REVIEW

Cellular Logistics 2:2, 59–68; April/May/June 2012; © 2012 Landes Bioscience

PAK family kinases

Physiological roles and regulation

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Keywords: Cdc42, Rac1, cytoskeleton, development

Abbreviations: ACK1, activated Cdc42 associated kinase 1; AID, autoinhibitory domain; PBD, p21 binding domain; PH, pleckstrin homology; PIX, PAK-interacting exchange factor; EGF, epithermal growth factor; SH3, Src homology domain 3

The p21-activated kinases (PAKs) are a family of Ser/Thr protein kinases that are represented by six genes in humans (PAK 1–6), and are found in all eukaryotes sequenced to date. Genetic and knockdown experiments in frogs, fish and mice indicate group I PAKs are widely expressed, required for multiple tissue development, and particularly important for immune and nervous system function in the adult. The group II PAKs (human PAKs 4–6) are more enigmatic, but their restriction to metazoans and presence at cell-cell junctions suggests these kinases emerged to regulate junctional signaling. Studies of protozoa and fungal PAKs show that they regulate cell shape and polarity through phosphorylation of multiple cytoskeletal proteins, including microtubule binding proteins, myosins and septins. This chapter discusses what we know about the regulation of PAKs and their physiological role in different model organisms, based primarily on gene knockout studies.

Background

PAKs were discovered 20 years ago by serendipity in the search for new GTPase-activating (GAPs) proteins for the small Rho-like G-proteins (p21s) Cdc42 and Rac1.[1] Protein bands of ~65 kDa, when immobilized on nitrocellulose filters, were found to stabilize the GTP-bound state of Rac1. The triplet of bands of apparent 62 kDa, 65 kDa and 68 kDa were seen in brain samples, from which PAK1 (αPAK in rat) was first purified and cloned.[2] Because the activity associated with the purified 68 kDa Ser/Thr kinase was directly stimulated by active Cdc42 and Rac1, this protein was coined a p21-activated kinase (αPAK). By coincidence the kinase had been worked on as a “protease-activated kinase” (PAK) for many years prior in reticulocyte lysate,[3] and the kinase was eventually purified and identified as PAK2.[4] The homology of PAK1 to the budding yeast sterile-20 (Ste20) in both the catalytic and the Cdc42/Rac interaction/binding (CRIB) led to the discovery that the mating pathway in yeast is directly regulated by Cdc42.[5] This region of PAK that binds to Cdc42 and Rac1 is related to the sequence in ACK1, a cytoplasmic Tyr- kinase,[6] and effectors such as PAKs and ACK1 stabilize the active form of Cdc42 and Rac1 from intrinsic hydrolysis of GTP.[2] Mammals contain at least three highly related PAKs, which are termed PAK1, PAK2 and PAK3.[2,7,8] The human PAK4 was cloned in a search for other human PAK-like kinases, and contains an N-terminal CRIB motif, but none of other features seen in human PAK1.[9,10]

Early studies focused on the potential role of group I PAKs (see next section) in regulating actin and microtubule networks downstream of Rac1.[11-13] Although PAK1 was linked to the activities of Cdc42 and Rac1 to induce actin-rich structures in cultured cells termed filopodia and lamellipodia, effector mutants of these GTPases essentially excluded PAK1 as a key driver of these activities.[11,14,15] However, PAK can act upstream of Rac1 by interacting with the Rac-GEF called PAK-interacting exchange factor (PIX), leading to enhanced neurite formation in PC12 cells.[16] Direct binding of group I PAKs to these PIX proteins,[17] generates a large > 1 mDa complex[18] that contains PIX trimer (s) and oligomeric forms of GIT1/2,[19] which are enriched at focal adhesions and at the leading edge of migrating cells.[20] Activation of PAKs leads to auto-phosphorylation of Ser199/Ser204 in the PIX-binding Pro-rich motif (residues 186–203).[21] This negative feedback loop prevents the accumulation of PAK at focal adhesions. PAK1 has been found to associate with the centrosome by binding PIX/GIT1 where it can regulate Aurora-A activity.[22] Again the kinase appears to reversibly associate with this complex through the same negative feedback mechanisms.

The ability of PAK1-like kinases to regulate cytoskeleton dynamics is underscored by the identification of a number of important targets including myosin light-chain kinase (MLCK), cortactin, the LIMK1, stathmin.[23] Unfortunately none of these targets are PAK-specific, and none offer a validated “readout” of PAK activity in vivo. The phosphorylation of Mek1 at Ser298 by group I PAKs is of significant interest to understanding PAK’s role in proliferation. However, although inhibition of PAK1 kinase activity does profoundly reduce MEK1 Ser298 phosphorylation in response to epithermal growth factor (EGF), this inhibition does not prevent MEK1 activation by EGF.[24] Inhibiting group 1 PAKs also reduces the Ser338 phosphorylation of c-Raf in

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Submitted: 03/23/12; Revised: 07/26/12; 08/21/12; Accepted: 08/21/12
http://dx.doi.org/10.4161/cl.21912

response to both PDGF and EGF; however the reduction in Ser338 phosphorylation was not accompanied by a significant decrease in c-Raf activity as expected.[24] It may be that these phosphorylation events are only relevant at low growth factor concentration or in the context of integrin-initiated signaling.

The Classification of PAKs

In addition to the conserved catalytic domain, all PAKs harbor an N-terminal regulatory domain of ~50 residues which contains a CRIB motif responsible for binding Cdc42 and Rac1-like GTPases. The vertebrate PAK1–3 are highly related to each other, while their catalytic domains are very similar to the group II kinases PAK4–6. The PAK kinase domain also resembles Mst kinases, which are essential regulators of the Hippo pathway,[25] though not direct targets of small G-proteins. The PAK1-like family are classified as "group I" PAKs while the PAK4-like kinases are termed "group II" or non-conventional PAKs,[26-28] primarily based on the observation that of the group II PAKs are not activated by Cdc42 binding.[10] Since it turns out that PAK4 is indeed activated by Cdc42, this distinction becomes less relevant. The group II PAKs differ from the group I PAKs in their mode of kinase regulation, intracellular localization and binding partners. These PAKs are found only in metazoans and perhaps evolved along with cadherin-based adherens junctions.

The metazoan PAK1-like (i.e., group I PAKs) all contain binding sites for Nck at the N-terminus and for the PIX SH3 in the region that lies between the AID and the catalytic domain (**Fig. 1**). Model invertebrates also contain a third class of PAK (denoted PAK3 in *C. elegans* and *D. melanogaster*), which resemble the more diverse protist PAKs. Many Drosophila species express compact versions of *D. melanogaster* PAK3, comprising (for example in *Drosophila grimshawi)* only 401 amino acids. The N-terminal regulatory domain includes a basic, CRIB and AID domain (residues 1–90) but no other protein-interaction motifs. The remainder of the protein (residues 92–401) comprises the catalytic domain. This might represent an ideal kinase species for structural determination of a full-length PAK.

Although the protozoan PAKs are suggested to belong to the group 1 PAKs they lack the key KYMS/T (Lys-Tyr-Met-Ser/Thr) inhibitory motif (**Fig. 2**) in the auto-inhibitory domain (AID) which is responsible for displacing the kinase activation loop.[29] The bulk of the AID is involved with contacts to the C-lobe of the kinase; however, this KYMS/T motif displaces the (activation) A-loop by interacting with the α-C helix, whose position is critical for catalytic activity. Because we do not have any structure of a protozoan-inhibited PAK, it is unclear if the auto-inhibition mechanism is identical. Certainly the core AID is well conserved, indicating that protozoan PAKs contain an AID that binds to the kinase C-lobe; however, the role of any KYMS/T like inhibitory motif is unknown. Therefore it might be prudent to regard the very diverse class of protozoan PAKs as a different class of "group III" PAKs.

Group I PAKs: Conserved Motifs and Domain Structure

Group I PAKs are domain-rich proteins with a large number of conserved evolutionarily features in all metazoans (**Fig. 1**). Protozoan PAKs are much more diverse, and can include addition features such as the lipid binding PH domain. Three conserved Pro-rich regions in group I vertebrate kinases (residues 12–18, 40–45 and 186–203 in human PAK1) bind to NCK, Grb2 and PIX, respectively.[17,30,31] The CRIB (Cdc42/Rac1 interactive binding, 75–95 in human PAK1) overlaps the AID (auto inhibitory domain, 83–149). The AID interacts with the kinase

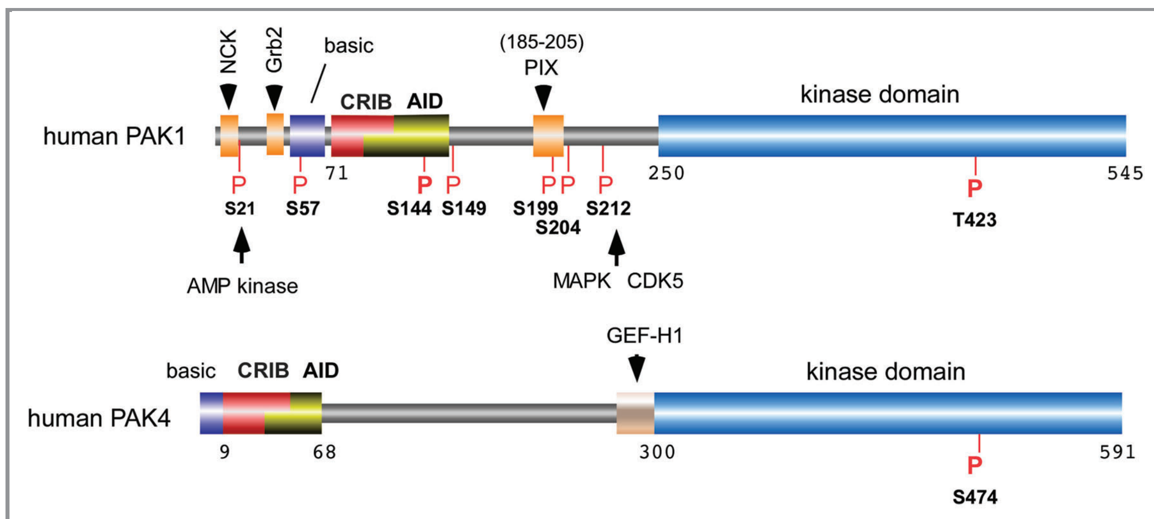

Figure 1. The domain structure of PAK1 and PAK4 highlighting the conserved features of group I kinases and the conserved sites of kinase phosphorylation. The presence of proline-rich SH3 binding sites are marked in orange. The p21-binding domain (PBD or CRIB) is indicated in red and overlaps the auto-inhibitory domain (AID) in yellow. The basic residue cluster required for phospholipid-mediated kinase activation is marked in purple. PAK1 phosphorylation sites that are conserved across other isoforms are marked in red. The activation-loop phopho-residue indicated: this is constitutively phosphorylated in the case of PAK4. Unless otherwise indicated these are auto-phosphorylation sites.

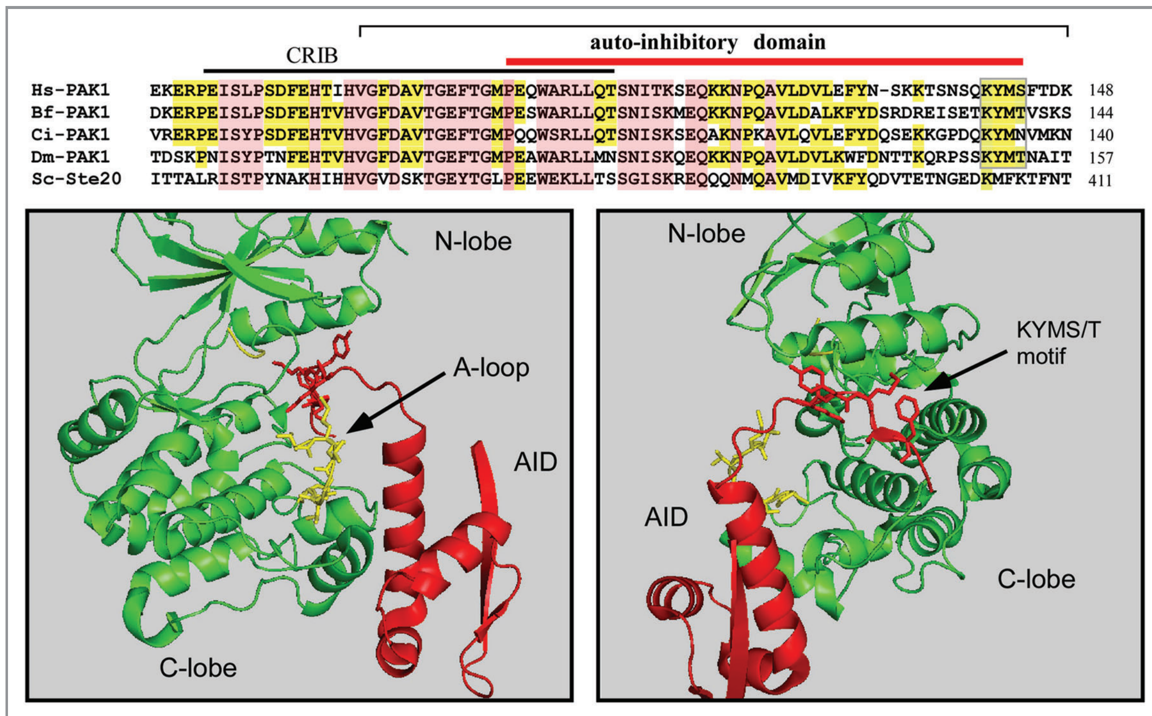

Figure 2. The structure of the PAK1 AID and sequence alignment among Group I PAKs from diverse phyla. PAK1 sequences are from human (Hs, Q13153), *Branchiostoma floridae* (Bf, XP_002595185), *Ciona intestinalis* (Ci, XP_002131099), dPAK1 from *Drosophila melangaster* (Dm, AAC47094) and Ste20p from *Saccharomyces cerevisiae* (Sc, AAA35038). Completely conserved residues are in pink and partial conservation in yellow. The interaction of the KYMS box is illustrated in the figure below, which shows a complex between human PAK1 and the AID (PDB: 1F3M). The A-loop in yellow is displaced by the presence of the KYMS sequence, which occupies a position under the α-C helix. The structure was prepared using Pymol.

domain[29] but when the kinase activated this cryptic helix can then bind fragile-X proteins[32,33] and the bacterial toxin EspG,[34] which maintains the kinase in an active state. The CRIB is preceded by a basic cluster, which allows the kinase to be responsive to acidic phospholipids.[35] The SH3-binding motif, flanked by a poly-acidic region (9–13 acidic residues) binds to the highly conserved SH3 domain found in PIX (PAK-interacting exchange factors) proteins,[17] which are found in metazoans. All PAKs contain a conserved Ser/Thr kinase domain (250–545 in human PAK1) with a single auto-phosphorylation site (T423), which is phosphorylated upon kinase activation. Mutation of certain residues in the AID lead to constitutive PAK1 activation in the absence of Cdc42.[36] However, the phospho-mimetic PAK1 (T423E), which has been used by a number of groups, fails to exhibit auto-phosphorylation characteristic of active kinase when expressed in mammalian cells.[37] This is consistent with the observation that PAK1 (T423E) catalytic domain can be tolerated by *E. coli*, which are growth-sensitive to active forms of PAK1.

Mechanisms of Activation of Group I PAKs

Biochemical and crystallization studies[29,36] revealed that Group I PAKs are regulated via a *trans* auto-inhibition mechanism. PAK1 can be activated by binding to small G-proteins (**Fig. 3**) or sphingolipids, phosphatidyl serine or phosphatidyl inositol phosphates.[38] The requirement for lipids in PAK activation is suggested by the slow kinetics of in vitro activation and the identification of multiple lipids that can activate PAK1 in the absence of Rac1 in vitro.[38] Recent experiments implicate two basic regions upstream of the CRIB in lipid binding.[35] The model proposed in this study is that binding of both PIP2 and Rac1 is needed for cellular PAK1 activation. The details of this cooperative activation require further study, but perhaps kinase recruitment to sites of Rac1.GTP (or other small G-proteins) can explain this behavior. The structure of the Rac3-PAK1 CRIB complex (protein database ID: 2QME) suggests a conformational change in the KID upon binding can drive its dissociation from the kinase domain, and subsequent trans-autophosphorylation at many sites, including the activation loop T423[39-41] and the AID Ser144.[42] A recent asymmetric dimer structure of PAK1 gives some clues as to the mode of trans-autophosphorylation of the activation loop T423 residue[43] which is needed for full kinase activity. Certain proteins such as hPIP (human PAK1-interacting protein 1) and CRIPAK (Cys-rich inhibitor of PAK1) may act to prevent such PAK kinase activation.[44-46] A small molecule inhibitor of PAK1 termed IPA-3 binds to the CRIB region and prevents Cdc42 interaction.[47] The role of adaptor proteins Nck1 and Grb2 in recruiting PAK to phospho-Tyr containing signaling complexes such as the ligand-activated EGF receptor (ErbB1) has not yet been tested in the relevant knockout cells.

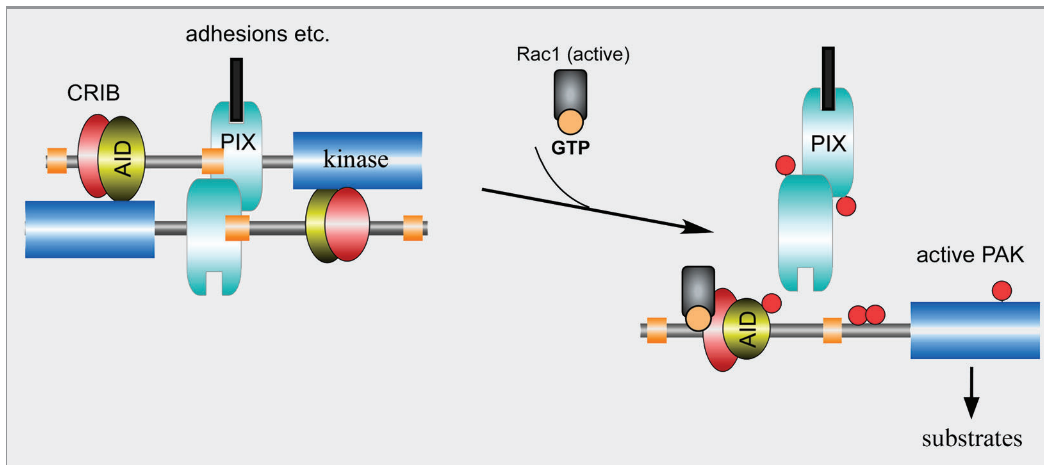

Figure 3. A model for group I PAK activation. The auto-inhibited kinase is inhibited in *trans* as shown. The AID directly contacts the catalytic domain in order to prevent kinase auto-phosphorylation. The PAK is targeted for activation by interaction with PIX, which binds to the central proline-rich sequence. The GTP.Rac1 binds to the CRIB region and a conformational change allows autophosphorylation (red circles). Phosphorylation of Ser144 serves to disable the AID-catalytic domain interaction, while phosphorylation of Ser198/203 reduces the affinity for βPIX (which itself is modified at Ser340). Phosphorylation of the activation-loop Thr423 likely occurs in *trans*.

Regulation of Group II PAKs

Aside from Cdc42 no other factors are known to activate group II PAKs. The structure of the kinase domains of human PAK4–6 resemble typical Ser/Thr kinases of the Ste20 class,[48,49] likely the auto-inhibited structure is quite different from PAK1.[50] Certainly the CRIB of PAK4 functions as an auto-inhibitory region to allow activation by Cdc42.GTP, but the activation loop is constitutively phosphorylated.[50] Generally Group II PAKs do not bind well, if at all, to Rac-family GTPases (i.e., Rac1–3 and RhoG) though the structure of a complex between Rac3.GTP and PAK4 has been solved (protein database ID: 2OV2).

It is notable that PAK4 requires Cdc42 interaction for its localization to cell-cell adherens junctions in epithelial cells[45] and in the Drosophila eye.[51] PAK4 likely operates downstream of Cdc42 in multiple contexts including the Met receptor.[52] Human PAK6 kinase activity is reported to be upregulated by binding of androgen receptor,[53,54] and the basis of this interactions requires further study. Although MKK6 can phosphorylate PAK5 and PAK6[55] it has not been shown that the p38 pathways is involved in kinase activation. If active Cdc42 is the key interactor that drives the association of PAK4 to cell-cell junctions[10,56,57] it would be interesting to find out how this is spatially restricted. PAK4 undergoes nuclear-cytoplasmic cycling and is suggested to promote intracellular translocation and signaling of β-catenin.[58] PAK4 can phosphorylate Ran on Ser135 during mitosis and both these proteins may dynamically associate with components of the microtubule spindle during mitotic progression.[59] A small region upstream of the PAK4 kinase domain has been found to bind substrates such as GEF-H1[60] and PDZ-RhoGEF.[61]

The Diverse PAKs of Protozoa

Saccharomyces cerevisiae has three conventional PAKs: Sterile 20 (Ste20), Cla4 and Skm1. These PAK members contain a typical CRIB/AID motif located N-terminal to the kinase domain (**Fig.** 4), but protozoa express forms of PAKs not found in metazoans that also contain a PH domain (i.e., Cla4p and Skm1p) as reviewed.[62] The Ste20 and Cla4 kinases play overlapping functions,[63] while Skm1 is not an essential gene.[64] Loss of Ste20 results in sterility, while deletion of Cla4 leads to aberrant cytokinesis; deletion of both kinases is lethal.[63] Ste20 was identified as an essential protein in the mating pathway upstream of this mitogen-activated protein kinase (MAPK) cascades.[65] The

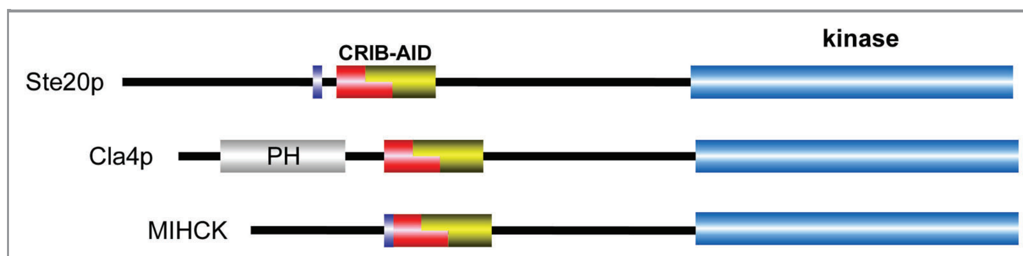

Figure 4. Schematic diagram indicating conserved features of protozoan PAKs. All organisms seem to include Ste20p-like kinases. Both fungi and amoeba have PH containing PAKs resembling Cla4p. None of these kinases have typical protein interaction domains found in metazoan kinases.

mating pheromone receptors Ste2–Ste4 activate heterotrimeric G-protein signaling, which requires co-activation of the small G-protein Cdc42 via Cdc24.[5,66] The Ste20 protein therefore acts upstream of the yeast MAPK pathway consists of Ste11 (MAP3K), Ste7 (MAP2K), Fus3 and Kss1 (MAPKs).[67]

In addition to mating, Ste20 plays an overlapping role with Cla4 in the regulation of filamentous growth and osmotolerance by activating MAP3K.[68] Mating, vegetative growth and filamentous growth all require cell polarization, which is an activity of the Cdc24/Cdc42 module acting through Ste20 and Cla4. Cla4 is involved in septin ring assembly, actin polymerization and mitotic entry and exit. Cla4 kinase activity peaks at mitosis, and in this context acts late in the cell cycle.[69,70] Clap4 participates in a negative feedback loop to end the polarized growth phase and thus loss of Cla4 leads to an exaggerated banana-like bud growth.[63] Interestingly Skm1 is expressed in meiotic cells. In addition to Cdc42, PAK kinases in yeast are responsive to the cell cycle-dependent kinase Cdc28.[71]

Fission yeast such as *Schizosaccharomyces pombe* are genetically divergent from budding yeast strains. *S. pombe* contains two PAK kinases: PAK1 (Shk1 or Orb2) and PAK2, which are effectors of Cdc42,[72,73] The loss of PAK1 leads to defects in actin and the microtubule cytoskeleton, loss of polarity and defects in mating.[74,75] *S. pombe* PAK1 most closely resembles *S. cerevisiae* Ste20 and is an essential gene. In this organism microtubules regulate polarized vegetative growth via a landmark involving the protein Tea1 that is directly phosphorylated by PAK1.[76] Consistent with a role for Tea1 as a critical downstream effector of PAK1 in this context, loss of Tea1 function rescues the PAK1 hyperactivity-induced lethal phenotype caused by loss of a PAK1 inhibitor, Skb15.[76] Remarkably all phenotypes associated with Skb15 loss (i.e., cells with hyper-active PAK1), including defects in actin cytoskeletal organization, chromosome segregation and cytokinesis, are suppressed by Tea1 loss of function.[76] PAK1 is also positively regulated by the SH3 domain-containing proteins Skb1 and Skb5,[77] neither of which have vertebrate counterparts. These stimulate PAK1 kinase activity to regulate cell polarity during hypertonic stress.[78,79] PAK2 is a PH domain containing kinase like Cla4,[73] and participates in the Ras1/Cdc42-dependent regulation of morphology as well as mating responses.[80]

The amoebae *Dictyostelium discoideum* is widely used as a model to study the chemotactic directional cell movement and differentiation. The PAKs in this organism are termed DdPAKa, DdPAKb (also termed myosin I heavy chain kinase, MIHCK) and DdPAKc. The DdPAKc belongs to the Cla4 class of kinases with N-terminal PH domain, thus demonstrating that this class of kinase is not restricted to fungi and probably evolved earlier. The biochemistry of the MIHCK is well understood; the kinase is activated by Rac only in the presence of acidic phospholipids.[81,82] Unlike mammalian PAK1,[38] MIHCK is not activated by sphingosine or other non-negatively charged lipids.[82]

The kinase activity of DdPAKa is regulated by phosphoinositide 3-kinase and Akt, which phosphorylates DdPAKa at Thr-579.[83] DdPAKa-null cells display defects in myosin II assembly which delay significantly the onset of chemotaxis.[84] The DdPAKb (MIHCK) is activated by Rac isoforms and acidic lipids and inhibited by Ca^{2+}-calmodulin, as also reported for Acanthamoeba MIHCK.[85] In response to chemo-attractant (cAMP) stimulation,[86] PAKc kinase activity is rapidly and transiently activated, with activity levels peaking at approximately 10 sec. PAKc preferentially binds the Dictyostelium RacB, which is essential for its function. Loss DdPAKb shows mild chemotaxis defects, but strains lacking both DdPAKb and DdPAKc exhibit severe loss of cell movement, suggesting that PAKc and PAKb cooperate to regulate a common chemotaxis pathway.[86] Remarkably, PTEN loss in Dictyostelium, which causes elevated PIP(3) levels, is rescued (with respect to defects in cytokinesis and chemotaxis) by the removal of PAKa.[87] Thus one is left with the surprising conclusion that in PTEN-null cells, excessive PIP3 levels cause cytoskeletal defects primarily through the hyper-activation of PAKa.

Function of Invertebrate Group I and II PAKs

Fruit flies are one of the best studied invertebrate models. There are three PAKs in *Drosophila melanogaster*: dPAK1, mushroom bodies tiny (Mbt)/dPAK2 and dPAK3. The N-terminal proline-rich domain of dPAK1 is genetically linked to Dock (Nck) since photoreceptor axons under the guidance of extracellular cues require the SH2/SH3 adaptor Dock for correct targeting of these axons into the fly CNS.[88] The guanine nucleotide exchange factor (GEF) Trio activates Rac, which in turn activates PAK1. Mutations in trio result in projection defects similar to those observed in both PAK and dock mutants, and Trio interacts genetically with dRac, dPAK1, and Dock.[89] dPAK1 also plays role in defining the size and shape of the Drosophila embryonic salivary gland lumen by regulating the size and elongation of the cells. PAK1 mediates these effects by decreasing and increasing E-cadherin levels at the adherens junctions and basolateral membrane.[90] During Drosophila oogenesis, basally localized F-actin bundles in the follicle cells covering the egg chamber are needed to drive elongation along the anterior-posterior axis. Mutations in dPAK1 disrupt the follicular epithelium that covers developing egg chambers due to failure in epithelial integrity and apical-basal polarity epithelial integrity and apical-basal polarity. Overall dPAK1 mutant egg chambers exhibit disorganized F-actin and remain spherical due to a failure to elongate. The removal of one copy of the Rho1 locus can suppress the dPAK1 phenotype, which provides strong evidence for an in vivo PAK1-Rho antagonism.[91]

Mushroom body tiny (Mbt) is involved in photoreceptor cell morphogenesis rather than photoreceptor axon guidance.[92] The mushroom body in adult Drosophila corresponds to the hippocampus in the human brain, which is critical for many learning behaviors. Mbt-null mutants have defects in the central brain connectivity and fewer neurons. The Mbt mutant flies also have a reduced number of photoreceptor cells in the eye and disorganized adherens junctions. Similarly, active forms of Mbt disturbs the actin cytoskeleton and affects adherens junction organization.[92] Cdc42 is required for recruitment of the kinase to adherens junctions.[93] Both dPAK1 and Mbt have distinct functions during eye development: a genetic screen identified Twinstar/Cofilin as one target of Mbt signaling.[94] However, this

study failed to link these effects to the cofilin kinase dLimK, suggesting another intermediary kinase.

In both Drosophila and humans, loss of Spastin function results in reduction of synaptic connections and disabling motor defects. It is interesting to note that dPAK3 mutants (which have has mild phenotypes) have potent genetic interaction with spastin mutations. Aberrant bouton morphology, microtubule distribution and synaptic transmission caused by spastin loss of function were all restored by simultaneous dPAK3 loss.[95]

C. elegans expresses three PAK isoforms: CePAK1, CePAK2 and CePAK3/MAX-2.[96,97] PAK-1 is a Group I kinase, PAK-2 belongs to Group II and MAX-2 is related to Drosophila dPAK3 (i.e., a peculiar form not found in vertebrates). CePAK-1 is expressed in the hypodermal cell boundaries during embryonic body elongation and co-localizes with CeRac1 and CeCdc42.[96] Both CePAK1 and MAX-2 function with the Rac GTPases during axon guidance and also function redundantly in P cell migration; this pathway includes the upstream Rac activator UNC-73/Trio and Rac-like CED-10 and MIG-2.[97] PAK1 scaffolding by the PIX and paxillin binding protein GIT1 are exemplified by such studies. The PAK/PIX/GIT complex can serve to allow PAKs to act independently of Rac or Cdc42 GTPases; the migration of the distal tip cells (DTCs) during morphogenesis of the *C. elegans* gonad requires the PAK1/CePIX/CeGIT complex, but does not require the small G-proteins.[98] Only PAK1 functions in the DTC GIT/PIX/PAK pathway, while both PAK1 and MAX-2 are used redundantly for Cdc42/Rac-coupled cell migration pathways.

Of considerable interest is the discovery of a mechanotransduction pathway operating between the body-wall muscles of *C. elegans* and the epidermis that requires PAK1/CePIX/CeGIT. Tension exerted by adjacent muscles or externally exerted mechanical pressure maintains GIT1 at hemidesmosomes (integrin linked desmosomes) and stimulates PAK1 bound to PIX1, in this case requiring Rac.[99] This pathway promotes the maturation of a hemidesmosome into a junction that can resist mechanical stress and contributes to coordinating the morphogenesis of epidermal and muscle tissues. An intermediate filament protein may be the direct kinase target of PAK1 in this context.

The Physiological Roles of Vertebrate Group I PAKs

The close connection between PAK, PIX and GIT as described above, is also seen in vertebrates. Standard blast searches indicate zebrafish have genes that encode six forms of PAKs termed PAK1, PAK2a, PAK2b, PAK4, PAK5(7) and PAK6. A mutant termed redhead (rhd) represents a hypomorphic mutation in PAK2a[100] in which blood leaks from vessels in the head region. Such PAK2a deficiency is the result of an autonomous endothelial cell defect in the vessels supplying the brain. In PAK2a-deficient embryos reducing PAK2b levels results in a more severe and penetrant hemorrhagic phenotype, confirming the functional overlap between the two genes. Zebrafish have three PIX genes (αPIX, βPIX and γPIX)[101] that are ubiquitous expressed and abundant in the nervous system.[102] The zebrafish bubblehead (bbh) mutant exhibits hydrocephalus and severe cranial hemorrhage during early embryogenesis.[102] Hemorrhages are associated with poor cerebral endothelial-mesenchymal contacts and an immature vascular pattern in the head. Thus in the context of the blood-brain barrier PAK2a lies downstream of βPIX and is required for cerebro-vascular stabilization. The ability of βPIX to stabilize vessels requires GIT1 binding,[103] and knockdown of GIT1 also leads to a similar hemorrhage phenotype, and is phenocopied by loss of integrin α(v) or integrin β(8). Thus in fish a PAK/βPIX/GIT complex regulates vascular stability and cerebral angiogenesis in the developing embryo.

The loss of PAK1 in fish gives a late developmental phenotype.[104] The atypical Rho protein Chp (also known as RhoV), has been found to act earlier in development upstream of PAK, during the process of epiboly.[101] The underlying cause is a failure to localize E-cadh and β-catenin at the adherens junctions. Loss of Chp results in delayed epiboly (and embryonic death) that can be rescued by mRNA co-injection, and closely phenocopies zebrafish E-cadh mutants. This pathway involves Chp activation of the group I PAKs, and involvement of the adaptor βPIX. These observations are consistent with studies in mammalian epithelial cells where PAK1 targeting to cell-cell junctions is required for proper contact inhibition.[105]

The number of PAKs expressed in *Xenopus laevis* is unresolved because of the lack of complete genome coverage and the tetraploid nature of this model organism. These are designated xPAK1, xPAK2, etc. in line with human numbering; however, xPAK5 is actually the ortholog of human PAK4. Active forms of xPAK1 promote oocyte arrest in the G_2/prophase of the cell cycle, thereby negatively regulating oocyte maturation. This is similar to the effects of active Cdc42, which likely acts via xPAK2.[106] Interestingly, xPAK2 is inactivated during maturation upon stimulation of the MPF and MAPK pathways however ectopic expression of inactive xPAK1 promotes apoptosis in the Xenopus oocytes[107] indicating in this context xPAK1/2/3 support cell survival. One target of PAK1 in later stages of development is reported to be tumorhead. Phosphorylation of tumorhead enhances its binding to the apical cortex and lateral cell membrane of neural plate epithelial cells, resulting in neural plate expansion and inhibition of neuronal differentiation.[108]

Some indication PAK biological action is indicated by knockout phenotype in mice.[26] Both PAK1-null and PAK3-null mice are viable, healthy and fertile, but the mice lacking the ubiquitous PAK2 are embryonic lethal due to development failures in multiple organs.[109] PAK3 loss of function is associated with certain X-linked nonsyndromic forms of mental retardation.[33] To date, five distinct point mutations have been found: three in the kinase domain (abolishing kinase activity), one in the Cdc42/Rac-binding domain and one in an intron, causing a premature stop.[110,111] Behavioral and cognitive impairments can be associated with abnormal neuron plasticity, which would fit with cytoskeletal functions of PAK1/PAK3 in neurons.[112] Loss of PAK1 alone gives rise to modest defects in long-term potentiation in hippocampal CA1 synapses,[113] similar to the effects reported for loss of PAK3.[109] However, combined PAK1- and PAK3-null mice exhibit pronounced loss of brain volume compared with that of wild-type mice, despite apparently normal brain organization.[114] In these animals the

morphology of neurons was much less complex, with reduced dendrite length and number of dendritic tips, showing that PAK1/PAK3 are involved in branch formation. The biochemical basis for these changes is not clear: of seven substrates tested, only cofilin was found to be significantly altered with respect to phosphorylation, which is a LIM kinase (LIMK) target. It should be noted, however, that in Drosophila (see previous section) LIMK1 is not the genetic link between Twinstar and dPAK.[94]

Forms of human non-syndromic X-linked mental retardation are associated with genetic mutations of βPIX,[115] which is needed for PAK targeting. When expressed in neurons, the AID of PAK1 (which can inhibit PAK1–3) affects synapse morphology and consolidation of long-term memory in mice.[116] Interestingly expression of this AID in the hippocampus of a mouse modeled for fragile-X syndrome (FXS) can rescue some aspects of the syndrome.[33] Greater spine density and elongated spines in the cortex, which are synaptic abnormalities commonly observed in FXS, are partially restored by postnatal expression of the AID in the forebrain. The deficit in cortical long-term potentiation observed in FMR1 KO mice is fully restored by this AID.[33] The connection between PAK1 and fragile-X proteins involves a direct interaction between the residue 122–135 within the AID of PAK1 and the second KH domain of FMR1.[32] PAK1 binds to FMR1 only when this kinase is in an open active state and phosphorylates the fragile-X related protein (FXR1) at Ser420, which appears to be required for its biological function. While FXS arises from a loss of the FMR1 protein (in males inheriting the defective X-chromosome), a rare null mutation of FMR1 (I304N) fails to interact with PAKs,[32] indicating that this interaction could be critical to the function of the fragile-X complex in the normal synaptic spine. Surprisingly the activation of both Rac1 and PAK1 is reported to be impaired at hippocampal synapses in the FMR1 knockout mice.[117] Stimulation-induced activation of synaptic Rac1 which stabilizes F-actin could not be seen in the FXS mutants. Thus a Rac1/PAK activation pathway appears to be "missing" for the rapid stabilization of activity-induced actin filaments in FXS. This observation apparently contradicts the notion that PAK inhibition can ameliorate fragile X defects in the brain.[33]

Mammalian βPIX is critical for the correct localization of PAKs to various cellular compartments and its loss in mice is embryonic lethal (Kerstin Schilling, thesis dissertation 2008). Although βPIX is widely described as a Rac1 and Cdc42 GEF[118] co-expression with PAK1 in mammalian cells does not lead to potent kinase activation, suggesting the PAK/βPIX complex does not locally activate these G-proteins, at least without other (unknown) signals. As described previously PAK1/PIX complex requires GIT1 in order to target to cell adhesions[119] and the centrosome where PAK1 is activated.[22] βPIX via its PDZ binding domain can interact independently of GIT1 with Scribble (Scb) at cell-cell junctions[120,121] and with Shank at post-synaptic densities.[122,123] Although PIX has homology to established Rac GEFs such as Vav, it has essentially no activity in vitro[124] because several contact residues (for Rac1) are missing. Whether the PIX partner GIT1 or others can "activate" the GEF activity is not known. The PIX proteins all have an SH3 domain that binds to a non-canonical binding sequence conserved in group I PAKs,[17] but other partners have been invoked including Cbl.[125]

In summary, vertebrate PAK1–3 loss of function mutants demonstrate phenotypes consistent with multiple roles for these kinases in different cell types. The underlying deficiencies in target(s) phosphorylation are not well understood, and may only be revealed through the use of unbiased large-scale phosphoproteomic analyses. It seems likely that the group I PAKs have non-overlapping functions with group II kinases, the details of which are covered in depth by Audrey Minden.[126]

Acknowledgments

We thank the GSK-IMCB fund for previous long-term support.

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