REVIEW ARTICLE

PAK and other Rho-associated kinases – effectors with surprisingly diverse mechanisms of regulation

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The Rho GTPases are a family of molecular switches that are critical regulators of signal transduction pathways in eukaryotic cells. They are known principally for their role in regulating the cytoskeleton, and do so by recruiting a variety of downstream effector proteins. Kinases form an important class of Rho effector, and part of the biological complexity brought about by switching on a single GTPase results from downstream phosphorylation cascades. Here we focus on our current understanding of the way in which different Rho-associated serine/threonine kinases, denoted PAK (p21-activated kinase), MLK (mixed-lineage kinase), ROK (Rho-kinase), MRCK (myotonin-related Cdc42-binding kinase), CRIK (citron kinase) and PKN (protein kinase novel), interact with and are regulated by their partner GTPases. All of these kinases have in common an ability to dimerize, and in most cases interact with a variety of other proteins that are important for their function. A diversity of known structures underpin the Rho GTPase–kinase interaction, but only in the case of PAK do we have a good molecular understanding of kinase regulation. The ability of Rho GTPases to co-ordinate spatial and temporal phosphorylation events explains in part their prominent role in eukaryotic cell biology.

Key words: Cdc42, MLK (mixed-lineage kinase), PAK (p21-activated kinase), Rac, Rho, ROK (Rho-kinase).

INTRODUCTION

The Rho GTPase cycle

The Ras-related Rho family of GTPases are molecular switches that use an apparently simple biochemical strategy to control complex cellular processes. They cycle between two conformational states, i.e. one bound to GTP (active state) and the other bound to GDP (inactive state), and of course they hydrolyse GTP to GDP. In the 'on' (GTP-bound) state, GTPases recognize target proteins (often referred to as effectors) and generate a response until GTP hydrolysis returns the switch to the 'off' state. Studies of such GTPases have provided important insights, because members of this family are master regulators of many cellular activities. This review focuses specifically on regulation of the kinases that bind GTPases of the Rho subfamily: their study has in turn provided important insights into the molecular mechanisms underlying cytoskeletal dynamics.

The first *Rho* gene was identified in 1985 [1], but it was not until 1992 that evidence emerged for specific cellular functions of Rho GTPases. Constitutively activated (GTPase-deficient) mutants of Rho and Rac were found to induce the assembly of contractile actin and myosin filaments (stress fibres) and actin-rich surface protrusions (lamellipodia) respectively when introduced into fibroblasts [2,3]. Later, Cdc42 was shown to promote the formation of finger-like membrane extensions termed filopodia [4]. The conclusion that Rho, Rac and Cdc42 regulate separate signal transduction pathways linking plasma membrane receptors to the assembly of distinct filamentous actin structures is complicated by the observations that these pathways are intimately linked, and that downstream effector proteins can interact with more than one Rho GTPase. For example, the prototype kinase effector PAK (p21-activated kinase) interacts with Rac1, Rac2, Rac3 [5–7] and Cdc42 [5], as well as the less studied GTPases CHP [8], TC10 [9], and Wrch-1 [10] proteins.

The Rho family in mammals represents a group of 23 gene products [11] that in general are activated by GEFs (guanine nucleotide exchange factors) and inactivated by GTPase-activating proteins [12]. Target (effector) proteins for Rho can contain recognizable sequence motifs involved with the GTPase interaction that allow identification via database searches [13]. Rho GTPases participate in the regulation of the cytoskeleton, cell polarity, gene transcription, cell cycle progression, microtubule dynamics, vesicular transport and a variety of enzymatic activities. This eclectic list of activities is confusing, but consistent with the now large number of target proteins identified. In this review we concentrate on those serine/threonine kinases that have been discovered to interact with Rho GTPases, and highlight the various mechanisms by which the kinases are regulated. These components (as well as non-kinase counterparts) are essential to co-ordinate the complex machinery underlying cellular functions such as cell movement and division.

How Rho modulates actin/myosin II dynamics

The way in which Rho signalling controls myosin II (Figure 1) represents the best understood regulatory system involving the kinases discussed here. RhoA, Cdc42 and Rac1 all provide input via their associated kinases to modulate contractile actin/myosin

Abbreviations used: ACC, anti-parallel coiled-coil; CRIB, Cdc42 and Rac interactive binding; CRIK, citron kinase; Crmp, collapsin response mediator protein; DMPK, myotonic dystrophy kinase; GEF, guanine nucleotide exchange factor; GIT1, G-protein-coupled receptor kinase-interacting target 1; Hsp90, heat shock protein 90; JNK, c-Jun N-terminal kinase; KI, kinase inhibitory; KIM, KI motif; LIMK, LIM domain kinase; MAPK, mitogen-activated protein kinase; MBS, myosin-binding subunit; MEKK, MAPK/ERK (extracellular-signal-regulated kinase) kinase kinase; MKK, MAPK kinase; MLK, mixed-lineage kinase; MRCK, myotonin-related Cdc42-binding kinase; PAK, p21-activated kinase; PBD, p21-binding domain; PDK1, 3-phosphoinositide-dependent kinase 1; PH, pleckstrin homology; PIX, PAK-interacting exchange factor; PKC, protein kinase C; PKN, protein kinase novel; PP1, protein phosphatase type 1; R-MLC, regulatory myosin light chain; ROK, Rho-kinase; SH3, Src homology 3.

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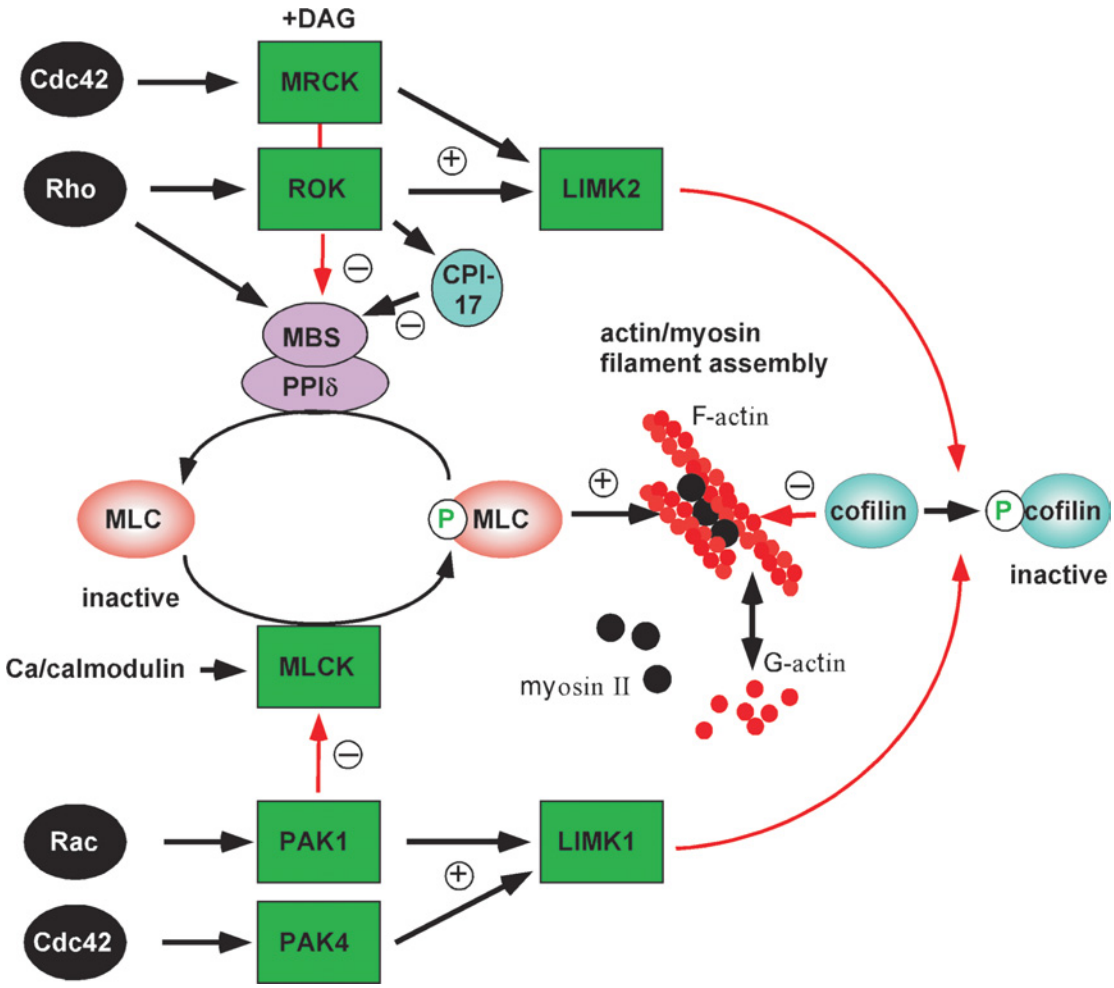

Figure 1 Involvement of Rho-associated kinases in the assembly and contractility of non-muscle myosin II/actin fibres

RhoA, Rac1 and Cdc42 interact with a variety of associated kinases (arrows). Myosin light chain kinase (MLCK) is a calcium/calmodulin-responsive enzyme that maintains the myosin heavy chain–MLC complex in an active state, but is negatively regulated by PAK [179]. ROK conversely blocks PP1δ, by phosphorylating the MBS, also referred to as the myosin phosphatase target subunit (MYPT). Both the N- and C-termini of MBS contain a myosin-binding site (reviewed in [15]). The C-terminal half contains a binding site for RhoA [15,180]. Phosphorylation of a central region of MBS results in direct inhibition of PP1 and a concomitant increase in phosphorylated R-MLC [21]. PKN (not shown) and ROK can also act via CPI-17 (protein kinase C-potentiated inhibitor of 17 kDa), an inhibitor of MBS/PP1, whose phosphorylation at Thr-38 potentiates its inhibitory activity [181]. Various studies have implicated ROK, MRCK and PAK in the regulation of LIMKs [84,182,183], which inactivate cofilin by phosphorylation at Ser-3. Once phosphorylated, cofilin/ADF (actin depolymerizing factor) can no longer bind effectively to F-actin, and the ability of these proteins to catalyse both F-actin depolymerization and severing is thus inhibited (reviewed in [184]). PAK1 is thought to modulate R-MLC function primarily via inhibition of MLCK activity [179]. Phosphorylation of MLCK occurs at Ser-439 and Ser-991; binding of calmodulin to MLCK is inhibited by modification of Ser-991 [185]. PAK1 has been shown to be able to bind to and regulate Ser-508 within the LIMK1 activation loop downstream of Rac1 [186]; Rho and Cdc42 are more closely linked to the effects of LIMK2 [187]. Thus the Rac and Cdc42 signalling pathways, acting via PAKs, can function either co-operatively with or antagonistically to Rho/ROK. DAG, diacylglycerol.

II filaments used by many cell types to induce rapid, reversible changes in shape. For example, vascular smooth muscle cells control blood flow by constricting and dilating in response to physiological stimuli requiring RhoA/ROK (Rho-kinase; also known as ROCK) [14]. In non-muscle cells, similar contractile components (often referred to as actin stress fibres) are assembled from bundles of actin filaments and myosin II; other accessory proteins such as tropomyosin and caldesmon are present, but are not shown in Figure 1. Myosin phosphorylation depends mainly on the balance of two activities: the Ca^{2+}-dependent R-MLC (regulatory myosin light chain) kinase, and R-MLC phosphatase. Phosphorylation of the R-MLC of myosin II induces its interaction with actin, which thereby activates the myosin ATPase, and results in enhanced cell contractility. R-MLC phosphatase is composed of three subunits: a catalytic subunit [PP1 (protein phosphatase type 1)], an MBS (myosin-binding subunit), and a small non-catalytic subunit. Both the N- and C-termini of the MBS have binding sites for myosin [15]. The recent crystal structure of the complex between PP1δ and MYPT1, a 34 kDa N-terminal domain of MBS, indicates that structural elements around the RVXF motif of MYPT1 reshape the catalytic cleft of PP1, contributing to the increased specificity of this complex towards R-MLC [16].

ROK phosphorylates Ser-19 of R-MLC efficiently *in vitro* [17], and perhaps also *in vivo* [18], but its ability to prevent the dephosphorylation of R-MLC via phosphatase inhibition is most critical. Phosphorylation of MBS130 by ROK occurs at Thr-697, Ser-854 and Thr-855 (as recently reviewed [19]). This affects the phosphatase in two ways: phosphorylation of Thr-855 induces its dissociation from myosin [20], while, more importantly, modification of Thr-697 in the phosphatase inhibitory motif promotes its direct binding to and inhibition of PP1 [21]. Myosin II assembly and activity is thus orchestrated by conventional second messengers such as calcium in combination with Rho GTPase activity. Like the PAKs, ROK can phosphorylate LIMKs (LIM

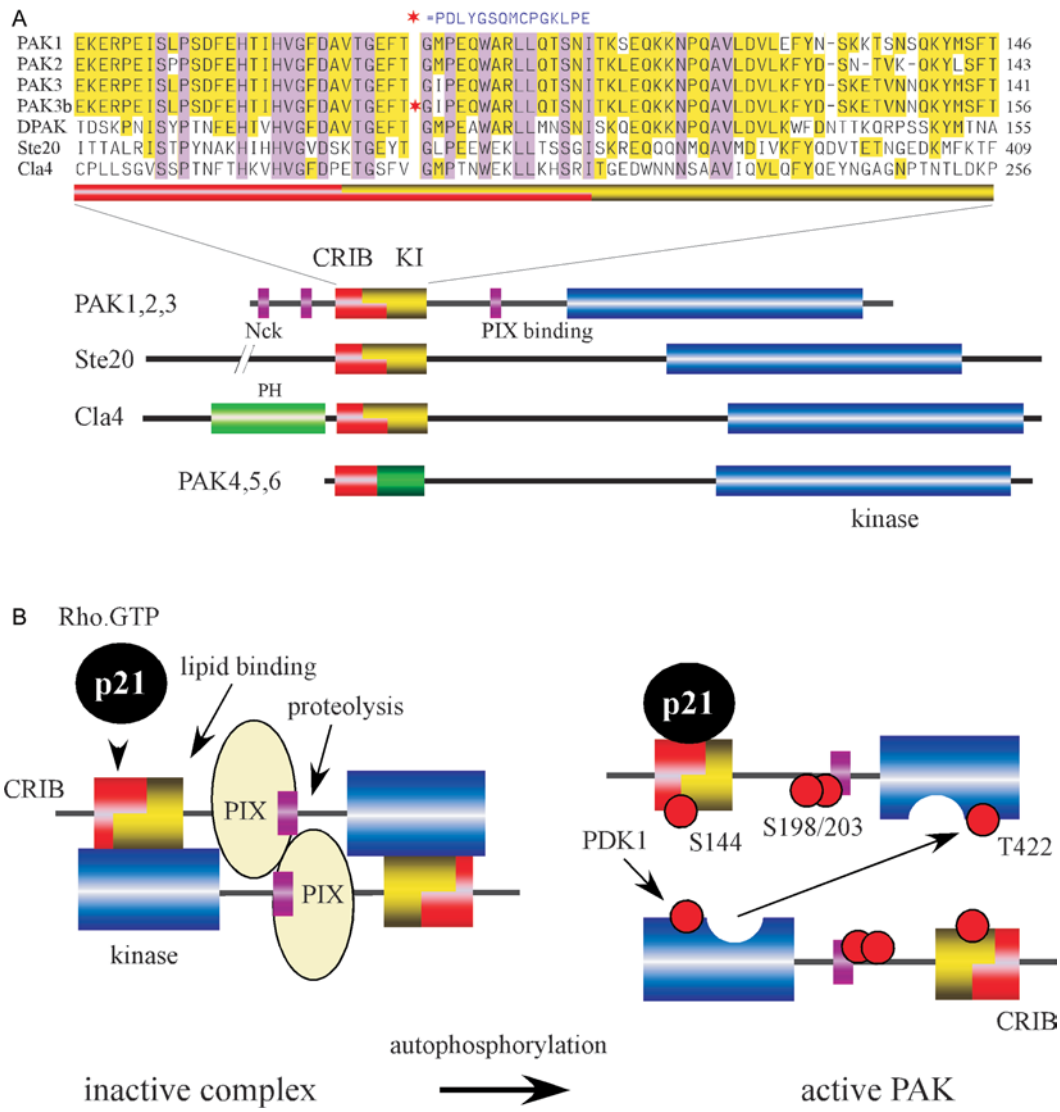

Figure 2 Schematic diagram indicating conserved features of PAKs

(**A**) The top part of the figure shows the domain structure and sequence comparison among the conventional PAKs represented by human PAK1–PAK3 (accession nos. Q13153, Q13177 and NP_002569 respectively), *Drosophila* DPAK (AAC47094), budding yeast Ste20p (AAA35038), and Cla4p (P48562), which is characteristic of fungal PAKs with a PH domain (light green). A unique 15-amino-acid insert in the CRIB domain of PAK3b (accession no. O75914) is highlighted with a red star and its sequence indicated above the alignment. The conventional PAK family contains a conserved Cdc42/Rac-interacting binding domain (CRIB; shown in red) that overlaps a KI domain (in yellow). Cdc42/Rac1 binding to the CRIB rearranges the KI domain and releases it from the catalytic domain (blue). The three purple boxes correspond to conserved proline-rich motifs that bind SH3 domain-containing proteins Nck, Grb2 and PIX (left to right respectively). The group II PAKs (PAK4–PAK6) contain a CRIB sequence that binds GTPases, but lack a KI domain, although unrelated conserved sequences (shown in dark green) are present. Identical residues are shaded pink, and conservative substitutions in yellow. (**B**) A model for PAK1 activation. The auto-inhibited kinase is arranged in head-to-tail fashion, in which the catalytic domain (blue) binds the KI domain (yellow) and is supported by associated PIX dimers. Upon Cdc42 (or related GTPase) binding, proteolysis [53] or lipid binding (arrows), the kinase undergoes a conformational change that allows autophosphorylation (red circles). Phosphorylation of Ser-144 serves to disables the KI-domain–kinase interaction, while phosphorylation of Ser-198/203 reduces the affinity for PIX. Phosphorylation of the activation-loop Thr-422 may occur in *trans* as indicated, or may involve a third-party kinase such as PDK1.

domain kinases; e.g. LIMK2 at Thr-505 [22]), and enhances the ability of LIMKs to phosphorylate cofilin [23].

At present, the role of protein phosphorylation in other established Rho-mediated events such as the production of lamellipodia or filopodia is not understood at a level that allows assessment of the roles of various Rho-associated kinases.

CONVENTIONAL PAKs (PAK1–PAK3)

Background

The PAKs were the first GTPase-regulated kinases to be identified, in a screen for Rho GTPase binding partners in rat brain cytosol [5]. GTP-bound forms of Rac or Cdc42, but not RhoA, were shown to interact in overlay assays with proteins of 68, 65 and 62 kDa. These protein targets turned out to be the three major PAK isoforms (Figure 2); the human forms are termed PAK1 (rat αPAK), PAK3 (rat βPAK) and PAK2 (rat γPAK) respectively [24,25]. The phosphotransferase activity of PAKs is stimulated dramatically *in vitro* by the GTP-bound forms of Rac1 and Cdc42, but also by a variety of GTPase-independent mechanisms that are not presently understood. PAKs have an ancient origin and serve as important regulators of cytoskeletal dynamics and cell motility. In addition, they are implicated in transcription through MAPK (mitogen-activated protein kinase) cascades, death and survival signalling, and cell-cycle progression (as

comprehensively reviewed in [26]). Consequently, PAKs are implicated in a number of pathological conditions and in cell transformation. The HIV Nef protein recruits a kinase variously identified as PAK1 and/or PAK2 [26]. The ability of PAK to promote the survival of infected cells and the production of viral particles may relate in part to the ability of PAK1 to phosphorylate and inactivate the pro-apoptotic protein Bad [27].

Ste20p was originally identified as a MEKK [MAPK/ERK (extracellular-signal-regulated kinase) kinase kinase] in the yeast pheromone signalling pathway, and is now known to function with a related PAK, Cla4p, in actin and septin cytoskeletal assembly, cytokinesis and polarized growth [28]. Cdc42p is the principal determinant in establishing correct cell polarity upstream of Ste20p and Cla4p [5,29]. Given the complexity of interactions identified for the yeast PAKs through genetic screens [30], it is likely that PAKs play multiple roles in vertebrate cell function. *Drosophila* dPAK has been genetically linked to axonal guidance in growth cones of photoreceptor (R) cells [31]. PAK's role in neuronal guidance in humans may be reflected in a non-syndromic X-linked mental retardation caused by point mutations in PAK3, the brain-enriched PAK isoform [32].

PAK1–GTPase interaction and primary structural features

PAK1 binds and is activated by Rac1, Rac2, Rac3 [5–7] and Cdc42 [5], as well as the less studied GTPases TC10 [9], CHP [8] and Wrch-1 [10] proteins, but does not bind RhoA/B/C/E/G nor other Ras superfamily members. Conserved residues within the N-terminal PBD (p21-binding domain) are involved primarily in binding and activation by these GTPases (see Figure 2). Rac and Cdc42 can bind minimally to the so-called CRIB (Cdc42 and Rac interactive binding [13]) domain [PAK1-(75–90)], but sequences in the flanking KI (kinase inhibitory) domain contribute to overall binding affinity [6,33,34]. Residues conferring GTPase selectivity within the overall PBD domain have been mapped [35]. A short lysine-rich tract (PAK1 residues 66–68) just upstream of the CRIB domain is reportedly required for effective Rac GTPase binding [6], but is not present in the *Drosophila* sequence. The C-terminal catalytic domains of PAK1–PAK3 are essentially identical. These mammalian PAKs have significant sequence similiarity in their catalytic domains to Ste20p and Cla4p (Figure 2), the budding-yeast PAKs [28,29,36], but Cla4p-like PAKs with PH (pleckstrin homology) domains do not exist in vertebrates.

Other distinguishing features of the regulatory domain illustrated in Figure 2 include two conserved canonical PXXP SH3 (Src homology 3) binding motifs, and a conserved non-classical SH3 binding site for PIX (PAK-interacting exchange factor; also known as Rac/Cdc42 GEF6) [37]. The first conserved SH3 binding site binds the adaptor protein Nck [38], while a second site can bind Grb2 [39], suggesting that all three PAKs can be recruited in a similar manner to a variety of signal transduction pathways.

Regulation of PAK activity

Of the kinases discussed here, only PAK regulation is understood at the molecular level. The partial crystal structure of PAK1 in an auto-inhibited conformation has been determined to 2.3 Å (0.23 nm) resolution [34]. PAK1 exists as a homodimer in solution and in cells, probably in a *trans*-inhibited conformation (Figure 2B), where the KI region of one PAK1 molecule packs against the C-terminal catalytic domain of the other [40]. The KI domain inhibits the catalytic domain with an K_i of $\sim$90 nM [41]. PAK1 KI domain residues, including Leu-107 (which is often mutated to generate active forms of the kinase), contribute to this inhibitory interface [34]. The KI segment of PAK stabilizes two structural components of the active site (helix C and the activation loop), as seen for other auto-inhibited kinases. In addition, a lysine from the KI segment blocks the active site by forming salt bridges with two catalytically important aspartate residues. This KI polypeptide can block PAK activation, even *in vivo*, although it must be borne in mind that this polypeptide does not affect the active (autophosphorylated) form of the kinase [41]. The binding constants for binding of Cdc42 to peptides encompassing PAK1 PBD are in the range 10–50 nM [42]. Such polypeptides are useful affinity reagents for assaying the nucleotide status of Rac and Cdc42 in cell lysates [43] and for FRET (fluorescence resonance energy transfer) experiments [44].

Structural data [34,45,46] and biochemical studies [41,47–53] suggest that GTPase binding causes a major change in the conformation of the KI domain that disrupts its interaction with the catalytic domain, allowing autophosphorylation that is required for full kinase activity [34]. These events are illustrated in Figure 2(B); regardless of the trigger for activation, PAK requires autophosphorylation to switch to an active state. Autophosphorylation of a single residue in the catalytic domain (equivalent to Thr-423 of PAK1) in the activation loop is important both for maintaining relief from auto-inhibition and for full catalytic function towards exogenous substrates [48–50]; modification by PDK1 (3-phosphoinositide-dependent kinase 1) is also suggested [54]. Autophosphorylation of αPAK at Ser-144 (a conserved residue in the KI domain) contributes to kinase activation [52], while autophosphorylation sites Ser-198/203 (PAK1) serve to down-regulate the PIX–PAK interaction. Interestingly, an alternatively spliced version termed PAK3b has been identified (also represented by a number of expressed sequence tags), which has an insertion in the PBD/KI sequence (Figure 2), allowing the kinase to be active in the absence of GTPase binding [55].

While PAKs are considered primarily as Rac and Cdc42 GTPase effectors, GTPase-independent activation mechanisms are known. Indeed, PAKs were first studied as kinases whose autophosphorylation and activity could be stimulated by limited protease-mediated digestion [47,56]. A physiological correlate came when PAK2 was found to be a substrate for caspase 3 at Asp-212, generating a catalytically activated kinase during apoptosis [57]. Membrane recruitment of PAK1 via SH3-containing Nck and Grb2 adaptor proteins (mimicked by the addition of membrane-targeting sequences) results in the stimulation of kinase activity [58,59]. This might involve phosphorylation at the critical Thr-423 residue by PDK1 [54] or interaction with lipids such as sphingosine, which can activate the kinase in a GTPase-independent manner [60]. GIT1 (G-protein-coupled receptor kinase-interacting target 1), which associates indirectly with PAK via PIX, can also potently activate PAKs through a mechanism that does not require Rho GTPases [61].

PAK1–PAK3 complex with the focal adhesion-associated protein PIX (also referred to as Cool), which is a Cdc42/Rac1 GEF. Multiple PIX proteins, derived from two different genes (αPIX and βPIX), bind PAK via their SH3 domains [37,62]. The PIX–PAK interaction might be expected to strongly activate the kinase due to local activation by Rac · GTP or Cdc42 · GTP [63,64], but PIX is a rather poor GEF [37]. Analysis of αPIX-deficient cells has suggested a key role for the PIX–PAK complex in the Cdc42-mediated direction sensing of chemotactic leucocytes [65]. PIX is tightly associated with GIT1, a 90 kDa protein (also known as PKL/CAT1 [66,67]) that targets focal adhesions by binding paxillin [66]. Overexpression of GIT1 causes disassembly of focal adhesions accompanied by loss of paxillin, which might be an indirect effect resulting from PAK activation by GIT1 [61]. Thus GIT1 and PIX are critical partners that both localize and

activate PAK at focal adhesions, at the leading edge of motile cells, and to cell–cell junctions [68–70]. Two closely related human protein phosphatases have been identified that efficiently dephosphorylate PAK1, including at Thr-423 [71]: POPX1 (partner of PIX 1) and POPX2 bind to various forms of PIX and form multimeric cellular complexes containing PAK. Overexpression of either of these PP2C-related phosphatases antagonizes the cellular effects of active PAK1 [71]. The presence of such a negative regulator in complex with the kinase explains the rapid activation/inactivation cycle of PAKs [72].

Other protein kinases might down-regulate PAK function: Akt phosphorylates PAK1 at Ser-21, and this modification decreases binding of Nck to the PAK1 N-terminus while increasing kinase activity [73,74], which would suggest inhibition of PAK when Nck recruits PAK to various phosphotyrosine-containing complexes. However, neither PAK nor Nck was identified in a recent comprehensive proteomics analysis of epidermal growth factor-enriched components, although GIT1 and GIT2 were found [75].

THE NON-CONVENTIONAL PAKs

Background

PAK4 was the first reported member of the non-conventional PAKs (also referred to as group II PAKs [76]), and was identified through similarity to the PAK2 kinase domain [77]. PAK4 is expressed ubiquitously, with highest levels in the prostate, testis and colon [77,78]. A kinase designated PAK6 was identified as an androgen receptor-interacting protein in a yeast two-hybrid screen [79]. PAK6 translocates to the nucleus, resulting in the repression of androgen receptor-mediated transcription; an active PAK6 mutant, S531N, appears to prevent receptor translocation [80]. The last PAK member to be described was PAK5, which is brain-enriched [81,82]. Database entries under 'PAK7' correspond to PAK5 gene products. Mutations in the *Drosophila* group II PAK (*mbt* gene product) cause severe defects in neural structures [83].

Expression of activated PAK4 results in decreased stress fibres and focal adhesions, and this activity may relate to the ability of PAK4 to bind to and phosphorylate LIMK1 [84], as for PAK1. An interaction with PDZ-RhoGEF has been reported, leading to down-regulation of RhoA, which could also contribute to these effects [85]. Unlike group I PAKs, overexpression of PAK4 can drive anchorage-independent growth and transformation [86]. Interestingly, one study reported that expression of PAK4 was elevated in a panel of tumour cell lines [78].

PAK4–PAK6: Rho interaction and primary structural features

The N-terminal GTPase-binding domains of non-conventional PAKs are $>60\,\%$ identical to each other. Additional conserved features can be seen flanking the CRIB motif; however, no KI sequence is detected resembling that in PAK1 (Figure 2A). Group II PAKs are $>75\,\%$ identical in their catalytic domains. The better studied PAK4 binds to Cdc42 · GTP and, to a much lesser extent, Rac · GTP [77]. Curiously, this study found that a Cdc42 effector mutant (Y40C), which is unable to bind group I PAKs, retains the ability to bind PAK4; perhaps a different structural interface exists between the GTPase and kinase. Non-conventional PAKs do not exhibit the SH3 binding sites discussed above for various protein partners.

Regulation of PAK4–PAK6

Binding of Cdc42 · GTP to PAK4 does not stimulate kinase activity [77], but rather can result in translocation of PAK4 to the Golgi apparatus [77]. Similarly, although PAK5 and PAK6 bind to both Cdc42 and Rac, their activities are not enhanced [82]. These results indicate that the two subgroups of PAKs are differently regulated, and perhaps that group II kinases have dispensed with the KI mechanism for kinase regulation in favour of a different one.

An activation loop phosphoserine (Ser-474 in PAK4) is critical for activity [77], but substitution with glutamic acid is not sufficient to render PAK4 constitutively active [86], although minor activation may occur [78]. Since N-terminally truncated versions of both PAK4 [77] and PAK6 [79] have greater activity than the full-length proteins, some form of intramolecular inhibition may occur. Most serine/threonine kinases, including group I PAKs, have a conserved Asn which stabilizes the catalytic loop by hydrogen bonding with a conserved Asp. A notable exception is the group II PAKs, which have Ser at this position (Ser-445 in PAK4); substitution with Asn results in constitutive activation of PAK4 [86], PAK5 [87] and PAK6 [80]. In summary, the group II PAKs may have common substrates with group I kinases, but their mode of regulation, localization and binding partners are apparently different.

THE MLKs (MIXED-LINEAGE KINASES; MLK1–MLK4)

Background

The naming of this kinase family has an interesting history: of the 11 conserved sequence blocks found in protein kinases, subdomains I–VII of the MLKs resemble serine/threonine kinases, particularly the MEKKs and Raf1, whereas subdomains VIII–XI more closely resemble tyrosine kinases, such as the fibroblast growth factor receptor and Src. Hence MLK ('mixed-lineage kinase') refers to their uncertain parentage [88]. Autophosophorylation of MLK3 was found to occur exclusively on serine and threonine residues [89], and the evidence thus far indicates that all MLKs are *bona fide* serine/threonine kinases. MLKs are considered primarily as kinases that act upstream of MAPK cascades, particularly the JNKs (c-Jun N-terminal kinases). Nonetheless, mammalian MLK3 is localized to the centrosome, and its activity is enhanced during G_2/M phase transition, at which time the JNK pathway remains inactive [90], suggesting a different role for MLK3 that is yet to be uncovered. Overexpressed MLK3 induces profound disruption of cytoplasmic microtubules and a nuclear distortion phenotype, indicating that MLK3 plays a role in cytoskeletal organization [90].

There is intense interest in MLK signalling because of the role of these kinase in neurodegenerative conditions. CEP-1347, a small-molecule derivative of K252a that inhibits MLKs, exhibits neuroprotective properties in many contexts, including primary neurons in culture undergoing neurotrophic-factor-withdrawal-induced apoptosis [91,92]. Both MLK2 and MLK3 are apparently important players in this context [93–95]. Furthermore, animal models of chemical-induced neural toxicity have shown that CEP-1347 is an effective neuroprotectant *in vivo* [96].

One particularly fascinating recent report indicates that silencing MLK3 can block mitogen-stimulated B-Raf activation and prevent serum- or Ki-Ras-stimulated cell proliferation [96a]. All known MLKs when overexpressed in mammalian cell lines act as MAPK kinase kinase kinases to activate JNK pathways. In *D. melanogaster*, the JNK pathway regulates the process of dorsal closure during embryogenesis (for a review, see Harden [97]), in which a cell sheet undergoes morphogenesis requiring Rho GTPases to co-ordinate the internal cytoskeleton. Studies focused on dorsal closure in the fly have identified a pathway that involves the GTPase dRac1, a MAP4K called *misshapen*,

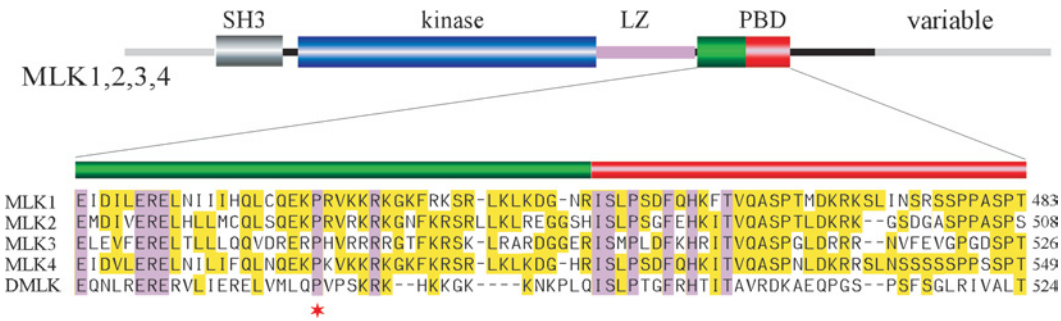

Figure 3 Domain structures of MLKs

Identified domains are illustrated schematically; coloured boxes represent the SH3 domain (grey), the kinase domain (blue), the leucine zipper (pink) and the CRIB domain (PBD; red). A conserved region N-terminal to the CRIB domain is boxed in green. The conserved proline residue (red star) is thought to be important for auto-inhibition by binding to the SH3 domain. Identical residues are shaded pink, and conservative substitutions in yellow. GenBank accession numbers for human MLK1, MLK2, MLK3 and MLK4 and *Drosophila* DMLK are AAG44591, S68178, A53800, NP_115811 and AAL08011 respectively.

the dMLK termed *slipper* (Slpr), the MKK7 (MAPK kinase 7) homologue *hemipterous* (Hep), and the JNK homologue *basket*. Slpr is implicated in JNK-dependent dorsal closure in the fly embryo [98]; the second messenger ceramide is a potential activator of Slpr, which in turn phosphorylates Hep directly [99]. Similarly, mammalian MLK3 is activated by ceramide and tumour necrosis factor α [100].

A *Xenopus laevis* MLK related to mammalian MLK2 (62 % similarity) has been shown to be expressed in a restricted fashion primarily in the cement gland, brain and pronephros [101]. In the differentiating cement gland, xMLK2 expression was correlated with cell elongation, and xMLK2 is required for normal cement gland development and pronephric tubule formation. Thus MLKs may have important morphological roles in the context of vertebrate development.

Rho interaction and primary structural features of MLKs

The MLKs are characterized by an N-terminal SH3 domain, followed sequentially by a kinase domain, a leucine-zipper region and a CRIB motif (Figure 3). These kinases were first identified *in silico* as Cdc42 targets by virtue of their PAK-related domain [13]. MLK3 was shown experimentally to interact with the GTP-bound forms of Cdc42 and Rac1 [102,103], and more recently RhoG [104]. MLK1–MLK4 share 75 % sequence identity within their catalytic domains and 65 % sequence identity within the CRIB motif and flanking autoregulatory SH3 binding domains (Figure 3). The C-termini of these proteins diverge, but all are proline-rich (and thus potential SH3 targets). MLK3 also has a Gly/Pro-rich N-terminus that is absent from MLK1, MLK2 or MLK4. MLKs are absent from yeast, but the *Drosophila* MLK Slipper (Slpr) contains a similar domain arrangement and shows significant sequence similarity.

Regulation of MLK activity

MLK1–MLK4 have N-termini that include an SH3 domain, which in the case of MLK3 has been shown to auto-inhibit its kinase activity [105], such that disruption of the SH3 domain by mutation (Y52A) increases MLK3 activity. The SH3 domain of MLK3 seems to bind intramolecularly to a conserved region between the leucine zipper and the CRIB motif (Figure 3), and mutation of the single proline prevents SH3 binding and increases kinase activity [105]. This auto-inhibition by binding of the SH3 domain to an autoregulatory sequence is similar to that seen for the SH3 domain in the Src family of tyrosine kinases [106]. The crucial proline in the SH3-binding region of MLK3 is conserved (Figure 3, star), probably indicating that members of this subfamily of kinases have a common mechanism of SH3-mediated auto-inhibition.

MLK3, which binds activated forms of both Cdc42 and Rac1 [102,103], is activated when co-expressed with Cdc42, and the GTPase potentiates MLK3-induced JNK activation. This is consistent with Rac and/or Cdc42 being upstream activators of MLK3 in the JNK pathway. The mechanism by which these activate MLKs is not understood, but does not appear to involve a KI domain as for PAK1. Co-expressing activated Cdc42 and MLK3 may promote MLK3 oligomerization [107,108]. Given the close proximity of the CRIB motif and the auto-inhibitory SH3-binding sequence of MLK3 (Figure 3), it would be of interest to establish if loss of SH3 binding [105] affects GTPase-mediated activation.

MLKs contain a leucine zipper (Figure 3) which mediates protein dimerization (or oligomerization), a recurring theme among GTPase-regulated kinases. The zipper-regulated stoichiometries of MLKs are not known, but certainly in the case of MLK3 appear to go beyond dimerization [109]. Deletion of the entire leucine zipper from MLK3 results in a kinase that fails to autophosphorylate and activate the JNK pathway [107], as for disruption of the MLK3 leucine zipper [109]. Interestingly, this mutant MLK3 undergoes Cdc42-mediated autophosphorylation, but fails to efficiently phosphorylate its target MKK4 on Thr-258 [109]. Thus Cdc42 regulation of MLK3 is leucine-zipper-independent, but kinase oligomers are required for proper substrate interaction and phosphorylation.

Among the kinases described here, MLK3 is unique in binding to Hsp90 (heat shock protein 90) and its kinase-specific co-chaperone p50cdc37; inhibition of Hsp90/p50 by geldanamycin can block JNK signalling by MLK3 [110], indicating that these cofactors are critical for MLK3 function. Recruitment of protein kinase clients to Hsp90 involves p50cdc37 acting as a scaffold, and, as for other kinases regulated by this complex, it is the catalytic domain of MLK3 that associates with Hsp90/p50. Therefore MLKs need be considered in the context of their association with chaperones, which might affect the validity of overexpression data.

The motif TTXXS (MLK3 residues 277–281) is found in the activation loop; mutagenesis studies support Thr-277 and Ser-281

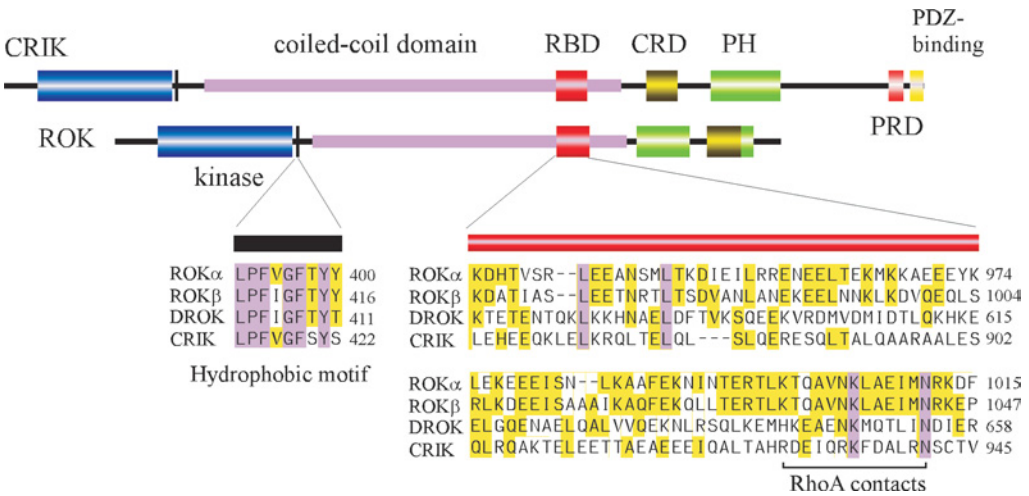

Figure 4 Domain organization and features of ROK and CRIK

The kinase domain (blue) is well conserved among family members: ROK exhibits 50 % and 45 % amino acid sequence identity with DMPK and CRIK respectively. An extensive region flanking the kinase domain forms coiled-coil structures (pink). The sequences of the Rho-binding domain (RBD; shown in red) of ROK and CRIK are weakly similar to each other. Residues involved in contacting RhoA are highlighted by a black line. The C-terminus of ROKs contains a PH domain interspersed with a cysteine-rich region/domain (CRD; dark yellow), whereas in CRIK these domains are separated. A hydrophobic motif at the C-terminal end of the kinase domain is marked in black, with corresponding sequence alignment below. Identical residues are shaded pink, and conservative substitutions in yellow. DROK, *Drosophila* ROK.

as positive regulatory (auto)phosphorylation sites [111]. These two residues are present in all mammalian MLKs. MS has identified 11 *in vivo* phosphorylation sites of MLK3, most of which cluster at the C-terminus [112]. Since a proline residue immediately follows seven of these identified sites, MLK3 is clearly a target of proline-directed kinases (cf. MAPKs and cyclin-dependent kinases). It seems likely that MLK3-activated JNK and p38 pathways feed back to phosphorylate MLK3.

The anti-apoptotic kinase Akt can phosphorylate and negatively regulate MLK3 [113]. An interaction between MLK3 and Akt1/protein kinase Bα occurs via the C-terminal half of MLK3 (residues 511–847), and Akt phosphorylates MLK3 on Ser-674 both *in vitro* and *in vivo*. The expression of activated Akt1 inhibits MLK3-mediated cell death in a manner dependent on this phosphorylation of Ser-674. POSH ('plenty of SH3s') can bind Rac [114] and also MLKs in addition to MKK4/7 and JNK1/2, but whether this modulates MLK activity is not really resolved [115].

THE RHO-ASSOCIATED ROK AND CRIK (CITRON KINASE)

Background

ROK was the first kinase effector of RhoA to be discovered, by groups working in Singapore and Japan [116–120]. ROK (or Rho-kinase) was found to be directly associated with the major activities of RhoA, namely actin stress fibre and adhesion complex formation (ROK does not phosphorylate Rho). Two ROK isoforms have been identified: ROKα [117] (also known as ROCK II [118,119]) and ROKβ (also termed ROCK I/p160ROCK [120]). ROKα/ROCK II was isolated as a Rho-binding protein by an overlay assay using GTP-bound RhoA as a probe for expression screening [116], and using a glutathione S-transferase-bound RhoA affinity column [119]. ROCK I was purified as a RhoA · GTP binding protein using a ligand overlay assay [121].

ROKα and ROKβ mRNAs are expressed in most mammalian tissues [117,118], with the ROKα transcript being most abundant in muscle and brain. In cell culture, ROKα can be immunolocalized with the vimentin intermediate filament network [122] or actin stress fibres, depending on fixation conditions [123]. ROKα is reported to localize at the cleavage furrow in late mitosis [124], while ROKβ can localize to centrosomes [125]. It is likely that ROKs play multiple roles in mitosis [126]; for example, ROKα phosphorylates several sites in the intermediate filament proteins vimentin, GFAP (glial fibrillary acidic protein) and NF-L [neurofilament protein (large subunit)], which allows the local intermediate filament disassembly [127,128] required for separation of daughter cells in late mitosis.

CRIK is a target of activated Rho [129,130] that shares similarities with both ROK and MRCK (myotonin-related Cdc42-binding kinase) (Figures 4 and 5). There are two variants, called citron-N and CRIK, both of which are produced by the same transcription unit, with CRIK including an N-terminal serine/threonine kinase domain. A shorter variant is expressed specifically in the nervous system and localized to the postsynaptic density, where it forms a stable complex with the adaptor protein PSD-95 [131,132]. CRIK clearly plays a role in cytokinesis [129,130], in which a role for RhoA has also been suggested. The involvement of CRIK in cytokinesis is also supported by the phenotype of CRIK-deficient mice, which show severe ataxia and epilepsy, probably as a result of abnormal cytokinesis and massive apoptosis in certain neuronal precursors [133].

Interaction with Rho and primary structural features

ROKs are multi-domain protein serine/threonine kinases of ~160 kDa (Figure 4). The N-terminal kinase domain is preceded by a conserved region that is critical for function. The amino acid sequences of the two ROK isoforms show highest similarity in their kinase domains (92 % identity) [121]. In the ROKs and CRIKs, a PBD overlaps the extensive coiled-coil region (Figure 4). The Rho-binding domain at the C-terminal end of the predicted coiled-coil region also shows some sequence similarity to the Rho-interaction domain of kinectin [134]. The crystal structure of a region of ROKα (residues 947–1015) indicates that the homodimer binds RhoA · GTP as a coiled-coil, as predicted [135],

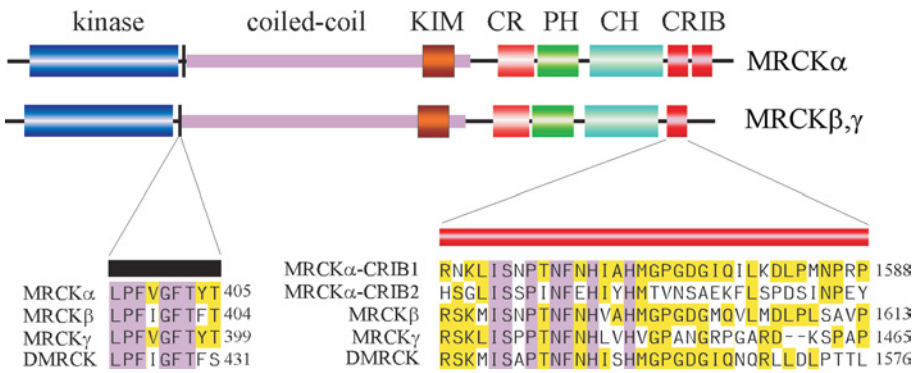

Figure 5 Schematic diagram showing features of MRCK

The kinase domains (blue) of MRCKs are located at the N-terminus and are preceded by a dimerization motif. A centrally located KIM (orange) plays a key role in MRCK autoregulation, but is not proximal to the CRIB. The cysteine-rich domain (CR; light red) is thought to regulate KIM upon binding of diacylglycerol. The lipid binding properties of the PH domain (green) have not been reported. The citron homology domain (CH; light blue) and CRIB (red) are located in the C-terminal region of the kinase. The isoform of MRCKα containing an exon encoding a second tandem CRIB domain [158] is shown. A coiled-coil leucine zipper (pink) is responsible for protein oligomerization. The 'hydrophobic motif' (in black) is thought to stabilize the catalytic domain. The CRIB domains of human and *Drosophila* MRCKs are compared. Identical residues are shaded pink, and conservative substitutions in yellow. The accession numbers are as follows: MRCKα, CAD57745; MRCKβ, AAD37506; MRCKγ, AAT67172; DMRCK (*Drosophila* MRCK), NP_523837.

but employs only ~10/13 residues present at the extreme C-terminal end of the extended parallel coiled-coil region to bind a predominantly hydrophobic patch assembled by the switch regions of RhoA [135]. The specificity of the interaction of ROK with RhoA (compared with Rac1 and Cdc42) appears to involve residues Phe-39, Tyr-66 and Leu-69 of RhoA. The ROK PH domain is unusually interrupted by an internal cysteine-rich domain. CRIK has a similar arrangement of PBD, but contains a flanking cysteine-rich zinc finger (that more closely resembles that in MRCK than in ROK) that might contribute to kinase activation.

Other small GTP-binding proteins may function as negative regulators of ROKs by interacting with other regions. Gem and Rad have been shown to bind to and inhibit ROK function in a region adjacent to the Rho-binding domain [136], and similarly RhoE has been described as a ROKβ inhibitor [137]. More work needs to be done to assess whether these GTPases target ROK under physiological conditions.

Regulation of ROK/CRIK activity

The wider ROK family are considered to be members of the AGC kinases, which include kinases such as protein kinase B, PKC (protein kinase C), p70S6 kinase and PKN (protein kinase novel) [138], whose regulation requires phosphorylation of the 'hydrophobic motif' present outside the catalytic domain and first identified in PKC and p70S6 kinase (as reviewed by Newton [139]). Phosphorylation of this hydrophobic motif (see Figure 4) allows it to form an ordered structure that packs into the hydrophobic pocket of the catalytic domain, which then reconfigures the kinase into an active bi-lobed state. The hydrophobic motif is also important for kinase function, because it is known to provide a docking site for PDK1 (see section describing PKN). In the case of ROK there is no evidence as yet for a PDK1 requirement, but substitution of the hydrophobic-motif threonine (Thr-405 in ROKα) does decrease kinase activity (T. Leung, personal communication), as for MRCK [140]. When expressed in *Escherichia coli*, the catalytic domain of ROKα is active, suggesting that autophosphorylation might be responsible for modification of this site.

Collective findings on ROK, MRCK and DMPK (myotonic dystrophy kinase) suggest that their activation requires coiled-coil-mediated multimerization and involves changes to the auto-inhibitory regions [135,140,141]. Unlike the PKN anti-parallel arrangement, the region involved in RhoA binding is itself formed of parallel coiled-coils from two ROK polypeptides [135,142], so monomeric ROK would be unable to bind RhoA. The kinase activity of ROKs is moderately enhanced after RhoA · GTP binding [119,121] relative to GTPase effects on PAK [5]. The auto-inhibitory domain of ROK was mapped to the C-terminal half of the protein [141], with Rho · GTP binding presumably releasing certain such inhibitory constraints. In common with other Rho-associated kinases, autophosphorylation is essential for their function; various C-terminal constructs or kinase-deficient forms of ROKs function as dominant-inhibitory forms when they are overexpressed in cells [117,121,141]. ROK constructs mutated in their phosphotransferase activity effectively inhibit endogenous ROK function, as assessed by the stability of actin stress fibres [17,143]. Some lipids, such as arachidonic acid, can activate ROK [144], and it could be that ROK activity, which is held in check by the auto-inhibitory C-terminal region [141], can be modulated by other protein players. Auto-inhibition requires the PH/cysteine-rich domains, which can bind to the N-terminal kinase region [123]. Since the N-terminal dimerization domain of ROKα is critical for kinase activity [117], it is envisioned that this is essential for productive kinase domain dimerization and trans-autophosphorylation. In line with these proposals, C-terminal deletion of ROK results in a constitutively activate kinase [117,121]. This occurs *in vivo* during apoptosis, when ROKβ is cleaved and activated by caspases [145].

An important ROK-modulating protein may be Crmp-2 (collapsin response mediator protein-2) [146], a neuronal protein that is involved in the semaphorin-3A-induced collapse of growth cones and in axon outgrowth [147]. Only two neuronal phosphoproteins of 62 and 80 kDa co-immunoprecipitated with ROKα from brain extracts [148]: p80 is a spliced form of Crmp-1 with an extended N-terminus bound to p62 Crmp-2. The p80 Crmp-1 binds the ROKα kinase domain, and inhibits its catalytic activity. In neuroblastoma N1E-115 cells, Crmp-2, regulated in turn by ROK, reverses the usual effects of RhoA and Rac1 on neurite outgrowth and collapse [149].

It is reported that binding of the cyclin-dependent kinase inhibitor p21Cip1/WAF1 to ROK can inhibit its activity [150], which may have physiological relevance, given the potential role of ROK in mitosis [126]. Several important synthetic compounds

inhibit ROK. The most widely used is Y-27632; this compound can also inhibit PKN2 *in vitro* at ~10-fold higher concentrations than that needed for ROK inhibition [151]. The physiological outcome of PKN inhibition is unclear, but such cross-reactivity (and with CRIK [151]) should be borne in mind when assessing experimental data.

MRCK

Background

The MRCKs (Figure 4) are Cdc42/Rac effectors that are related to PAK in the GTPase-binding domain, but to the Rho effectors ROK/CRIK in the kinase domain [130], and therefore act on related substrates [21]. MRCKα was isolated through its interaction with Cdc42, and was later found to contain a functional binding site for PMA (an analogue of diacylglycerol) by virtue of its similarity to the zinc finger domains of PKC and chimaerin [152]. The smaller DMPK is not GTPase-regulated, but has been extensively studied because of its disease link. The human gene for MRCKα is located at chromosome band 1q42.1 and extends over approx. 250–300 kb [153]. The newly described *MRCK*γ gene is far more compact, but its product contains all the features of the α- and β-isoforms [154]. In mammals MRCK expression is ubiquitous, but highest in the brain. In *Drosophila*, mutations of *gek* (a *Drosophila* homologue of MRCK) cause abnormal F-actin accumulation and, as expected, *gek* interacts genetically with *Drosophila* Cdc42 [155].

Both the p130 MBS of PP1δ and the p85 isoform are substrates of MRCK [21]. Both of these are regulated by phosphorylation of a centrally located conserved motif termed the phosphatase inhibitory motif which allows binding and inhibition of PP1δ. How MRCK is coupled to a distinct subset of MBS/myosin II, as suggested by experiments in PC12 cells [156], remains to be seen. MRCKα activity can enhance the ability of Cdc42 to induced filopodia [152], which may relate to the ability of the kinase to activate ERM proteins [157]. Interestingly, MRCK (like Cdc42) is permissive for neurite outgrowth in PC12 cells [156], which is opposite to the behaviour of ROK, but MRCK behaves similarly to activate LIMKs [157]. It should be noted that the function of MRCK in phorbol ester-induced morphological effects is unclear, since dominant-inhibitory constructs of MRCK do not observably alter PMA-dependent membrane ruffling (T. Leung, personal communication). To date, no non-GTPase protein partners for MRCK have been reported.

MRCK–Cdc42 interaction and primary structural features

The combined analyses of genomic and cDNA sequences have allowed characterization of the human *MRCK*α gene '*in silico*' [158]. Uniquely among CRIB-containing effectors, MRCKα can exist as a product with two PBDs (Figure 5), while the other two isoforms are restricted to a single such CRIB motif. MRCKα CRIB1 (encoded by exon 36) and CRIB2 (exon 37) can appear in the same transcript [158]. Interestingly, CRIB1 binds preferentially to Cdc42 · GTP [152], but the addition of CRIB2 increases the interaction of MRCK with Rac · GTP. In addition, MRCKα exons 21–24 represents a region of alternate splicing, with at least 13 isoforms detected [158]. The conserved stretch of about 70 amino acids N-terminal to the kinase domain (termed the leucine-rich domain in DMPK) is essential for kinase activity [156]. The extended central coiled-coil domain drives oligomerization, and within this sequence a KIM (KI motif) has recently been identified [159]. The biochemical roles of the PH and citron homology domains have not been established, but these regions, when expressed in isolation, are also inhibitors of the biological actions of MRCK [156].

Regulation of MRCK activity

Unlike ROK, the regulation of MRCK activity does not appear to involve the PH domain; instead, a small region within the extensive MRCK coiled-coil region, termed the KIM (see Figure 5), acts as a negative autoregulatory domain by complexing to and inhibiting the catalytic domain [159]. Co-expression of the MRCKγ KIM is sufficient to inhibit the activity of either MRCKα or MRCKγ. This current model of activation involves diacylglycerol binding to the zinc finger region, relieving kinase inhibition by KIM, thereby allowing trans-autophosphorylation upon appropriate N-terminal interactions. An active (phosphorylated) MRCK kinase domain also exhibits reduced affinity towards the inhibitory region [140]. Although in co-transfection experiments no enhancement of MRCK activity is seen when Cdc42V12 is introduced with the full-length kinase [140], under physiological conditions it seems likely that Cdc42 might help to target MRCK to its activators.

MRCKα forms oligomers which by gel filtration chromatography correspond primarily to tetramers of ~900 kDa [140]. The coiled-coil domain arrangement indicates parallel intermolecular interactions, as for ROK [135], rather than the anti-parallel structures of PKN. Hetero-oligomers containing wild-type and kinase-inactive MRCK are completely inactive, showing that trans-autophosphorylation is required for activation [140]. Phosphorylation of key residues within the activation loop and the 'hydrophobic motif' provides the means for activation; thus activation-loop residues corresponding to MRCKα Ser-234, Thr-240 and the hydrophobic motif Thr-403 are implicated in kinase phosphorylation and activation [140]. Because relatively little attention has yet been paid to the MRCK family, no information is available at present on the potential for other kinases to regulate MRCK.

PKN

Background

PKN, also known as PRK (PKC-related kinase), is similar to PKCs with regard to the C-terminal kinase domain [160,161], with N-termini containing repeated domains that bind Rho (Figure 6). The different nomenclatures are still in use: here we refer to members of the family as PKN1 (=PKNα or PRK1), PKN2 (PRK2) and PKN3 (PKNβ). Overall, the domain organization most resembles that of yeast PKC-related proteins [162]. Green fluorescent protein-tagged PKN1 translocates in a reversible manner to a vesicular compartment following hyperosmotic stress or addition of the inhibitor HA1077 [163]; this is suggested to be an important site of action in association with RhoB. PKN1 targeting by RhoB to such an endosomal compartment can affect the kinetics of receptor traffic [164,165]. Interestingly, the co-activator PDK1 (which is required for the activity of many AGC kinases) becomes recruited to the same PKN1-positive compartment [163]. In *Drosophila*, Rho1/Pkn plays a distinct role from the MLK/JNK pathway (see relevant section) in regulating dorsal closure [166], indicating that the kinase has morphological roles in development.

Rho interaction and primary structural features of PKN

The domain structure of PKN isoforms is shown schematically in Figure 6. The C-terminal region of PKN contains the Ser/Thr kinase domain, while an N-terminal RhoA-interacting region contains three ACC (anti-parallel coiled-coil) repeats, of which

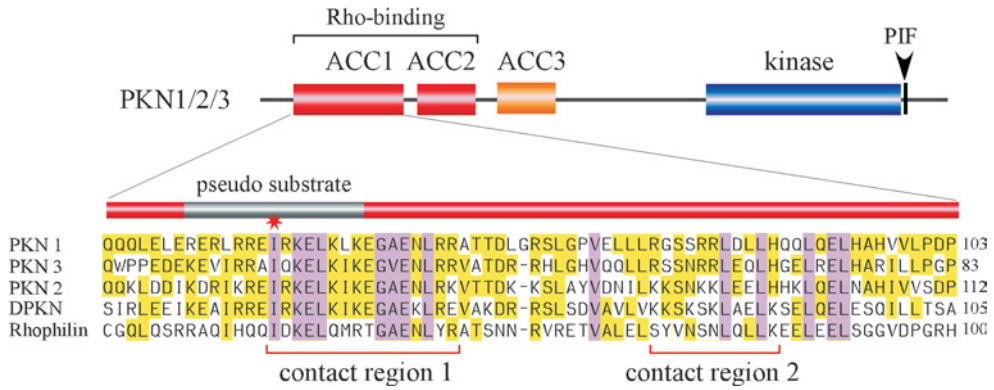

Figure 6 Domain structures of PKN family kinases

PKNs contain three ACC structures at their N-termini and a catalytic domain at the C-terminus (blue). The hydrophobic 'PIF' motif is unusual in not requiring phosphorylation, unlike those in ROK and MRCK. The first two ACCs form the RhoA-binding domain (shown in red) that overlaps a pseudo-substrate region which is indicated in grey, and (based on structural data) two RhoA contact regions marked by red lines. The RhoA-binding domain of PKN family kinases resembles that of a different RhoA effector, Rhophilin, as shown. A critical and conserved isoleucine residue that mimics the phosphoralatable residue is marked by a red star. Identical residues are shaded pink, and conservative substitutions in yellow. The accession numbers for sequences shown in the alignment are BAA05169 (human PKNα/PKN1), BAA85625 (human PKNβ/PKN3), NP_006247 (human PKNγ/PKN2), NP_788291 (*Drosophila* DPKN) and AAL89809 (Rhophilin).

the first two appear to participate directly in Rho binding [17,167,168]. The structure of PKN1 residues 13–98 complexed to RhoA · guanosine 5′-[γ-thio]triphosphate at 2.2 Å resolution reveals a short helix (α1) and two long helices (α2 and α3) in an ACC fold that form a platform for the interaction [169]. In the crystal structure a second mode of packing between RhoA and PKN/ACC1 involves a hydrophobic patch that interacts more closely with the switch II region of RhoA. PKN/PRK1 binds with a similar affinity to RhoA and Rac1, and the binding contacts of the ACC2 domain with Rac1 have been mapped by NMR [170]. Residues adjacent to switch I/II and in helix α5 are required for the interaction [170]. At present it is thought that ACC1 and ACC2 can simultaneously contact RhoA or Rac1 via different and complementary binding modes, but this must await a structure containing both ACC domains.

Mammalian PKNs can interact with RhoA/B/C and perhaps Rac1 (reviewed in [171]). *Drosophila* PKN shows relatively little similarity in the Rho-binding domain (see Figure 6), but binds *Drosophila* Rho1, Rac1 and Rac2, with concomitant increases in catalytic activity [166]. The PKN ACC domains are reported to bind other proteins, such as the anchoring protein CG-NAP (centrosome- and Golgi-localized PKN-associated protein [172]). A stretch of about 130 amino acids proximal to the catalytic domain of PKN (PKN1 residues 455–511) has weak similarity to the C2 domain of PKC, and may be auto-inhibitory and sensitive to a number of lipids, for example arachidonic acid [171].

Regulation of PKN activity

There is strong structural resemblance of the catalytic domains of PKN and PKC, and indeed PKN efficiently phosphorylates peptide substrates RXS/TXR/K, based on the pseudosubstrate sequence of PKC. In common with other GTPase-associated kinases, the interaction of Rho with the Rho-binding domain in ACC1/2 appears to disrupt an auto-inhibitory intramolecular interaction, thereby allowing activation, i.e. an open conformation [173]. The first ACC finger overlaps a putative pseudosubstrate site (Figure 6) corresponding to PKN1 residues 39–53, and the I46S substitution generates a potent substrate for PKN [173]. Thus competitive RhoA binding to the ACC1 finger domain could unmask an active catalytic domain of PKN [173]. The interaction of Rho with PKN1 has been demonstrated to facilitate phosphorylation of the PKN1 activation loop by PDK1 [174,175]. Activation loop phosphorylation (on Thr-774 of PKN1 and Thr-816 of PKN2) is required for activity. Based upon co-transfection experiments, an *in vivo* ternary complex of Rho–PKN1–PDK1 was shown to be required for catalytic activation of PKN1 [175]. PDK1 plays a role in the phosphorylation of equivalent residues on many other AGC kinases, such as PKCs (as reviewed by Newton [139]). Thus current models suggest that Rho binds to PKN and induces a conformational change that is permissive for binding and phosphorylation by PDK1. The recruitment of PDK1 is likely to involve binding to the 'PIF' motif (FXXFDY) [176]. This hydrophobic motif is unusual, since the acidic residue is occupied by a phosphorylatable Ser/Thr residue in most AGC kinases [139] (also see Figures 4 and 5). Whether this 'PIF' region (cf. residues 957–980 of PRK2) is designed to interact with PDK1 in order to modulate the behaviour of other AGC kinases is unclear. It is observed that overexpression of C-terminal fragments of PKN can inhibit the interaction of PDK1 with other AGC kinases.

Surprisingly little is known of interacting proteins that regulate PKNs; however, a proline-rich region between the C2-like region and the catalytic domain can bind various SH3 domain-containing proteins, including GRAF, a GTPase-activating protein for RhoA [177], which is suggested to be a feedback loop to down-regulate RhoA.

CONCLUDING THOUGHTS

It seems likely that new Rho-associated kinases will be uncovered with time. Some kinases such as p70 S6 kinase are known to complex with Rho GTPases [178], but binding site(s) are not well defined. Those that contain conserved CRIB-like motifs have probably already been identified through bioinfomatic searches; however, a significant number of mammalian Rho GTPases are poorly characterized [11] and are likely to interact with different subsets of kinases. The analysis of a growing number of signal transduction pathways controlled by Rho GTPases has yielded new insights into kinase regulation and downstream phosphorylation pathways. The spatially localized activation of Rho GTPases is clearly critical, and may allow the GTPases to select the correct target(s) from the large range of effectors present in the same cell. As such, the recent introduction of tools such as phospho-specific antibodies directed towards regulatory sites on these

kinases allows us to 'see' where and when the kinases function. We can already find evidence of feedback loops, co-operative signalling pathways and scaffold proteins playing roles in compartmentalization. Whether the GTPases themselves are the main determinants of (active) kinase localization remains an important but open question.

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