REVIEW ARTICLE
Rho GTPases and their effector proteins

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Rho GTPases are molecular switches that regulate many essential cellular processes, including actin dynamics, gene transcription, cell-cycle progression and cell adhesion. About 30 potential effector proteins have been identified that interact with members of the Rho family, but it is still unclear which of these are responsible for the diverse biological effects of Rho GTPases. This review will discuss how Rho GTPases physically interact with, and regulate the activity of, multiple effector proteins and how specific effector proteins contribute to cellular responses. To date most progress has been made in the cytoskeleton field, and several biochemical links have now been established between GTPases and the assembly of filamentous actin. The main focus of this review will be Rho, Rac and Cdc42, the three best characterized mammalian Rho GTPases, though the genetic analysis of Rho GTPases in lower eukaryotes is making increasingly important contributions to this field.

Key words: actin reorganization, autoinhibition, CRIB motif, HR1 motif.

THE RHO FAMILY

Rho GTPases are members of the Ras superfamily of monomeric 20–30 kDa GTP-binding proteins. Ten different mammalian Rho GTPases, some with multiple isoforms, have been identified to date: Rho (A, B, C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE, RhoD, RhoG, TC10 and TTF [1]. The most extensively characterized members are Rho, Rac and Cdc42. Each of these GTPases act as a molecular switch, cycling between an active GTP-bound, and an inactive GDP-bound, state. In the GTP-bound form they are able to interact with effector or target molecules to initiate a downstream response, while an intrinsic GTPase activity returns the proteins to the GDP-bound state, to complete the cycle and terminate signal transduction.

Regulation

Cycling between the GTP- and GDP-bound states is regulated by numerous cellular proteins [2]. Although still poorly characterized, over 30 guanosine nucleotide exchange factors (GEFs) have been identified that facilitate the exchange of GDP for GTP [3,4]. All Rho GEFs contain a Dbl-homology (DH) domain which encodes the catalytic activity [5,6] and an adjacent pleckstrin homology (PH) domain. The PH domain is thought to mediate membrane localization through lipid binding [7,8], but, in addition, structural and biochemical evidence suggests that it might also directly affect the activity of the DH domain. For example, a comparison of the crystal structures of the DH-PH domains of Sos GEF, in the absence of lipid ligand, and the PH domain of phospholipase Cδ bound to inositol 1,4,5-trisphosphate, suggests that a ligand binding to Sos PH domain is likely to affect the conformation of the neighbouring DH domain [9]. In Trio, a GEF with two DH-PH modules, one specific for Rac and one for Rho [10], the N-terminal DH-PH domains together have a 100-fold higher Rac GEF activity than does the DH domain alone [11]. Finally, the binding of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to the PH domain of Vav GEF inhibits activity, whereas phosphatidylinositol 3,4,5-triphosphate binds more strongly and stimulates Vav activity [12]. Additional domains specific to each GEF may provide variations in subcellular localization and activation mechanisms [13].

About 20 GTPase-activating proteins (GAPs) which increase the intrinsic rate of GTP hydrolysis of Rho GTPases have been identified to date [14]. The human genome sequencing project reveals that chromosome 22 alone potentially encodes eight GAPs [15]. A comparison of the crystal structures of a ground-state complex between RhoGAP and Cdc42·guanosine 5′-[β,γ-imido]triphosphate (guanosine 5′-[β,γ-imido]triphosphate, also known as 'GMPPNP', is a non-hydrolysable GTP analogue) and a transition-state-mimicking complex of Rho-GAP with RhoA·GDP·AlF$_4^{4-}$, along with NMR analysis of a Cdc42·RhoGAP complex, has provided insight into the mechanistic details of GAP-facilitated GTP hydrolysis [16–18]. The most striking feature is that a 20° rotation between GTPase and GAP, from ground state to transition state, allows an arginine residue in the GAP protein, the 'arginine finger', to enter the GTPase active site and participate in the stabilization of the transition state.

Rho GTPases are able to interact with membranes via a post-translational C-terminal geranylgeranyl lipid modification

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Table 1   Summary of the cellular activities which involve Rho, Rac and Cdc42

These activities refer to biological pathways which can be induced by the activated Rho GTPases indicated and/or which can be inhibited by dominant-negative constructs of the appropriate Rho GTPases. SRF and NF-κB are both transcription factors; JNK and p38 are MAP kinase pathways; the NADPH oxidase complex is present only in professional phagocytic cells; secretion has only been shown to involve Rho GTPases in mast cells.

	Actin	SRF	JNK/p38	NF-κB	NADPH oxidase	G_1 cell-cycle progression	Cell–cell contacts	Secretion	Cell polarity	Transformation
Rho	+	+	−	+	−	+	+	+	−	+
Rac	+	+	+	+	+	+	+	+	−	+
Cdc42	+	+	+	+	−	+	+	?	+	+

(farnesyl for RhoD and RhoE; either modification for RhoB) [19,20]. However, in the unactivated state, Rho and Rac can be isolated as a soluble complex associated with RhoGDI (guanosine nucleotide dissociation inhibitor). RhoGDI appears to sequester GDP-bound Rho GTPases in the cytoplasm and inhibit their spontaneous GDP$\leftrightarrow$GTP exchange activity, similar to what has been proposed for $\beta\gamma$ subunits and the α subunits of heterotrimeric G-proteins, but the precise role of GDI is still poorly understood [21]. NMR and crystal structures of GDI alone, a low-resolution crystal structure of a RhoA·GDI complex (no geranylgeranyl lipid attached to RhoA) and a recent high-resolution [0.26 nm (2.6 Å)] crystal structure of a Cdc42·GDI complex (geranylgeranyl lipid present on Cdc42) all agree that the binding of GDI to a Rho GTPase occurs through an immunoglobulin-like C-terminal domain, and that a hydrophobic pocket in this domain can accommodate the geranylgeranyl lipid, whilst a flexible N-terminal domain inhibits GDP$\leftrightarrow$GTP exchange [22–25]. Cerione's group reported the use of fluorescence resonance energy transfer ('FRET') to monitor the removal of Cdc42 from membranes by GDI in real time. They detected two phases which appear to represent a rapid membrane-associated GTPase–GDI binding step followed by a slow transfer of the geranylgeranyl moiety from membrane to GDI [26].

Experimental manipulation

The biological activities of individual Rho GTPases (Table 1) have been elucidated using a variety of methods, including the use of activated and dominant-negative mutants, as well as bacterial toxins. Amino acid substitutions of Val for Gly at codon 12 or of Leu for Gln at codon 61 (Rac numbering) have been extensively used to generate constitutively active Rho GTPases, since both of these mutations prevent intrinsic and GAP-induced GTP hydrolysis. The crystal structure of RhoGAP complexed with RhoA·GDP·AIF$_4^-$ confirms an essential role for Gln[63] (equivalent to Gln[61] in Rac) in stablizing the γ-phosphate during GTP hydrolysis [17]. The crystal structure of a [Val[14]]RhoA·guanosine 5′-[γ-thio]triphosphate (GTP[S]) (equivalent to the Val[12] mutant in Rac) (GTP[S] is a non-hydrolysable GTP analogue) shows how the larger Val side chain forces Gln[63] away from the γ-phosphate, so preventing it from facilitating GTP hydrolysis [27]. Interestingly Rnd1-3 and TTF Rho family members do not contain Gly and Gln at positions 12 and 61 respectively and thus appear to be constitutively GTP-bound [28–30]. This raises interesting questions as to how these proteins are regulated, but so far nothing is known.

A substitution of Asn for Thr[17] (Rac numbering) allows this mutant GTPase to compete with the corresponding endogenous GTPase for binding to cellular GEFs, but this leads to a non-productive complex unable to generate a downstream response [31]. These dominant-negative proteins appear to be rather specific for individual Rho GTPases, despite the complexity of the RhoGEF family [3].

A variety of bacterial toxins have been used to modify the activity of Rho GTPases. The exoenzyme C3 transferase, an ADP-ribosyltransferase from *Clostridium botulinum* [32], is relatively specific and inactivates only RhoA, RhoB and RhoC. *C. difficile* toxin B inactivates most, if not all, members of the Rho GTPase family and is particularly useful in assessing the involvement of a Rho GTPase in a particular biological process [33]. The prevalence of Rho GTPase modifications by bacterial toxins illustrates the fundamental importance of these proteins to cellular regulation.

Biological activities

The major function of Rho GTPases is to regulate the assembly and organization of the actin cytoskeleton [34]. The effects of Rho, Rac and Cdc42 were initially described using quiescent Swiss3T3 fibroblasts, a cell line in which serum starvation creates a very low background of organized F-actin structures. Addition of lysophosphatidic acid induces the formation of contractile actin–myosin stress fibres and associated focal adhesions, and this can be blocked by C3 transferase [35]. Growth factors, such as platelet-derived growth factor, insulin or epidermal growth factor (EGF) induce the formation of actin-rich lamellipodia and membrane ruffles associated with focal contacts and the dominant-negative [Asn[17]]Rac specifically inhibits this response [36]. Finally bradykinin induces the formation of peripheral microspikes or filopodia, which are also associated with focal contacts, and this can be inhibited by expression of dominant-negative [Asn[17]]Cdc42 [37]. These types of experiment have lead to the conclusion that Rho, Rac and Cdc42 regulate three signal-transduction pathways linking various membrane receptors to the assembly of actin–myosin filaments, lamellipodia and filopodia respectively. It is not surprising, therefore, that Rho GTPases have been found to play a role in a variety of cellular processes that are dependent on the actin cytoskeleton, such as cytokinesis [38–40], phagocytosis [41,42], pinocytosis [36], cell migration [43,44], morphogenesis [45] and axon guidance [46].

One of the most interesting aspects of this family of regulatory proteins is that, in addition to their effect on the actin cytoskeleton, they also regulate a variety of other biochemical pathways (see Table 1) including serum response factor (SRF) and nuclear factor κB (NF-κB) transcription factors [47,48], the c-*jun* N-terminal kinase (JNK) and p38 mitogen-activated protein kinase pathways [49,50], the phagocytic NADPH oxidase complex [51], G_1 cell-cycle progression [52], the assembly of cadherin-containing cell–cell contacts [53,54], secretion in mast cells [55],

cell polarity [56] and cell transformation [3]. Therefore, although Rho GTPases are best characterized for their effects on the actin cytoskeleton, there is now much interest in their ability to affect cell proliferation and gene transcription, and the contributions of all of these activities to malignant transformation is an important field of study.

THE INTERACTION OF Rho GTPases WITH EFFECTOR PROTEINS

Given the involvement of Rho GTPases in such a wide variety of important cellular processes, it is not surprising that a great deal of effort has been put into identifying their cellular targets or effector proteins. To date 30 or so potential effectors for Rho, Rac and Cdc42 have been identified (Table 2), primarily using affinity chromatography and the yeast two-hybrid system. These proteins interact specifically with the GTP-bound form of the GTPase. A comparison of the RhoA·GDP and [Val^{14}]RhoA·GTP[S] crystal structures, reveals that the conformational differences between the GTP and GDP-bound forms are restricted primarily to two surface loops, named switch regions I and II (Cdc42/Rac amino acids 26–45 and 59–74 respectively) [27,57]. Effector proteins must, therefore, utilize these differences to discriminate between the GTP- and GDP-bound forms, though they also interact with other regions of the GTPase (see below).

Numerous point mutations have been introduced into Switch I of Rho, Rac and Cdc42, often referred to as the 'effector region', and have rather interesting effects preventing the binding of some, but not all, target proteins [58–60]. For example, the interaction of PAK (p21-activated kinase) with Cdc42/Rac is blocked by a Tyr^{40}-to-Cys mutation, but not by a Phe^{37}-to-Ala mutation [58]. Whereas the interaction of Mlk (mixed-lineage kinase) 2 and 3 with Rac is blocked by either of these mutations [61]. Substitution of different amino acids at the same position in switch I can also have varied effects upon target protein binding; for instance, [Cys^{40}]Rac can bind to p67^{phox} (a component of the NADPH oxidase complex), whereas [Lys^{40}]Rac cannot [58]. Such results suggest that different effectors interact with different residues, even within the switch I region.

Further studies using GTPase mutants and chimaeras have implicated regions outside of switch I in the binding of certain effectors. For instance, Rac–Rho chimaeras indicate that a region close to the C-terminus of Rac (amino acids 143–175) may be important for binding to p67^{phox} and for PAK activation [62]. An α-helical region present in all Rho-family GTPases (except *Drosophila* RhoL), but not in Ras, referred to as the 'insert region' (Rac amino acids 123–135), is required for Rac activation of the NADPH oxidase complex and for binding to an effector protein called IQGAP, but not for its interaction with PAK [63,64]. So far there are no known examples of Rho effectors

Table 2   Potential effector proteins for Rho, Rac and Cdc42

Proteins shown with an asterisk (*) are those which appear to be activated by GTPases in a GTP-dependent manner, but where the interaction is GTP-independent. Proteins shown with a superscript plus sign ($^+$) are those for which a functional relationship with GTPase, but not a direct interaction, has so far been shown. Note that Rho is reported to inhibit diacylglycerol kinase (DAG kinase), whereas all the other targets listed are thought to be activated by Rho GTPases. Common GTPase-binding motifs, assigned according to sequence homology are: CRIB, REM (also known as class-1 Rho-binding motif) and RKH (also known as class-2 Rho-binding motif). Where a function has been reported this is noted, but in many cells these are far from clear; for further discussion, see the text and key references given below. Further abbreviations: PKN, protein kinase N; PLC, phospholipase C; PLD, phospholipase D; IP3, inositol trisphosphate; PA, phosphatidic acid.

Potential effector protein	Type of protein	Functions	Selectivity of Rho GTPase binding			GTPase-binding motif	Key references
ROKα, ROKβ	Ser/Thr kinase	Actin/myosin	Rho			RKH	[91,128,129]
PKN/ PRK1, PRK2	Ser/Thr kinase	Unknown	Rho			REM	[90,208]
Citron kinase (citron)	Ser/Thr kinase	Cytokinesis	Rho				[102,103]
p70 S6 kinase	Ser/Thr kinase	Translation regulation		Rac	Cdc42?		[209]
Mlk2, 3	Ser/Thr kinase	JNK		Rac	Cdc42	CRIB	[61,200,201]
MEKK1, 4	Ser/Thr kinase	JNK		Rac	Cdc42	CRIB for MEKK4	[202]
PAK1, 2, 3	Ser/Thr kinase	JNK/actin		Rac	Cdc42	CRIB	[79]
PAK4	Ser/Thr kinase	Actin			Cdc42	CRIB	[81]
MRCKα, MRCKβ	Ser/Thr kinase	Actin			Cdc42	CRIB	[104]
Ack1, 2	Tyr kinase	Unknown			Cdc42	CRIB	[73,210]
MBS	Phosphatase subunit	MLC inactivation	Rho				[128]
PI-4-P5K	Lipid kinase	PIP$_2$ levels/actin	Rho*$^+$	Rac*			[144,147,169,171]
PI3K	Lipid kinase	PIP$_3$ levels		Rac	Cdc42		[211–213]
DAG kinase	Lipid kinase	PA levels	Rho$^+$	Rac*			[169,214]
PLD	Lipase	PA levels	Rho	Rac	Cdc42		[215–218]
PLC-β2	Lipase	DAG/IP$_3$ levels		Rac	Cdc42		[219]
Rhophilin	Scaffold	Unknown	Rho			REM	[76]
Rhotekin	Scaffold	Unknown	Rho			REM	[77]
Kinectin	Scaffold	Kinesin binding	Rho			RKH	[220]
Dia1, Dia2	Scaffold	Actin organization	Rho				[91,141]
WASP, N-WASP	Scaffold	Actin organization			Cdc42	CRIB	[94,96,97]
WAVE/ Scar	Scaffold	Actin organization		Rac$^+$			[94,95,172]
POSH	Scaffold	Unknown		Rac			[61]
POR-1	Scaffold	Actin organization		Rac			[166]
p140Sra-1	Scaffold	Actin organization		Rac			[168]
p67^{phox}	Scaffold	NADPH oxidase		Rac			[51,63,207]
MSE55, BORGs	Scaffold	Unknown			Cdc42	CRIB	[221,222]
IQGAP1,2	Scaffold	Actin/cell–cell contacts		Rac	Cdc42		[54,96,98,115]
CIP-4	Scaffold	Unknown			Cdc42		[223]

(a)

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ACK (505–531)    L S A Q D I S Q P L Q N S F I H T G H G D S D P R H C
WASP (236–258)   I S K A D I G A P . . S G F K H V S H V G W D P Q N G
PAK1 (70–94)     K E R P E I S L P . . S D F E H T I H V G F D A V T G
PAK2 (71–91)     K E R P E I S P P . . S D F E H T I H V G F D A V T G
PAK4 (6–30)      K K R V E I S A P . . S N F E H R V H T G F D Q H E Q
CRIB consensus         *   I S * P     S * F * H * * H * G * D
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(b)

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PKN/PRK1 HR1a (34–63)   Q L E L E R E R L R R E I R K E L K L K E G A E N L R R A T
PRK2 HR1a (44–73)       K L D D I K D R I K R E I R K E L K I K E G A E N L R K V T
Rhophilin (39–68)       Q L Q S H R A R L H Q Q I S K E L R M R T G A E N L Y R A T
Rhotekin (20–49)        A L S L E D T E L Q R K L D H E I R M R Q G A C K L L A A C
HR1 consensus             L       . . *     * L   *       I   * E L * * *     G A * * L     * A *
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PKN/PRK1 HR1a (64–93)   T D L G K S L G P V E L L L R G S S R K L D L L H Q Q L Q E
PRK2 HR1a (74–102)      T D K . K S L A Y V D N I L K K S N K K L E E L H H K L Q E
Rhophilin (69–97)       S N . T W V R E T V A L E L S Y V N S N L Q L L K E E L A E
Rhotekin (50–79)        S Q R E Q A L E A T K S L L V C N S R I L S Y M G E L Q R R
HR1 consensus                       *       *       * L           L   L       *       *
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PKN/PRK1 HR1a (94–103)  L H A H V V L P D P
PRK2 HR1a (103–112)     L H A H I V V S D P
Rhophilin (98–107)      L S T S V D V D Q P
Rhotekin (80–89)        K E A Q V L E K T G
HR1 consensus           *         V       * *
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Figure 1   Sequence comparisons for (a) the CRIB motifs from ACK, WASP and three PAKs and (b) the HR1 motifs from PKN/ PRK1 (HR1a), PRK2 (HR1a), rhophilin and rhotekin, which are the essential Rho-binding regions within the REM domain of each protein (see the text)

(**a**) Other mammalian GTPase effectors which contain CRIB motifs are listed in Table 2. Conserved CRIB consensus residues are shown in red; * indicates less conserved residues within the CRIB motif. (**b**) Conserved HR1 motif consensus residues are shown in red; * indicates important HR1 motif residues that are less conserved. Charged amino acids which are particularly important for PKN binding to Rho, according to structural data, and are less conserved in the other two PKN HR1 motifs (HR1b and HR1c) are underlined (see the text for discussion).

requiring this insert region for binding. Two amino acids in loop 6 (just C-terminal to switch II) of Rho are acidic (Asp[87] and Asp[90]), but are hydrophobic (Val and Ala) in Rac, and these appear to be important for Rho binding to two effectors PRK2 and ROK [65]. This same region of Rac (amino acids 74–90), on the other hand, has been implicated in the binding of GAPs [62] and an overlapping region of Cdc42 (amino acids 84–120) appears to be involved in binding to Wiskott–Aldrich-syndrome protein (WASP) and IQGAP, but not to PAK1 [66]. These mutational studies indicate that distinct regions of Rac, Cdc42 and Rho outside of switch I are required to make essential contacts with effector proteins.

The data obtained from the many mutational studies that have been undertaken have lead to a complex, and sometimes con-tradictory, picture of the mechanisms of Rho GTPase–effector interactions. However, the recently reported NMR structures of Cdc42 bound to **a**ctivated **C**dc42-associated tyrosine **k**inase (ACK) (amino acids 504–545) and WASP (amino acids 230–288) have provided much clarification [67,68]. ACK and WASP both contain the conserved GTPase-binding consensus site, the CRIB (**C**dc42/**R**ac-**i**nteractive **b**inding) motif, which is present in many, though not all, Rac- and Cdc42-binding proteins (see Table 2) [69]. The CRIB sequences for ACK and WASP, and a consensus

sequence, are shown in Figure 1(a). This motif is necessary, but not sufficient, for strong binding to the GTPase, as shown by binding studies for Cdc42 with different WASP fragments [70]. The NMR structures of ACK and WASP CRIB-containing peptides bound to Cdc42 show that Asp[38] in Switch I interacts with the two His residues conserved in all CRIB proteins (Figure 1a). Interestingly Switch I and II are almost identical in Rho, Rac and Cdc42, except for position 38 which is Asp in Rac/Cdc42 and Glu in Rho. Mutation of Cdc42 Asp[38] to Glu decreases the affinity for PAK3 (a CRIB protein which binds to both Rac and Cdc42) by 50-fold [71], and it seems likely that all CRIB proteins may use Asp[38] to distinguish Rac/Cdc42 from Rho.

Whereas PAK1 binds with similar affinity to Rac and Cdc42 [72], ACK and WASP are relatively selective for Cdc42 [73,74]. To achieve this selective binding, ACK and WASP must interact with amino acids that differ between Cdc42 and Rac, and these contacts have been revealed in the NMR studies [67,68]. [Leu[174]]Cdc42, for instance, forms a strong hydrophobic contact with both ACK and WASP, but in Rac residue 174 is Arg, which would weaken this interaction. The ACK CRIB residue which interacts with Leu[174] is Leu[505], but the equivalent residue in PAK is Lys, which would not pack well against Leu[174] and is, presumably, therefore, not involved in the

interaction (see Figure 1a). The equivalent residue in WASP is Ile, which should pack almost as well as Leu (see Figure 1a). Thus, a picture of how different effector proteins recognize different combinations of Rho GTPases emerges. However, while ACK and WASP are clearly Cdc42 targets, it is not clear whether, *in vivo* and under physiological conditions, PAKs 1–3 are targets for Cdc42 or Rac or both.

A further insight gained from these NMR structures is that the GTPase induces significant conformational changes in ACK and WASP. The CRIB-containing fragments of ACK and WASP used in these experiments (amino acids 504–545 and 230–288 respectively) have no discernable structure in solution, but when bound to Cdc42 they form a tight intermolecular β-sheet across the GTPase switch regions. Presumably Cdc42 stabilizes this key conformation, which is only one of many that the free peptide can adopt in solution. WASP sequences C-terminal to the core CRIB motif form an anti-parallel β-hairpin and a short α-helix only when bound to Cdc42. Equally, the switch regions of free Cdc42 in solution are flexible, but become rigid when bound to an effector.

These NMR data have certainly provided much insight into how CRIB motif-containing proteins, in general, may interact with Rac and Cdc42. However, one must remember that not all Rac/Cdc42 effector proteins contain a CRIB domain (see Table 2), so cannot be assumed to bind to GTPases in the same way. IQGAP, for instance, appears to require the insert region for binding to Rac and Cdc42 (see above) which has not been shown to be required for the binding of any of the known CRIB-containing proteins. Such observations imply that a very different set of interactions may be formed between Rac and Cdc42 and their numerous effectors.

Rho binding to its effector proteins also appears, from mutational studies, to require quite different GTPase regions compared with Rac and Cdc42 (see above). Rho effectors protein kinase N (PKN)/PRK1 and PRK2 bind to Rho via an N-terminal Rho effector homology (REM) region which contains three repeats of a leucine-zipper-like motif named HR1 (amino acids 40–91, 129–184 and 216–271). The first two HR1 repeats (HR1a and HR1b) can each independently bind to Rho, but only HR1a binding is GTP-dependent (Figure 1b shows PKN HR1a sequence and the HR1 motif consensus sequence) [75]. The scaffold-like Rho effectors rhophilin and rhotekin also contain one N-terminal HR1 motif, very similar to PKN HR1a, which is required for their GTPase binding (see Figure 1b) [76,77]. Recently the crystal structure of PKN amino acids 7–155 (HR1a and half of HR1b) bound to RhoA·GTP[S] has been solved [78]. When bound to Rho the HR1a region of PKN forms an antiparallel coiled-coil (ACC) finger fold, a very different conformation to the CRIB peptides of ACK and WASP bound to Cdc42. Rho residues within switch I, particularly Lys[27] and Gln[29], make strong contacts with PKN, and allow the effector to specifically recognize the GTP-bound form of Rho; Lys[27] in particular is inaccesible in the GDP–Rho structure. These are switch I residues different from those which appear to be important for Cdc42 recognition by ACK and WASP (see above) [67,68]. A hydrophobic patch at the end of the PKN HR1a ACC finger, plus hydrogen-bond contacts from PKN Lys[51], Lys[53], Arg[61] and Asp[85] (see Figure 1b), form important contacts with Rho switch I, the C-terminal end of switch II (amino acids 66–76) and some adjacent amino acids. The polar residues are less conserved in PKN HR1b and HR1c, and the hydrophobic patch is partially replaced by charged residues in HR1c, which may explain why HR1b has low affinity for Rho and why HR1c does not bind to Rho at all, despite their similar overall sequences. Sequence analysis suggests that similar ACC

finger folds will be present in other REM proteins, such as rhopilin and rhotekin, and also perhaps in non-REM Rho effectors such as ROK and kinectin [78].

THE ACTIVATION OF EFFECTORS BY Rho GTPases

The structural analysis of Rho GTPase–effector interactions has so far made use of discrete binding domains derived from effectors. When considering how a GTPase might regulate the activity of a target protein, and how these proteins subsequently transduce signals to the cell, the whole effector protein must be considered.

The most common mechanism of effector activation by Rho GTPases appears to be the disruption of intramolecular auto-inhibitory interactions, to expose functional domains within the effector protein (see the general model in Figure 2). For example the Rac/Cdc42 targets PAK1–3 and Ser/Thr kinases, have an intramolecular regulatory domain that inhibits kinase activity. Upon GTPase binding, the inhibitory sequence is displaced, leaving the kinase domain free to bind to substrates [79,80]. Interestingly, a more distantly related PAK family member recently cloned, PAK4, does not contain an autoinhibitory domain, and this PAK is not significantly stimulated by GTPase binding [81]. In agreement with the model a peptide from the PAK1 regulatory region (amino acids 83–149) can be used as a dominant-negative inhibitor of PAK activity both *in vitro* and *in vivo* [82], and mutants of the PAK inhibitory domain are found to have constitutive kinase activity [82,83]. Equally, cleavage of PAK-2 (and of PKN, see below) by caspases, which occurs during apoptosis, also removes its regulatory domain and creates a constitutively active protein [84,85].

Two kinases which are Rho effectors have also been reported to contain autoinhibitory domains, ROK and PKN. A region of the ROKα autoinhibitory domain (amino acids 941–1388) includes the GTPase-binding and PH domains, and when mutated so as to disrupt binding to Rho, will inhibit endogenous ROK [86]. In the case of PKN the binding of Rho–GTP appears to co-operate with binding of lipids such as arachidonic acid and with autophosphorylation to remove an autoinhibitory inter-action and to fully activate its protein kinase C-like kinase domain [87–90].

A similar principal may also apply to activation of the many scaffold-like targets of GTPases (see Figure 2). Dia is thought to act as a scaffold protein that can be activated by Rho and then interacts with profilin/actin. Recent work has revealed that the N-terminal 389 amino acids interacts with a region at the C-terminus (amino acids 1116–1255), which makes full-length Dia inactive. Binding of Rho to the N-terminal sequence releaves this inhibition and, in agreement with the model, Dia becomes constitutively active when the N-terminal inhibitory region is removed [91]. Interestingly a human genetic disease, non-syndromic deafness, results from amino acid substitutions in the C-terminal 52 amino acids of human Dia1, and the experimental work suggests that this might create constitutively active protein [92]. WASP and N-WASP, two related Cdc42 targets, also appear to be regulated by an intramolecular interaction. The regions of WASP which bind to each other have recently been identified, using a tryptophan-fluorescence-quenching assay, as the N-terminal GTPase-binding domain (amino acids 230–310) and a cofilin-homology region at the C-terminus (amino acids 461–492) [93]. Cdc42–GTP competes with WASP C-terminus for binding to the N-terminus and, according to NMR data, induces a conformational change in the WASP N-terminus quite different from its conformation when autoinhibited [93]. Owing to auto-inhibitory interactions, full-length N-WASP has decreased ac-
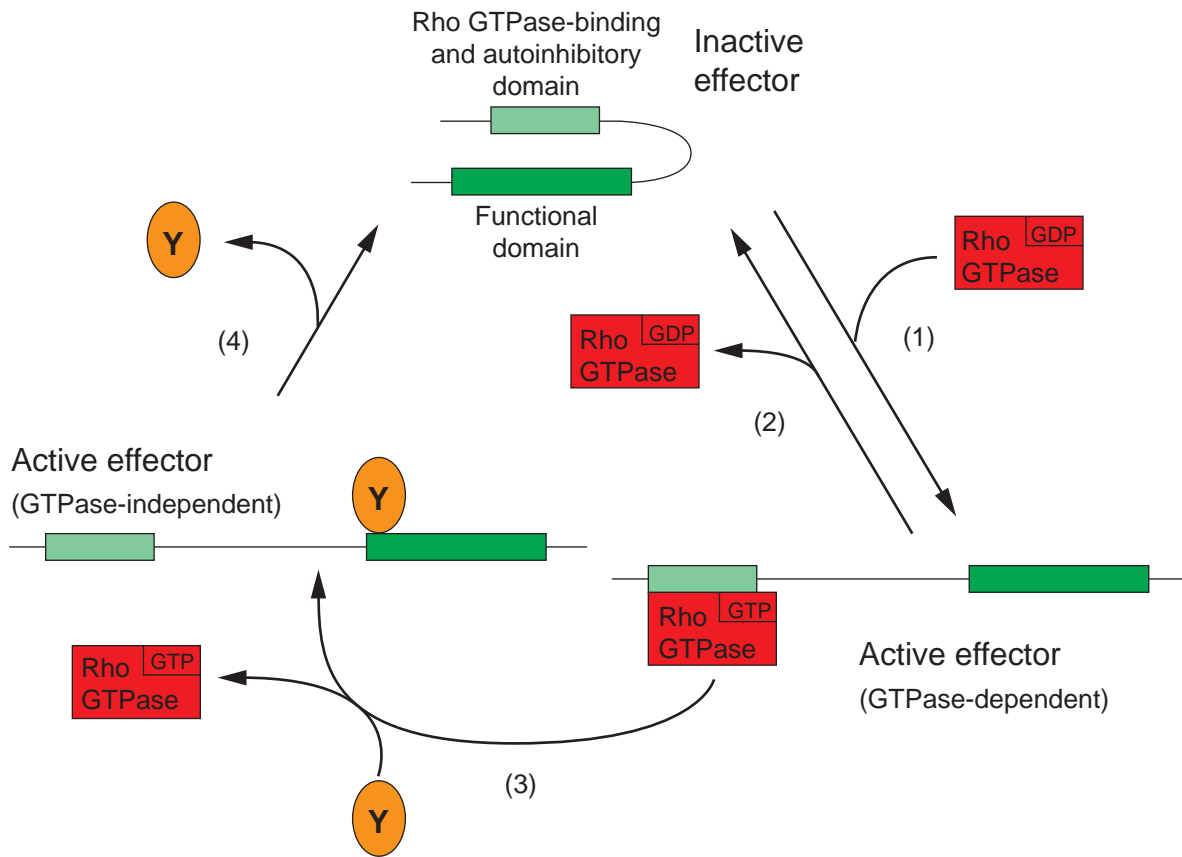
Figure 2   General model for activation of effector proteins

(1) Binding of Rho GTPase to effector relieves an antoinhibitory intramolecular interaction (this is clearly the case for the kinase PAK [80] and for the scaffold proteins Dia [91] and WASP [93], and maybe also for other effectors which contain autoinhibitory domains; see the text). (2) The effector remains active until GTP hydrolysis takes place. (3) Alternatively a modification of the effector (Y, orange ellipses) (e.g. autophosphorylation, as is the case for PAK [72], phosphorylation by a separate kinase or binding to a different activating protein) may maintain activity even after dissociation of the GTPase. (4) Inactivation of the effector occurs though removal of modification Y (e.g. dephosphorylation or removal of a bound activating protein), allowing the effector to re-enter its inactive conformation.

tivity compared with the functionally important C-terminal acidic region alone, and Cdc42-GTP can activate N-WASP by releasing intramolecular interactions [94–97]. A variation on this mode of effector activation has been suggested for the Cdc42-binding protein IQGAP. In this case a seperate protein, calmodulin-Ca^{2+}, binds to IQGAP (via its IQ motif) and inhibits its binding to actin and Cdc42 [98,99]. This might provide a calcium-sensitive regulatory mechanism for controlling the activation of IQGAP [56].

Almost all Rho GTPase effectors have multiple domains, and some of these might regulate their activity. For example, the PH domains present in WASP and N-WASP are thought to promote association with membrane, through lipid binding, where they may encounter activated Rho GTPases [100]. The Rho effectors ROK and citron/citron kinase and the Cdc42 effectors MRCKα/β also contain PH domains [101–104] (MRCKs are myotonic dystrophy kinase-related cdc42-binding kinases). Protein–protein, as well as protein–lipid, interactions can regulate the subcellular localization of GTPase effectors. For example, PRK2 (though not the related PKN/PRK1), WASP and PAK all contain Pro-Xaa-Xaa-Pro (Xaa is any amino acid) classic proline-rich SH3-binding motifs which have been reported to bind to the adaptor Nck [105–108]. Since Nck also has an SH2 domain, it could recruit these effectors to activated receptor

tyrosine kinases. PAK also interacts with another SH3-containing protein, αPIX (also known as Cool-2), though this interaction is via a non-classical SH3 domain-binding region of PAK (amino acids 175–206) [109,110]. αPIX, which is a Cdc42/Rac GEF, can potentially activate PAK, and can also localize the kinase to specific sites within the cell ([79,110,111], but see [111a]); for example, paxillin recruits Cat/Git/Pkl to focal contacts which, in turn, binds to PIX [112], while free G$\beta\gamma$ subunits released by activated G-protein-coupled receptors bind to G protein-coupled receptor kinases, which can also recruit Cat/Git/Pkl and therefore PIX [113]. Interestingly, another member of the PIX family, p50Cool-1, inhibits PAK, while p85Cool-1/βPIX appears neither to inhibit nor activate PAK [114]. Exactly how the PIX–PAK interaction contributes to Rac/Cdc42 activity requires further investigation.

It is noteworthy that many of the Rho GTPase effector proteins contain coiled-coil regions (ROK, citron, IQGAP, Dia) which in some proteins have been shown to facilitate oligo-merization, and indeed there is evidence that this is the case for IQGAP [115–117]. Effector oligomerization could add yet another level of complexity to target activation by Rho GTPases.

Once PAK has been activated by its interaction with Rac/Cdc42, it then autophosphorylates, which both decreases its affinity for GTPase (freeing the GTPase to bind to more effectors

or to be inactivated by GAPs) and also maintains PAK in an open, activated conformation even when dissociated from the GTPase [72]. Similar mechanisms may exist for scaffold proteins, though in this case a separate kinase activity would obviously be needed to phosphorylate the effector (see Figure 2). WASP may be an example of this; it is a substrate for two tyrosine kinases found in lymphocytes, Lyn and Bruton's tyrosine kinase (Btk) [118,119]. WASP shows increased Tyr phosphorylation in the presence of constitutively active Cdc42, indicating that Cdc42 may recruit WASP to the site of Lyn and Btk activity [118]. The tyrosine residue phosphorylated by Btk (Tyr[291]) [119] is predicted to disrupt the autoinhibited fold of WASP N-terminus, so may stabilize WASP in an active conformation even in the absence of Cdc42 [93]. The presence, or not, of kinases which phosphorylate scaffold-like effectors, and the activity of phosphatases (such as protein phosphatase 2A which co-precipitates with PAK1 [120]) which dephosphorylate effectors which are held in an active conformation, may prove to be a general mechanism for regulating the kinetics of effector activity (see Figure 2). Also, in keeping with the model, binding of other proteins to an activated effector, in addition to or instead of phosphorylation, could stabilize effectors in an active conformation after GTPase dissociation (see Figure 2), although no clear example of an effector utilizing such a mechanism has yet been identified.

EFFECTORS WHICH MEDIATE Rho GTPase FUNCTIONS

Along with an understanding of how Rho GTPases interact with, and are activated by, effector proteins, there has been much interest in identifying the contributions of specific effectors to different GTPase functions. So far significant progress has only been made in identifying effectors involved in actin reorganization.

Rho-induced actin reorganization

At least two effectors, ROK and Dia, appear to be required for Rho-induced assembly of stress fibres and focal adhesions (Figure 3). p164ROKα (also known as Rho-associated kinase) and p160ROKβ (also known as Rho-associated coiled-coil-containing protein kinase or ROCK) are Ser/Thr kinases, whose kinase domains resemble that of myotonic dystrophy kinase (52 % identity). They also contain a coiled-coil region, a ROK–kinectin homology (RKH) Rho-binding domain, a PH domain and a Cys-rich region. ROKs are activated by binding to Rho–GTP [121,122]. Expression of the constitutively active catalytic domain of ROKα induces stellate actin-myosin filaments in HeLa and Swiss3T3 cells [101,123]. Inhibition of ROK, on the other hand, using a pharmacological inhibitor (Y-27632) causes loss of serum and [Val[14]]Rho-induced stress fibres [124]. These results indicate that ROK is necessary, but not sufficient, for Rho-induced stress-fibre assembly.

Two substrates of ROK which are likely to be key players in actin–myosin filament assembly are myosin light chain (MLC) [125] and the myosin-binding subunit (MBS) of MLC phosphatase [126]. MLC phosphatase is inhibited by phosphorylation, indirectly leading to an increase in MLC phosphorylation (see Figure 3). Phosphorylation of MLC occurs at Ser[19], which stimulates the actin-activated ATPase activity of myosin II and promotes the assembly of actomyosin filaments [125–127]. Interestingly Rho-GTP also binds directly to the MBS of MLC phosphatase, but why Rho should bind to both kinase and substrate is unclear at present [128].
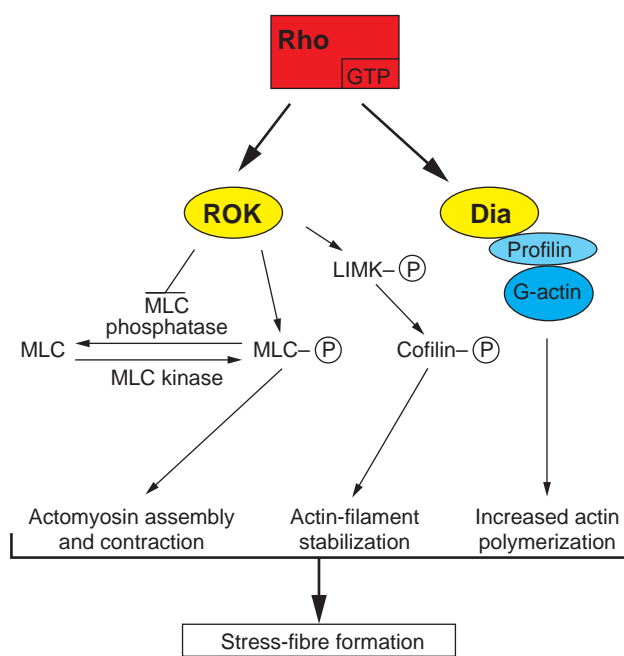

Figure 3 Signal-transduction pathways involved in Rho-induced stress-fibre assembly (see the text for details)

Another ROK target is LIM kinase (LIMK), which when phosphorylated is able to inhibit (by phosphorylation) cofilin, leading to stabilization of filamentous actin structures (Figure 3) [129,130]. ROK has also been reported to activate a ubiquitous Na^+/H^+ exchange protein (NHE1) and this contributes, via an unknown mechanism, to stress fibre and focal adhesion formation [131–134]. Other substrates for ROK which could contribute to actin assembly are adducin, which is phosphorylated by ROK both *in vitro* and *in vivo*, causing it to bind more strongly to F-actin [135,136], and the ERM (ezrin/radixin/moesin) family of proteins, which link actin to the membrane [137,138]. However, recent data has questioned whether ROK is in fact an *in vivo* ERM-activating kinase [139].

While the catalytic domain of ROK alone does not induce correctly organized stress fibres, it has been reported that, when combined with an activated (N-terminal truncation) version of Dia, stress fibres are induced [91,140,141]. This has lead to the idea that activation of both Dia and ROK by Rho are required in order to induce stress fibres. Dia (Dia1 and 2 in mammals) is a member of the formin-homology (FH) family of proteins and contains two FH domains [142]. The FH1 sequence contains multiple proline-rich motifs which bind to the G-actin-binding protein, profilin. This interaction somehow allows Dia to contribute to actin polymerization and F-actin organization into stress fibres [91,142].

Many observations point to a role for lipids, particularly PIP_2, in actin cytoskeleton rearrangements. For instance, the binding of PIP_2 to capping proteins such as gelsolin can induce their release from actin-filament barbed ends, providing a mechanism whereby PIP_2 could increase actin polymerization [143]. There are some data to suggest that PIP_2 is involved in Rho effects. Injection of antibodies against PIP_2, for example, has been reported to inhibit serum-induced assembly of stress fibres [144]. Furthermore, overexpression of phosphatidylinositol-4-phosphate 5-kinase (PI-4-P5K) (to produce PIP_2) induces massive

actin polymerization in COS-7 cells [145]. It is noteworthy that mutation of the gene for a yeast PI-4-P5K, *mss4*, results in defects in the formation of actin cables, which can be repressed by overexpression of Rho2p, a Rho-like GTPase [146]. A physical association between Rho and a type I (PI-activated) PI-4-P5K activity has been detected in Swiss-3T3-cell lysates, though this interaction is not GTP-dependent and may not be direct [147]. PIP_2 also binds to vinculin, increasing its talin-binding ability; therefore PIP_2 synthesis could also help to induce focal adhesion formation [144]. PI-4-P5K could, therefore, provide a link between Rho and the stimulation of both new actin polymerization and of focal adhesions assembly. Additionally, [Val¹⁴]Rho and PI-4-P5K both induce ERM phosphorylation when overexpressed in fibroblasts, suggesting that Rho activation of PI-4-P5K may also be a mechanism by which Rho activates ERM proteins [139].

Cytokinesis also depends on the assembly of contractile actin–myosin filaments and has been shown to be Rho-dependent in *Xenopus* and sand-dollar (flat sea-urchin) embryos [38,39]. Rho is recruited to the cleavage furrow of Swiss3T3 cells during cytokinesis [148] and may utilize some of the same effector proteins required for stress-fibre formation. ROK, for example, phosphorylates and disassembles the intermediate filament protein glial fibrillary acidic protein to allow cleavage to occur [149], and a dominant-negative ROK construct (containing a mutant Rho-binding domain fused to the PH domain) inhibits cytokinesis when injected into *Xenopus* embryos and mammalian cells [150].

Mammalian Dia may be involved in cytokinesis, since it has been detected at the cleavage furrow in mitotic Swiss3T3 cells [141], and, in lower eukaryotes, FH-domain proteins are commonly involved in cytokinesis. For example, the *Drosophila* homologue, diaphanous, is essential for cytokinesis [151], and two yeast formin-family proteins, Bni1p and Bnr1, are both required downstream of Rho GTPases for bud formation and *bni1 bnr1* double mutants are often multinucleate [152].

Citron kinase, which appears not to play a role in stress-fibre assembly, has been localized to the cleavage furrow in HeLa cells [103]. Citron kinase, like ROK, contains an N-terminal myotonic dystrophy kinase-like kinase domain (46 % identity with ROKα), a central coiled-coil region, a Rho-binding domain (with no homology with ROK), a Cys-rich region, a PH domain and, unlike ROK, a Pro-rich SH3 domain-binding region [102,103]. Truncations of citron kinase lacking the Rho-binding domain inhibit cytokinesis in HeLa cells, producing multinucleate cells [103], suggesting that this kinase acts downstream of Rho specifically at the cleavage furrow. Whether citron phosphorylates a distinct subset of proteins from ROK, in order to fulfil its role in cytokinesis, is unknown.

Cdc42 effectors implicated in actin reorganization

WASP is expressed in haematopoetic cells, and is the product of the X-linked immunodefficiency gene found in Wiskott–Aldrich syndrome ([153,154], but see [153a]). The more ubiquitously expressed (neuronally enriched) N-WASP, like WASP, also binds to Cdc42 [100,155,156]. WASP and N-WASP each contain an N-terminal PH domain, followed by WASP-homology domain 1 (WH1), a CRIB domain, an SH3-binding proline-rich sequence, a WH2 domain containing verprolin-like sequences and an acidic C-terminus to which Arp2/3 binds (see below). It was observed that overexpression of N-WASP plus Cdc42 induces very long microspikes, like an exaggeration of Cdc42 activity [97], suggesting that these proteins may be involved in the formation

of filopodia downstream of Cdc42. N-WASP binds to profilin, and WASP and N-WASP bind directly to actin monomers through their verprolin-like WH2 domains [94,157,158]. A WASP binding partner, WASP-interacting protein ('WIP'), which also binds to profilin and causes actin polymerization in BJAB cells may also be involved [159]. Recently it has been found that WASP/N-WASP, the yeast homologue Bee1p and the WASP-like Scar proteins (which do not contain a Cdc42-binding domain), bind directly to, and activate, the Arp2/3 complex via their acidic C-terminus [94,95,160]. The Arp2/3 complex binds to actin monomers and acts as a nucleation site for new actin polymerization [161,162]. Furthermore, Cdc42-GTP has been shown to induce actin polymerization *in vitro*, in extracts from *Xenopus*, the cellular slime mould *Dictostelium discoideum* or polymorphonuclear leucocytes [163,164], and biochemical disection of this process has revealed that the Arp2/3 complex and N-WASP are required [96]. Thus, WASP/N-WASP is activated by Cdc42 (plus lipids) and can then act as a scaffold to recruit the machinery required for new actin polymerization, actin monomers/profilin, and the Arp2/3 complex (Figure 4).

Cdc42 also interacts with two Ser/Thr kinases that are thought to be involved in actin reorganization and filopodia formation, MRCKs α and β. MRCKs are Cdc42-specific effector proteins which contain a PH domain and a ROK-like kinase domain which can phosphorylate MLC. Kinase-dead MRCKα inhibits Cdc42-induced filopodia, and overexpression of MRCKα synergizes with Cdc42 to induce large filopodia in HeLa cells [104]. The *Drosophila* homologue of MRCK, Genghis Khan ('GEK') is known to be required for cytoskeletal regulation during oogenesis [165]. Another protein that is able to synergize with Cdc42 to produce large filopodia-like structures is the new PAK family member PAK4 [81]. PAK4 has a CRIB motif (see Figure 1a) and a kinase domain similar to PAKs 1–3, but its sequence is divergent outside of these regions. PAK4 acts quite differently to PAKs 1–3, in that it binds to Cdc42 and not to Rac, and it is not activated by GTPase binding.

Rac effectors implicated in actin reorganization

To date there are few examples of unique Rac effectors that have been implicated in actin reorganization. POR-1 (**P**artner **o**f **R**ac) has been implicated in Rac-induced lamellipodia formation, since truncations act as dominant negative constructs [166,167], and p140Sra-1 (**S**pecific **R**ac1-**a**ssociated protein) co-sediments with F-actin, implying a role in Rac-induced actin reorganization [168]. However, little more is known about the cellular functions of these two proteins. A better characterized target of Rac is PI-4-P5K. Rac interacts directly with PI-4-P5K, though this interaction is not GTP-dependent [169]. It has been established, using permeabilized platelets, that thrombin-induced actin-filament assembly requires actin-filament uncapping, which is absolutely dependent upon an increase in PIP_2 levels, and that this is mediated by Rac activation of a type I PI-4-P5K [170,171].

Interestingly a WASP-like Verprolin-homologous protein (WAVE, also known as Scar) can be precipitated with Rac, though it is not known if this is through a direct interaction. WAVE localizes to membrane ruffles, and its overexpression causes actin clusters, an activity which requires the profilin-binding and verprolin-homology (actin-binding) domains. A verprolin-homology-domain mutant of WAVE inhibits Rac-induced ruffling, further suggesting an *in vivo* link between Rac and WAVE [172]. WAVE, like WASP/N-WASP, has been shown to interact with, and activate, the Arp2/3 complex through its C-terminal acidic domain (see Figure 4) [94,95], but so far it
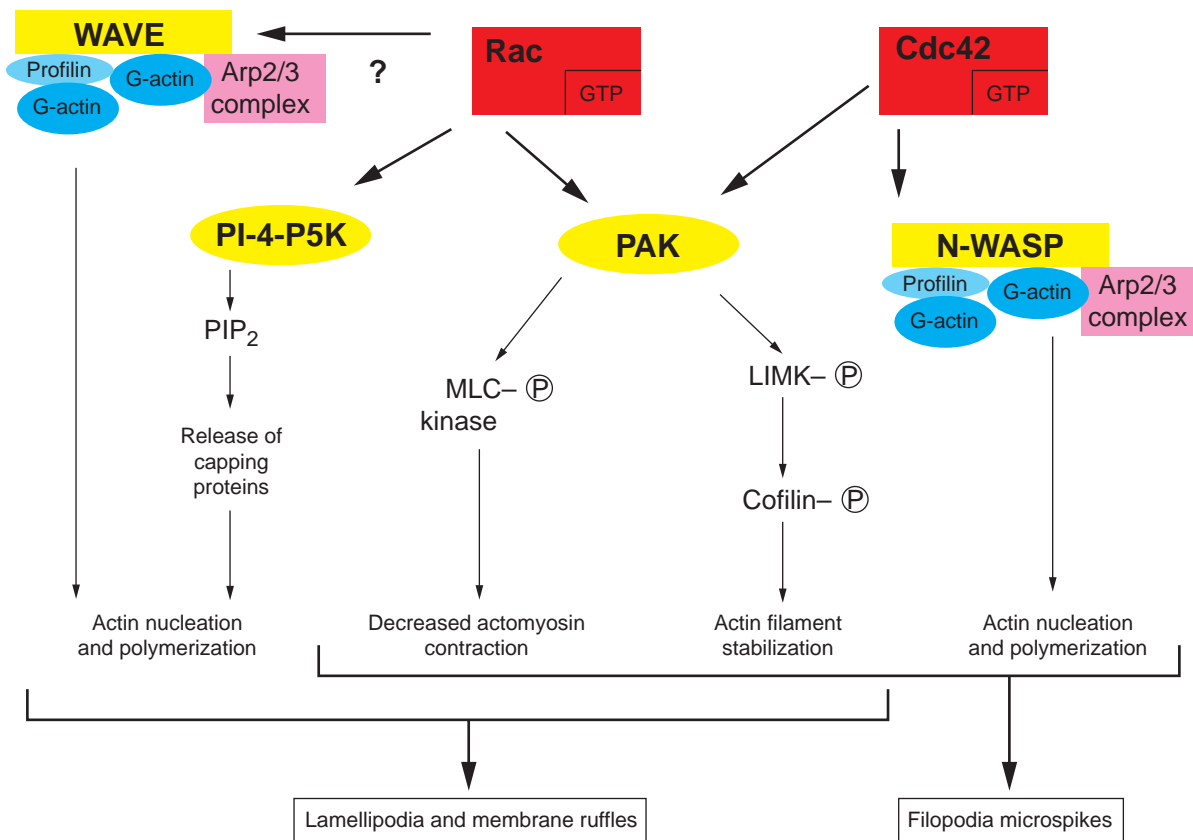
Figure 4   Signal-transduction pathways induced by Rac and Cdc42 (shown in red), which are thought to contribute to the formation of actin-containing lamellipodia and filopodia respectively (see the text for details)

has not proved possible to induce actin polymerization in cell-free extracts with Rac, as has been done with Cdc42 [164].

Rac and Cdc42 effectors implicated in actin reorganization

Some common target proteins appear to be utilized by both Rac and Cdc42 in the induction of lamellipodia and filopodia respectively. PAK1,2,3 are Ser/Thr kinases, related to yeast Ste20, which have received a great deal of detailed attention [72,173,174]. *In vitro*, PAK1 and 3 bind equally well to Cdc42 and Rac, though Cdc42 simulates PAK1 activity more strongly than does Rac [173]. PAK2 only inhibits the intrinsic GTPase activity of Rac, and not Cdc42, suggesting that it may be a Rac target [175]. However, it is difficult to decide whether PAK1,2,3 are targets for Rac, Cdc42 or both *in vivo* from these *in vitro* interactions. There have been conflicting reports linking PAKs to actin changes. Activated mutants of PAK1 have been reported to induce both filopodia and membrane ruffles in Swiss3T3 cells and to cause neurite outgrowth in PC12 cells, similar to the effects of constitutively active Cdc42 and Rac [176,177]. PAK1 has been seen to localize to membrane ruffles, as well as phagocytic actin-containing cups, in *N*-formylmethionyl-leucyl-phenylalanine-stimulated neutrophils [178]. Interestingly, PAK-induced cytoskeletal changes are partly independent of its kinase activity, but require membrane targeting [176,177,179]. Other groups, however, have failed to find any effects of PAK over-expression on the actin cytoskeleton ([58,59], but see [59a]), and, taken together, these results suggest that PAKs are capable of

affecting the actin cytoskeleton but that they only do so in co-operation with other signals that may or may not be present in the cell lines tested.

A variety of substrates for PAKs have been identified that could affect the actin cytoskeleton. Rac, like Rho, induces phosphorylation of LIMK [180], and PAK1 has been shown to phosphorylate LIMK *in vitro* [181]. Also, an inactive form of LIMK has been shown to inhibit both Cdc42 and Rac induced actin changes [182], suggesting that cofilin phosphorylation may be a general requirement in Rho GTPase pathways (Figures 3 and 4). PAK has been reported to phosphorylate and inactivate MLC kinase, decreasing MLC phosphorylation and reducing actomyosin assembly (Figure 4) [183]. There has also been a report of Rac-induced phosphorylation of myosin II heavy chain, which potentially would also lead to loss of actomyosin filaments [127]. Dominant-negative PAK inhibits this phosphorylation, but active PAK does not reconstitute the effect, suggesting that multiple effectors may mediate this activity [184].

IQGAP1 and 2 (named GAPs because of some homology with Ras GAP) are effectors for Rac and Cdc42 and may be involved in actin polymerization [63,185,186]. IQGAP has been detected in a complex with F-actin and Cdc42, which is enhanced by EGF and disrupted by dominant-negative Cdc42 [99]. IQGAP is able to oligomerize and to cross-link F-actin *in vitro*, an activity which is enhanced by GTP[S]–Cdc42 [115]. It has, therefore, been suggested that IQGAP oligomers may form upon binding to GTPase after dissociation of calmodulin, and that somehow this facilitates cross-linking of F-actin. In yeast an IQGAP homo-

logue, Iqg1/Cyk1p, is involved in actin recruitment to the bud neck [187], and in *Dictyostelium* DdGAP1, also IQGAP-like, regulates cytokinesis [188]. Whether IQGAP is involved in cytokinesis in mammalian cells is unknown [189].

Effectors involved in actin-independent activities of Rho GTPases

Overexpression of activated Rac and Cdc42 leads to activation of the JNK and p38 MAP kinase pathways in a variety of cell types [49,50,190]. JNK activation by certain growth factors and cytokines [e.g. EGF, tumour necrosis factor α (TNFα) and transforming growth factor β] has been reported to be inhibited by dominant-negative Rac and Cdc42 [49,191], but it is still not clear how general the role of Rac/Cdc42 is in JNK activation. In Swiss3T3 cells, for example, TNFα induction of JNK is not dependent on Cdc42 [192]. Which Cdc42/Rac effectors mediate JNK activation in mammalian cells is still unclear. In *Drosophila* there is good genetic evidence for a link from DRac1 and DCdc42, via mis-shapen (PAK-like), to hemipterous (a JNK kinase), a pathway that is essential for dorsal closure [193,194]. Also, in yeast, a PAK-like kinase, Ste20, acts downstream of Cdc42 and is required for activation of the Kss1p/Fus3p MAP kinase cascade in the mating-pheromone response [195,196]. While mammalian PAK1 has been observed to enhance p38 activation when co-expressed with Rac and Cdc42, and constitutively active PAK1 and PAK3 has been reported to activate JNK in COS cells [197,198], many other groups have failed to find PAK activation of JNK or p38 ([57,59,61,199], but see [59a]).

Additional Rac/Cdc42 targets which may mediate JNK activation are: (1) Mlks 1, 2 and 3, which contain CRIB motifs and act as MAP kinase kinase kinases (MEKKs) [60,69,200,201]; (2) MEKK1 and MEKK4, which also interact with Rac and Cdc42 (MEKK4 has a CRIB domain). All are strong activators of JNK when overexpressed, and inhibitory-kinase-dead MEKK1 or MEKK4 mutants block Rac/Cdc42-induced JNK [202]. However, to date no physiological link has been made between Rac/Cdc42 and Mlk1, Mlk2 and Mlk3 or MEKK1 and MEKK4.

All three Rho GTPases have been implicated in the assembly and/or maintenance of cell–cell contacts [53]. In general the mechanisms mediating these effects are unknown; however, a recent report suggests that IQGAP1 may be involved [54]. IQGAP1 appears to interact with β-catenin, preventing its association with α-catenin; free IQGAP (i.e. not bound to Cdc42 or Rac) could, therefore destabilize cell–cell contacts [203,204].

Finally, Rac is known to be a regulator of the NADPH oxidase complex, a specialized enzyme of phagocytic cells that generates oxygen radicals to kill internalized micro-organisms [51,205,206]. A cytoplasm-derived component of the oxidase complex, $p67^{phox}$, binds directly to Rac [207]. Data suggest that the role of Rac is not to promote relocalization of this cytoplasmic protein to the membrane-bound catalytic components, as was first thought, but rather to act as an allosteric regulator by inducing a conformational change in the preformed complex to promote catalytic activity [63].

Conclusions

In conclusion, the wide range of Rho GTPase activities identified to date (see above and Table 1) is reflected in the large number of cellular targets with which they interact. Over 30 have now been identified (see Table 2), and it is likely that the list is not complete. The three-dimensional analysis of GTPase-effector complexes has increased our understanding of how a Rho GTPase can interact with many different effector proteins in a

specific manner. However, to date only three effectors, PKN, WASP and ACK, have been analysed in this way, and these studies have all used isolated GTPase-binding domains and not full-length effector proteins. An emerging theme is that effectors can be activated by Rho GTPases through the relief of auto-inhibitory sequences. Clearly an analysis of the full-length effector proteins, carried out in solution, will be required to fully characterize the consequences of GTPases binding to their targets. Another potentially common feature, already demonstrated for PAK, is that additional cellular activities, such as kinases or other protein–protein interactions, can influence effector activity (see Figure 2). Progress has been made in identifying the cellular contributions of a handful of effectors. The clearest links downstream of Rho GTPases are in the area of cytoskeletal reorganization, where some targets appear to be essential (see Figures 3 and 4), while others have been shown to have the potential to be involved (see above). Another interesting possibility is that a Rho GTPase might interact with different subsets of effectors depending upon the cellular context and possibly upon the mechanism of activation; the large family of Rho GEFs could possibly contribute to such variations. A future challenge, so far not really addressed, will be to understand the spatial and temporal control of GTPase activity and the co-operation of Rho GTPases with other signalling networks. Finally, added to this complexity it should be remembered that Rho, Rac and Cdc42 are only three members of the Rho GTPase family: the functions of the seven other members awaits serious investigation.

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