis of GST–CIP4$_{293–481}$ (lane 2) run alongside molecular weight markers (lane 1).

**Figure 5**



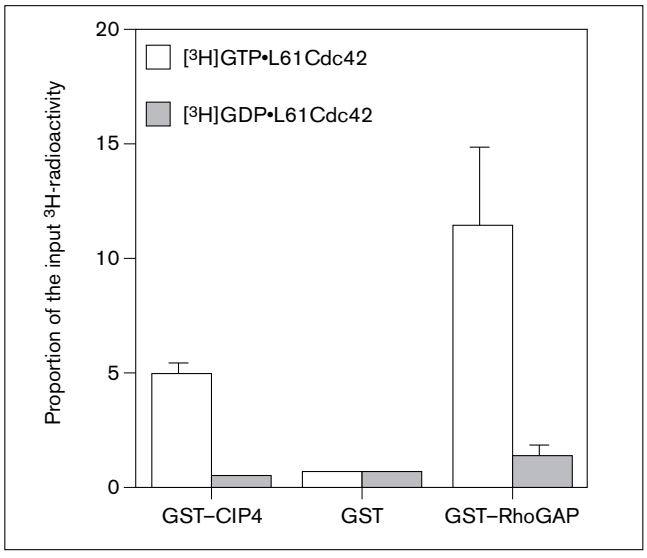

Interaction of GST–CIP4$_{293–481}$ with Cdc42, Rac and Rho in the dot-blot assay. GST fusion proteins were spotted onto nitrocellulose filters and incubated in the presence of GTPases preloaded with [$^{32}$P]GTP and the filters were visualised after either **(a)** normal washing or **(b)** a further wash in buffer containing 0.5 M NaCl.

beads were then added to the samples and the radioactivity remaining bound to the beads after washing was determined by collecting the samples onto nitrocellulose filters and counting them in a scintillation counter. About 5% of the added [$^{3}$H]GTP·L61Cdc42 remained bound to GST-CIP$_{293-481}$, which was well above the background estimated using free GST as a control (Figure 6). Under similar conditions, 11% of the added radioactivity remained bound to GST–RhoGAP. No binding of GST–CIP4$_{293–481}$ to [$^{3}$H]GDP·L61Cdc42 was detected. The interaction between GST–CIP4$_{293–481}$ and L63Rho was not strong enough to be detected in this type of assay (data not shown). In addition, the ability of CIP4 to increase the intrinsic GTPase activity of Cdc42 was determined by incubating GST–CIP4$_{293–481}$ with wild-type Cdc42 which had been preloaded with [$^{32}$P]GTP. No effect of the CIP4 fragment on the hydrolysis activity of Cdc42 could be detected (data not shown).

### Intracellular localisation of CIP4

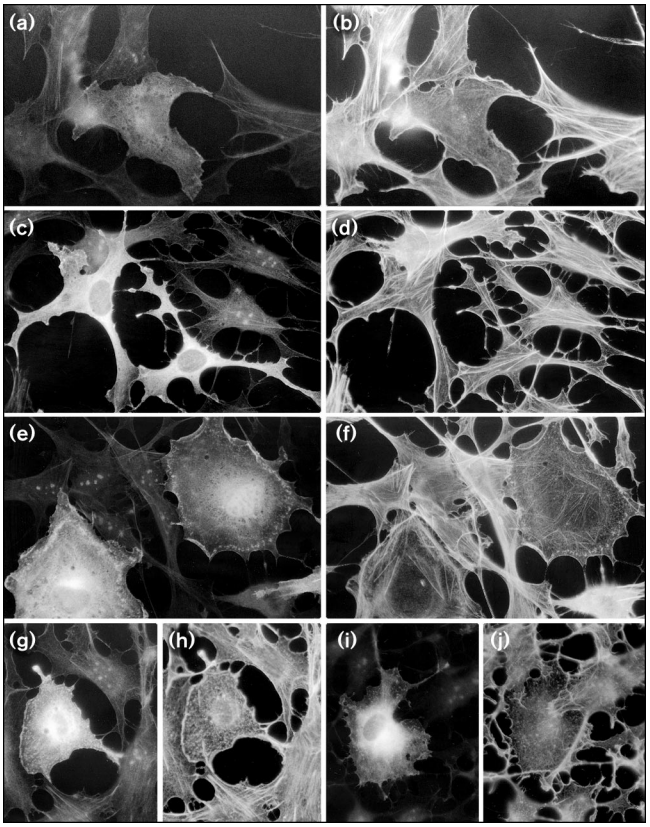
Swiss 3T3 fibroblasts were seeded on coverslips and transiently transfected with haemagluttinin-tagged CIP4 (HA–CIP4) and the proteins' subcellular localisation was detected using an anti-HA antibody (Figure 7). HA–CIP4 appeared to be present throughout the cell body, particularly in cells expressing high levels of the protein. An accumulation of HA–CIP4 protein was often found at the cell periphery, particularly in areas that exhibited membrane ruffling (Figure 7a,c). In addition, the edges of the cells expressing HA–CIP4 had a more undulated appearance than cells in the same sample expressing background levels of CIP4 (Figure 7a,c). Expression of HA–CIP4 correlated with a decrease in the general tetramethylrhodamine isothiocyanate (TRITC)-phalloidin staining and with a decrease in stress-fibre content, suggesting that CIP4 either caused breakdown of the pre-existing stress-fibres or interfered with their formation (Figure 7b,d). The inhibitory role of CIP4 was also tested in transfected cells that had been serum-starved for 12 hours and then stimulated by the addition of 10% fetal calf serum (FCS) before fixation. This procedure effectively triggered the formation of stress fibres in control cells; however, the stress-fibre content was reduced in HA–CIP4-expressing cells and the actin filaments still visible appeared thinner and less organised than in the cells expressing background levels of CIP4 (Figure 7e,f). In addition, a reduction of the stress-fibre content was detected in HA–CIP4-expressing cells stimulated with either the B-chain of PDGF (PDGF-BB; Figure 7g,h) or bradykinin (Figure 7i,j).

**Figure 6**




GTP-dependence of the interaction between Cdc42 and CIP4 determined using the glutathione–Sepharose bead assay. Results are the mean of four separate experiments measuring the binding of GST, GST–RhoGAP$_{230–439}$ and GST–CIP$_{293–481}$ to [$^{3}$H]GTP·L61Cdc42 and [$^{3}$H]GDP·L61Cdc42.

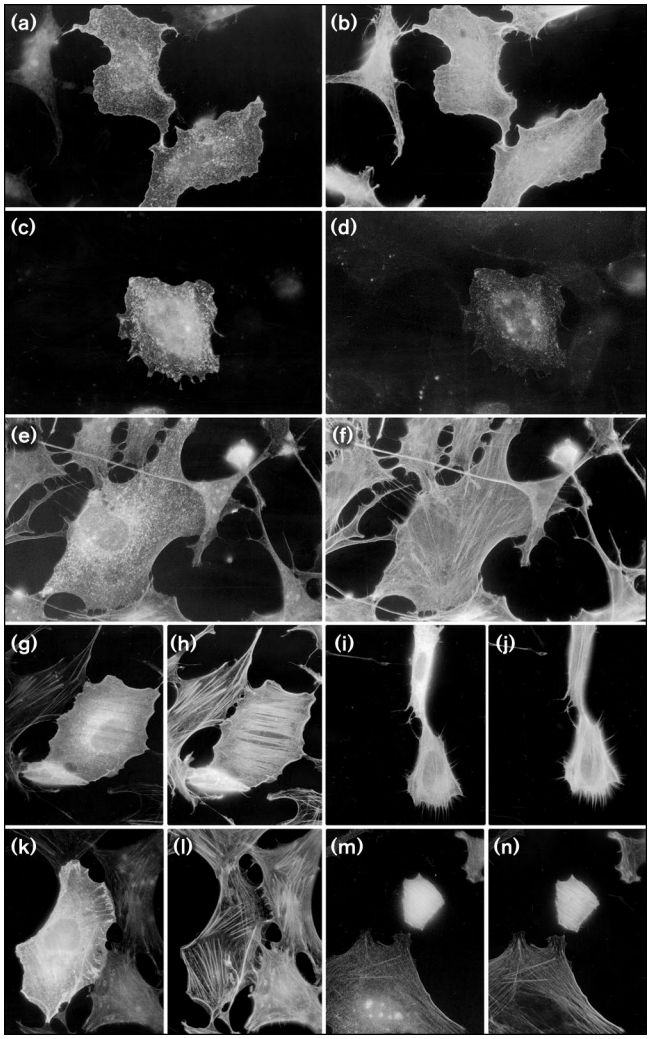
**Figure 7**




Subcellular localisation of HA–CIP4 in Swiss 3T3 fibroblasts transfected with pJ3H–CIP4. **(a,b)** Cells were left for 48 h without removal of serum before fixation and detection of either (a) HA–CIP or (b) filamentous actin, as described in Materials and methods. For other treatments, cells were either **(c,d)** serum-starved for 12 h, or serum-starved and then stimulated with either **(e,f)** 10% FCS for 30 min, **(g,h)** 100 ng PDGF-BB for 15 min, or **(i,j)** 100 ng bradykinin for 1 h before fixation and detection of either (c,e,g,i) HA–CIP4 or (d,f,h,j) filamentous actin.

Cotransfection of L61Cdc42 with HA–CIP4 caused a clustering of HA–CIP4 into a large number of brightly shining dots which were found to be predominantly on the dorsal side of the cells (Figure 8a,b). These dots appeared to co-localise with Cdc42 (Figure 8c,d); however, such a distribution of Cdc42 was not detected in cells expressing only L61Cdc42, in which the staining appeared uniform (Figure 8g,h). Surprisingly, a similar spotted organisation of CIP4 protein was also visible in cells expressing both N17Cdc42 and HA–CIP4 (Figure 8e,f). The reason for this is unclear, but it suggests that the clustering of CIP4 is dependent on factors other than the direct interaction between Cdc42 and CIP4. This is further emphasised by the fact that CIP4 clustering can be induced by L61Rac (Figure 8k,l), or by stimulation of cells transfected with HA–CIP4 either with PDGF-BB (Figure 7g,h) or with bradykinin (Figure 7i,j), suggesting that both Cdc42 and Rac regulated pathways are needed to trigger CIP4 clustering.

**Figure 8**




Subcellular localisation of HA–CIP4 in Swiss 3T3 cells cotransfected with pJ3H–CIP4 and vectors directing expression of **(a–d)** myc-tagged L61Cdc42, **(e,f)** myc-tagged N17Cdc42, **(k,l)** myc-tagged L61Rac, or **(m,n)** myc-tagged L63Rho. Cells transfected with vectors directing expression of either **(g,h)** myc-tagged L61Cdc42 or **(i,j)** myc-tagged N17Cdc42 alone are also shown. HA–CIP4 was detected using an anti-HA antibody and either (a,e,k,m) FITC-conjugated or (c) TRITC-conjugated anti-mouse antibodies. Myc-tagged Cdc42 was detected using either (g,i) anti-myc or (d) anti-Cdc42 antibodies followed by FITC-conjugated anti-mouse and anti-rabbit antibodies, respectively. Filamentous actin was detected using TRITC-conjugated phalloidin.

## Discussion

Data from Swiss 3T3 fibroblasts has demonstrated that Cdc42 is of particular importance for the formation of protrusions at the cell periphery, known as microspikes or filopodia. These protrusions are composed of tightly packed bundles of actin filaments which are organised with their fast growing, or barbed, ends towards the cell edge [43]. The formation of microspikes precedes the formation of lamellipodia or membrane ruffles and their increased activity usually correlates with an increased motile activity

of the cells [1,2]. Microinjection of the constitutively active Cdc42 mutant, V12Cdc42, into Swiss 3T3 cells effectively triggers the formation of filopodia [12]. Furthermore, treatment of cells with bradykinin induces a similar response, an effect which can be blocked by introducing the dominant-negative N17Cdc42 mutant protein into the cells [13]. This suggests that seven transmembrane domain G-coupled receptors can act upstream of Cdc42 in a pathway that eventually leads to the formation of filopodia.

The isolation and characterisation of proteins that are recognised by activated Cdc42 have not yet increased the understanding of the mechanisms underlying the effect of Cdc42 on the actin cytoskeleton. The function of the SH3-domain-containing tyrosine kinase $p120^{ACK}$ is largely unknown [24], whereas the Ser/Thr kinases PAK and SPRK/MLK3 appear to be components of the signalling cascade that eventually leads to JNK activation [44–47]. Work using effector mutants of Cdc42 and Rac has shown that the downstream pathways bifurcate at the level of Cdc42 and Rac, and that the activation of JNK and cytoskeleton rearrangements appear to be regulated by distinct pathways [31,32]. These reports suggest that the proteins containing the CRIB (Cdc42/Rac-interactive binding) motif are not responsible for the organisation of the actin cytoskeleton, but some data suggest that PAK1 could still promote cytoskeletal rearrangements via a pathway that requires the adaptor protein Nck [48].

CIP4 represents a novel class of potential Cdc42 effector proteins; members of this class lack a CRIB motif and at least CIP4 appears to have an effect on the organisation of the actin cytoskeleton. Swiss 3T3 cells transfected with HA–CIP4 had a more rounded or undulated appearance than control cells, which suggests that they have elevated membrane ruffling activity. The CIP4 protein seems, at least to an extent, to be accumulated in those areas at the periphery of the cells that ruffle. It is, however, unlikely that CIP4 binds directly to actin as it does not appear to contain regions with homology to known actin-binding domains. Instead, an inhibitory effect of CIP4 on stress-fibre formation might occur by CIP4 activating actin depolymerising factors that could disassemble pre-existing filaments. Alternatively, CIP4 might bring about sequestration of actin monomers leading to a local increase in the pool of unpolymerised actin. It remains to be established which of these two possible mechanisms is effective here.

Proteins related to CIP4 have so far been found in mouse (FBP17), *S. pombe*, *S. cerevisiae* and *C. elegans*. These proteins are likely to share several features; however, whether these features include the Cdc42-binding motif that is presumably present in CIP4 remains to be investigated. The identity between CIP4 and FBP17 is about 50%, which makes it unlikely that FBP17 is the mouse homologue of CIP4. It is therefore likely that there are at least two isoforms of CIP4 in mammalian cells. FBP17 was cloned from an expression library as a protein that could bind to a proline-rich peptide derived from formin. Formins are proteins with a role in mouse development and, in particular, limb formation [36]. The proline-rich peptide used in the study bound to several proteins containing either SH3 domains or WWP/WW domains. Interestingly, an observation that also suggests that Rho proteins can have a role in the development of limbs comes from a study of the product of the fasciogenital dysplasia gene (FGD1), a Cdc42 exchange factor. This gene is absent or mutated in the developmental disorder Aarskog–Scott syndrome and patients with this rare disorder suffer from characteristic facial, skeletal and urogenital anomalies [49,50]. It is thus tempting to speculate that Cdc42, via downstream target proteins like CIP4, is involved in signalling events leading to correct development of the limbs.

The primary sequences of the first 300 amino acids of CIP4-related proteins show homology to a similar domain in FER and Fes/Fps. The amino-terminal 60 amino acids appears to contain a domain structure present in all CIP4-related proteins with the exception of Cm19c6. This domain was subsequently found in a number of proteins, and I propose that it is named the FER–CIP4 homology (FCH) domain. Several of the FCH-domain-containing proteins have potential roles in organising Rho proteins and the actin cytoskeleton. The X-chromosome-linked gene product, p115, contains a RhoGAP domain and has an inhibitory effect on stress-fibre organisation [39]. *Cdc15* is an *S. pombe* gene involved in mediating the actin filament reorganisation required for cytokinesis [40]. Previous studies have implicated the noncatalytic amino-terminal domain of Fes/Fps in activating the protein by translocating it to the plasma membrane [38]. The target molecules responsible for this translocation have not been characterised so far. It is a possibility that the amino termini of the CIP4-related proteins can bind to related target molecules via their FCH domains.

The members of the ERM family of protein have been shown to co-localise with actin filaments and to bind actin [51,52]. Moesin and ezrin are present at the apical microvilli of epithelial cells and ezrin has been suggested to be involved in the assembly of the microvilli in the intestinal brush border [53]. The region of homology between CIP4 and the ERM proteins is outside the actin-binding domain, which makes it unlikely that CIP4 binds directly to actin molecules. The overall similarity between them suggests, however, that CIP4 and moesin participate in similar signalling pathways.

## Conclusions

It is concluded that CIP4 is a downstream target of activated Cdc42 *in vitro* as well as *in vivo*. Cdc42 has a specific effect on fibroblasts in tissue culture — it induces the

formation of filopodia at the periphery of cells. This process is likely to be complex and the work on Cdc42 effector proteins has not yet been able to explain how the transfer of information from activated Cdc42 to the actin cytoskeleton to form filopodia is achieved. None of the Cdc42 effector proteins isolated so far has been able to induce the formation of filopodia on their own. Overexpression of WASP in pig endothelial cells and NRK epithelial cells causes an accumulation of polymerised actin into large conglomerates at the perinuclear area [26]. Overexpression of CIP4 has a different effect on the organisation of the actin cytoskeleton — it causes a reduction in the stress-fibre content. The clustering of CIP4 into a large number of foci, triggered by a simultaneous overexpression of Cdc42 or Rac, or by treatment with PDGF-BB or bradykinin, might be needed for the initial stage in the formation of a filopodium.

## Materials and methods

### *Yeast two-hybrid screen*

The yeast strain and library used in the screen have been described previously [25]. Analysis of the three cDNAs encoding CIP4 showed that it had not been previously identified and that one of the clones encoded the full-length 545 amino-acid protein. The two additional cDNAs encoded amino-acid residues 112–545 and 293–545 of CIP4. The carboxy-terminal amino-acids encoding the GAP domain of RhoGAP (230–439) were subcloned into pACTII. L61Rac, N17Rac (Rac1 isoform), L63Rho, N19Rho (RhoA isoform) and L87R-Ras were introduced into pYTH6 and transformed into Y190 as described [25,42]. The ability of the GAL4DB–GTPases to bind to GAL4AD–CIP4 and GAL4AD–RhoGAP was analysed by transforming pACT–CIP4$_{1-545}$ and pACTII into Y190 cells expressing the various GALDB–GTPase constructs. The cells were grown on medium lacking histidine containing 25 mM 3-aminotriazole, as described previously [25,42]. The β-galactosidase production from the LacZ reporter gene was analysed using a membrane transfer assay or the solution assay as described before [42].

### *DNA work, sequencing and northern blot analysis*

The DNA work followed standard procedures [54]. The nucleotide sequence of pACTCIP4$_{1-545}$ was determined by the method of Sanger [54] or using a Perkin Elmer Genetic Analyzer 310. Nested oligonucleotide primers were used to perform double-stranded sequencing and both strands of CIP4 were each sequenced at least three times. The nucleotide sequence and the translated amino-acid sequence were analysed for homology to known proteins using the FASTA or BLAST functions in the Genetic Computer Group (GCG) package or via internet on GeneBank. For northern blot analysis, a hybridisation-ready northern blot (Human Multiple Tissue Northern Blot; Clonetech) containing mRNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas was hybridised using a probe (consisting of nucleotides 817–2000 of CIP4 labelled with [$^{32}$P]CTP) according to the manufacturer's instructions (rediprime, Amersham).

### *Protein production, dot-blot assay and glutathione–Sepharose binding assay*

A vector for the production of GST–CIP4$_{293-490}$ was constructed by generating an *Nco*I–*Hin*dIII PCR fragment of CIP4 and ligating it into pGEX-KG (Pharmacia). For production of the protein, pGEX-KG–CIP4$_{293-481}$ was transformed into BL21 cells and the fusion protein was produced essentially by following the manufacturer's protocol, eluted using 5 mM reduced glutathione and then dialysed overnight at 4°C against 20 mM Tris-HCl pH 7.5, 50 mM NaCl , 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). GTPases were isolated from GST fusion proteins by thrombin cleavage [8].

For dot-blot assays, GST fusion proteins were spotted onto nitrocellulose filters and incubated in blocking buffer (5% dried milk, 5% FCS, 1 M glycine and 1% ovalbumin) for 1 h at room temperature. The filters were washed twice in buffer A (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT). The filters were then incubated on ice for 10 min in 5 ml buffer A containing 0.1 mM GTP, 1 mg/ml BSA and 0.5 μg GTPase preloaded with 10 μCi γ-[$^{32}$P]GTP (Amersham, 5000 Ci/mmol). The filters were washed three times with 0.1 M Tris-HCl pH 8, 0.15 M NaCl, 0.1% Tween 20, wrapped in Saran-wrap and developed on X-ray film (Hyperfilm-MP, Amersham), for 1 h at −70°C.

For glutathione–Sepharose binding assays, 1 μg L61Cdc42 was preloaded with 2 μCi [$^3$H]GTP or [$^3$H]GDP (7.8 Ci/mmol and 13.3 Ci/mmol, respectively, Amersham) and incubated with GST–CIP4$_{293-481}$ or GST (40 μg of each) for 10 min on ice (the input counts were 110,000 cpm for [$^3$H]GTP·L61Cdc42 and 70,000 cpm for [$^3$H]GDP·L61Cdc42). Thereafter, 40 μl glutathione–Sepharose beads (Pharmacia) were added and the mixture incubated end-over-end for 30 min at 4°C. The beads were washed 3 times with buffer A containing 0.1% Triton X-100, collected on nitrocellulose filters and subjected to scintillation counting.

### *Cell cultivation, transfection and immunohistochemistry*

Swiss 3T3 fibroblasts were cultured in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% FCS and penicillin/streptomycin and incubated at 37°C in an atmosphere of 10% $CO_2$. The cells were seeded on coverslips and transfected by the lipofection method using the LipoFECTAMINE reagent (GIBCO, BRL) following the manufacturer's protocol. Plasmids encoding the myc-tagged proteins L63Rho and L61Rac (generous gifts from N. Lamarche), L61Cdc42 and N17Cdc42, the parental plasmid pRK5myc (a generous gift from M. Olson), and pJ3H–CIP4 (the parental plasmid pJ3H was a generous gift from J. Chernoff) were transfected alone or in combinations. After incubation for 48 h, the cells were harvested and analysed for expression of the introduced cDNAs.

For immunohistochemistry, the Swiss 3T3 cells were fixed in 2% paraformaldehyde for 15 min and then permeabilised in 0.2% Triton X-100 for 15 min. HA–CIP4 was visualised using an anti-HA monoclonal antibody (12CA5, a generous gift from S. Souchelniskyi) and myc-tagged Rho GTPases were detected using an anti-myc monoclonal antibody (9E10, Santa Cruz), followed by incubation for 1 h with fluorescein-isothiocyanate (FITC)-conjugated (Cedarlane Laboratories Ltd) or TRITC-conjugated (DAKO) anti-mouse antibodies. Filamantous actin was detected by incubating the cells with 0.1 μg/ml TRITC-conjugated phalloidin (Sigma) for 1 h. Cells were photographed using a Zeiss Axiovert 100 fluorescence microscope and T-Max 400 films.

### *Accession numbers*

The following gene products are referred to in the text: FBP17 (U40751), YHR4 (U00059), YA65 (Z54140), Cm19c6 (U41749), h74 (X85124), YM9973.05 (Z49213), F0910.9 (U41749), growth-arrest-specific gene product (U19860), Cdc15 (X86179), p115 (X78817), and F45E1.7 (U28732).

### *Supplementary material available*

Figures showing the nucleotide and deduced amino-acid sequences of CIP4 and the alignment of various regions of CIP4 with homologous domains of other proteins are published with this paper on the Internet.

## Acknowledgements

C-H. Heldin and M. Cross are greatly acknowledged for their critical comments on the manuscript. Susanne Grimsby and Jill Sandström are acknowledged for their technical support with the automated sequencer. P.A. was supported in part by grants from the Swedish Cancer Foundation. The initial part of this work was done at the Department of Zoological Cell Biology, Stockholm University.

## References

1. Bretscher A: **Microfilament structure and function in the cortical cytoskeleton.** *Annu Rev Cell Biol* 1991, **7:**337-374.
2. Mitchison TJ, Cramer LP: **Actin-based cell motility and cell locomotion.** *Cell* 1996, **84:**371-379.
3. Mellström K, Höglund A-S, Nistér M, Heldin C-H, Westermark B, Lindberg U: **The effect of platelet derived growth factor on morphology and motility of human glial cells**. *J Muscle Res Cell Motil* 1983, **4:**589-609.
4. Chinkers M, McKanna JA, Cohen S: **Rapid induction of morphological changes in human carcinoma cells A-431 by epidermal growth factor**. *J Cell Biol* 1979, **83:**260-265.

5. Hall A: **Small GTP-binding proteins and the regulation of the actin cytoskeleton.** *Annu Rev Cell Biol* 1994, **10:**31-54.
6. Foster R, Hu K-Q, Lu Y, Nolan KM, Thissen J, Settleman J: **Identification of a novel human Rho protein with unusual properties: GTPase deficiency and *in vivo* farnesylation.** *Mol Cell Biol* 1996, **16:**2689-2699.
7. Cerione RA, Zheng Y: **Dbl family of oncogenes.** *Curr Opin Cell Biol* 1996, **8:**216-222.
8. Lamarche N, Hall A: **GAPs for Rho-related GTPases.** *Trends Genet* 1994, **10:**436-440.
9. Ridley AJ, Paterson HF, Johnston CL, Diekman D, Hall A: **The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling.** *Cell* 1992, **70:**401-410.
10. Ridley AJ, Hall A: **The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors.** *Cell* 1992, **70:**389-399.
11. Johnson DI, Pringle JR: **Molecular characterization of *Cdc42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity.** *J Cell Biol* 1990, **111:**143-152.
12. Nobes CD, Hall A: **Rho, Rac and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibres, lammelipodia and filopodia.** *Cell* 1995, **81:**53-62.
13. Kozma R, Ahmed S, Best A, Lim L: **The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts.** *Mol Cell Biol* 1995, **15:**1942-1952.
14. Coso OA, Chiariello M, Yu J-C, Teramoto H, Crespo P, Xu N, *et al.*: **The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway.** *Cell* 1995, **81:**1137-1146.
15. Minden A, Lin A, Claret F-X, Abo A, Karin M: **Selective activation of the JNK signalling cascade and c-Jun transcriptional activity by the small GTPases rac and Cdc42Hs.** *Cell* 1995, **81:**1147-1157.
16. Hill CS, Wynne J, Triesman R: **The Rho GTPases RhoA, Rac1 and Cdc42Hs regulate transcriptional activation by SRF.** *Cell* 1995, **81:**1159-1170.
17. Olson MF, Ashworth A, Hall A: **An essential role for Rho, Rac and Cdc42 GTPases in cell cycle progression through $G_1$.** *Science* 1995, **269:**1270-1272.
18. Qui R-G, Chen J, Kirn D, McCormick F, Symons M: **An essential role for Rac in Ras transformation.** *Nature* 1995, **374:**457-459.
19. Qui R-G, Chen J, McCormick F, Symons M: **A role for Rho in Ras transformation.** *Proc Natl Acad Sci USA* 1995, **92:**11781-11785.
20. Khosravi-Far R, Solkski PA, Clark GJ, Kinch MS, Der CJ: **Activation of Rac1, RhoA and mitogen-activated protein kinases is required for Ras transformation.** *Mol Cell Biol* 1995, **15:**6443-6453.
21. Manser E, Leung T, Salihuddin Zhao ZS, Lim L: **A brain serine/threonine protein kinase activated by Cdc42 and Rac1.** *Nature* 1994, **367:**40-46.
22. Gallo KA, Mark MR, Scadden DT, Wang Z, Gu Q, Godowski PJ: **Identification and characterisation of SPRK, a novel src-homology 3 domain-containing proline-rich kinase with serine/threonine kinase activity.** *J Biol Chem* 1994, **269:**15092-15100.
23. Ing LY, Leung IWL, Heng HQH, Tsui L-C, Lassam NJ: **MLK-3: identification of a widely-expressed protein kinase bearing an SH3 domain and a leucine zipper-basic region domain.** *Oncogene* 1994, **9:**1745-1750.
24. Manser E, Leung T, Salihuddin H, Tan L, Lim L: **A non- receptor tyrosine kinase that inhibits the GTPases activity of $p21^{cdc42}$.** *Nature* 1993, **263:**364-367.
25. Aspenström P, Lindberg U, Hall A: **Two GTPases, Cdc42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott–Aldrich syndrome.** *Curr Biol* 1996, **6:**70-75.
26. Symons M, Derry JMJ, Karlak B, Jiang S, Lemahieu V, McCormick F, *et al.*: **Wiskott–Aldrich syndrome protein, a novel effector for the GTPase Cdc42, is implicated in actin polymerization.** *Cell* 1996, **84:**723-734.
27. Kolluri R, Tolias KF, Carpenter CL, Rosen FS, Kirchhausen T: **Direct interaction of the Wiskott-Aldrich syndrome protein with the GTPase Cdc42.** *Proc Natl Acad Sci* 1996, **93:**5615-5618.
28. Burbelo PD, Drechsel D, Hall A: **A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases.** *J Biol Chem* 1995, **270:**29071-29074.
29. Hart MJ, Callow MG, Souza B, Polakis P: **IQGAP1, a calmodulin-binding protein with a RasGAP-related domain, is a potential effector for Cdc42Hs.** *EMBO J* 1996, **15:**2997-3005.
30. Chou MM, Blenis J: **The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins Cdc42 and Rac1.** *Cell* 1996, **85:**573-583.
31. Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenström P, Bridges T, *et al.*: **Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of $p65^{PAK}$ and the JNK/SAPK MAP kinase cascade.** *Cell* 1996, **87:**519-529.
32. Joneson T, McDonough M, Bar-Sagi D, Van Aelst L: **RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase.** *Science* 1996, **274:**1374-1376.
33. Aitken A: **14-3-3 proteins on the MAP.** *Trends Biochem Sci* 1995, **20:**95-97.
34. Lee JW, Choi H-S, Gyuris J, Brent R, Moore DD: **Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor.** *Mol Endocrinol* 1995, **9:**243-254.
35. Kozak M: **Structural features in eukaryotic mRNAs that modulate the initiation of translation.** *J Biol Chem* 1991, **266:**19867-19870.
36. Chan DC, Bedford MT, Leder P: **Formin binding proteins bear WWP/WW domains that bind proline-rich peptides and functionally resemble SH3 domains.** *EMBO J* 1996, **15:**1045-1054.
37. Hao G-L, Heisterkamp N, Groffen J: **Isolation and sequence analysis of a novel human tyrosine kinase gene.** *Mol Cell Biol* 1989, **9:**1587-1593.
38. Sadowski I, Stone JC, Pawson T: **An noncatalytical domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami Sarcoma virus p130gag-fps.** *Mol Cell Biol* 1986, **6:**4396-4408.
39. Tribioli C, Droetto S, Bione S, Cesareni G, Torrisi MR, Lotti LV, *et al.*: **An X chromosome-linked gene encoding a protein with characteristics of a RhoGAP predominantly expressed in hematopoietic cells.** *Proc Natl Acad Sci USA* 1996, **93:**695-699.
40. Frankhauser C, Reymond A, Cerutti L, Utzig S, Hofmann K, Simanis V: **The *S. pombe cdc15* gene is a key element in the reorganization of F-actin at mitosis.** *Cell* 1995, **82:**435-444.
41. Arpin M, Algrain M, Louvard D: **Membrane-actin microfilament connections: an increasing diversity of players related to band 4.1.** *Curr Opin Cell Biol* 1994, **6:**136-141.
42. Aspenström P, Olson MF: **Yeast two-hybrid system to detect protein–protein interactions with Rho GTPases.** *Meth Enzymol* 1995, **256:**228-241.
43. Small JV: **The actin cytoskeleton.** *Electron Microscop Rev* 1988, **1:**155-174.
44. Brown JL, Stowers L, Baer M, Trejo JA, Coughlin S, Chant J: **Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway.** *Curr Biol* 1996, **6:**598-605.
45. Frost JA, Xu S, Hutchinson MR, Marcus S, Cobb MH: **Actions of Rho family small G proteins and p21-activated protein kinases on mitogen-activated protein kinase family members.** *Mol Cell Biol* 1996, **16:**3707-3713.
46. Teramoto H, Coso OA, Miyata H, Igishi T, Miki T, Gutkind JS: **Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway.** *J Biol Chem* 1996, **271:**27225-27228.
47. Tibbles LA, Ing YL, Kiefer F, Chan J, Iscove N, Woodgett JR, Lassam NJ: **MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6.** *EMBO J* 1996, **15:**7026-7035.
48. Sells MA, Knaus UG, Bagrodia S, Ambrose DM, Bokoch GM Chernoff J: **Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells.** *Curr Biol* 1997, **7:**202-210.
49. Pasteris NG, Cadle A, Logie LJ, Porteous MEM, Schwartz CE, Stevenson RE, *et al.*: **Isolation and characterization of the Faciogenital dysplasia (Aarskog–Scott syndrome) gene: a putative Rho/Rac guanine nucleotide exchange factor.** *Cell* 1994, **79:**669-678.
50. Olson MF, Pasteris NG, Gorski JL, Hall A: **Faciogenital dysplasia protein (FGD1) and Vav, two related proteins required for normal embryogenetic development, are upstream regulators of Rho GTPases.** *Curr Biol* 1996, **6:**1628-1633.
51. Turunen O, Whalström T, Vaheri A: **Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family.** *J Cell Biol* 1994, **126:**1445-1453.
52. Pestonjamasp K, Amieva MR, Strassel CP, Nauseef WM, Furthmayr H, Luna EJ: **Moesin, ezrin and p205 are actin-binding proteins associated with neutrophil plasma membranes.** *Mol Biol Cell* 1995. **6:**247-259.
53. Berryman M, Franck Z, Bretscher A: **Ezrin is concentrated in the apical microvilli of a wide variety of epithelial cells whereas moesin is found primarily in endothelial cells.** *J Cell Sci* 1993, **105:**1025-1043,
54. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: a Laboratory Manual,* 2nd edn. Plainview, New York: Cold Spring Harbor Laboratory press; 1989.


**Because *Current Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published on the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cub – for further information, see the explanation on the contents page.**