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P21 activated kinases
Structure, regulation, and functions

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The p21 activated kinases (Paks) are well known effector proteins for the Rho GTPases Cdc42 and Rac. The Paks contain 6 members, which fall into 2 families of proteins. The first family consists of Paks 1, 2, and 3, and the second consists of Paks 4, 5, and 6. While some of the Paks are ubiquitously expressed, others have more restrictive tissue specificity. All of them are found in the nervous system. Studies using cell culture, transgenic mice, and knockout mice, have revealed important roles for the Paks in cytoskeletal organization and in many aspects of cell growth and development. This review discusses the basic structures of the Paks, and their roles in cell growth, development, and in cancer.

Introduction to the Pak Kinases

The p21 activated kinase (PAK) family of serine/threonine kinases are effector proteins for the Rho GTPases Cdc42 and Rac. They bind to Cdc42 and Rac through a GTPase Binding Domain (GBD), also sometimes known as a Cdc42 Rac Interactive Binding (CRIB) domain. The Paks fall into 2 categories, group A and group B, also referred to as group I and group II, based on their sequences and functions (see **Fig. 1**).

Group A includes mammalian Pak1, Pak2, and Pak3.[1-3] Homologs can also be found in other organisms including *C. elegans*, *Xenopus*, and *Drosophila*. In fact, the Paks are considered to be related to the yeast protein Ste20, which is also a serine/threonine kinase with a GBD domain. Each of the group A Paks has an N-terminal regulatory domain and a carboxyl terminal kinase domain. Within the regulatory domain is the GBD, which binds to activated Rac or Cdc42. The group A Paks also have several other conserved motifs, as illustrated in **Figure 1**. Binding to Cdc42 or Rac stimulates the Paks' kinase activities by relieving an intramolecular interaction between the kinase domain and an autoinhibitory domain (AID), as discussed below. Paks 1, 2, and 3 share a high level of sequence homology, but they all have different tissue specific expression patterns. Pak2 is found in all tissues, whereas Paks 1 and 3 have more restricted expression patterns. Pak1 is found in several tissues including mammary gland, muscle, and spleen,[4,5] and all of the group A Paks are highly expressed in the nervous system.[5]

The group B Paks, like the group A Paks, also have N-terminal GBD and carboxyl terminal kinase domains, and they have a sequence that is related to the AID, but they do not contain the other conserved domains found in the group A Paks (see **Fig. 1**). Furthermore, the GBD and kinase domains of the group B Paks have only approximately 50% identity with those of Paks 1, 2, and 3, and the regulatory domains outside of the GBD are completely different from the group A Paks. PAK4 is the founding member of the group B Paks.[6] PAK4 binds most strongly to Cdc42, and less efficiently to Rac. PAK4 and the other group B Paks share some substrates in common with the group A Paks, but also have some of their own unique substrates.[7,8] PAK4 is often considered to be ubiquitous because it can be found in all tissues. In many adult tissues, however, PAK4 levels are low, while in embryogenesis its levels are quite high. Pak5 and Pak6, in contrast, have more of a tissue restricted expression pattern and they are especially high in the adult brain.[9-12] Pak6 is also found in testes and prostate, and it has an important role in androgen receptor signaling,[11,13,14] indicating that in addition to functioning as Rho GTPase targets, the group B Paks also have Rho GTPase independent functions.

Pak Structure and Activation

The group A and group B Paks are regulated by different mechanisms,[15,16] and the current models for the regulation of the 2 groups of Paks are shown in **Figure 2**. The group A Paks are notable for having an autoinhibitory domain (AID), which overlaps the GBD, and plays an important role in the control of Pak activation.[17,18] Group A Paks function as dimers, where the AID binds in trans to the Pak catalytic domain on the dimerizing Pak. This interaction prevents autophosphorylation at the activation loop (A-loop), and subsequent activation of Pak's kinase activity. Activation occurs when Pak binds to Cdc42 or Rac, along with lipids. Cdc42/Rac binding to the Pak GBD disrupts the interaction between the AID and the dimerizing Pak. This in turn leads to a conformational change and causes the Pak to become a monomer, which subsequently becomes autophosphorylated

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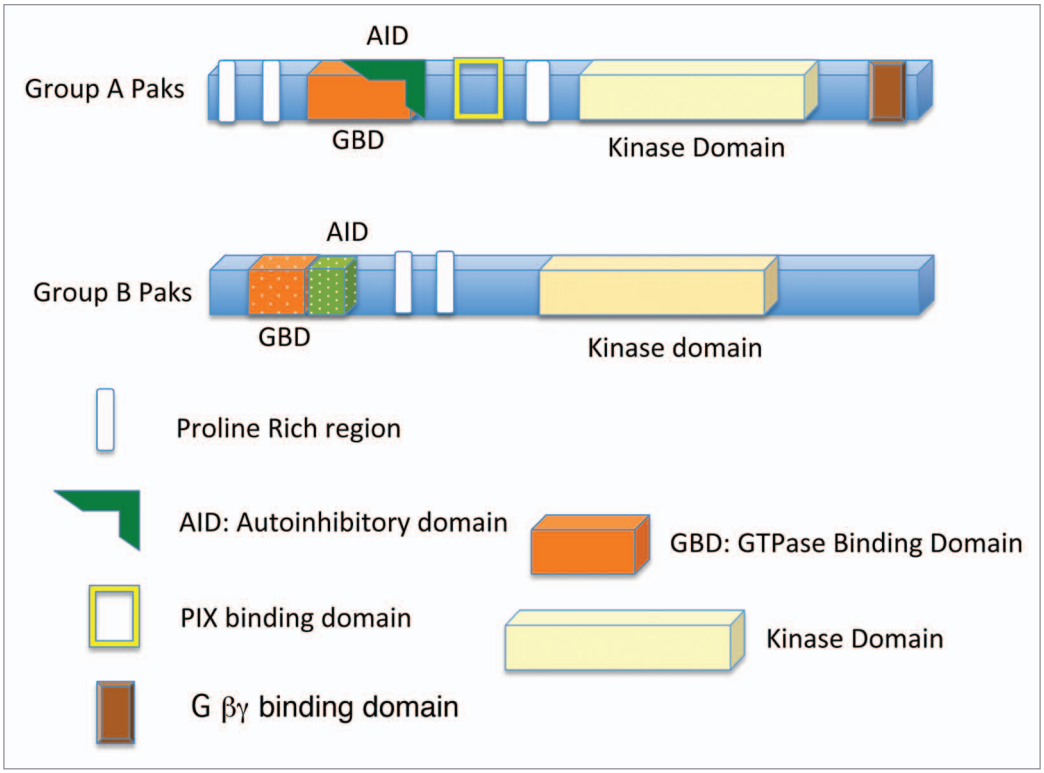

Figure 1. Basic structures of the group A Paks (Paks 1, 2, and 3), and group B Paks (Paks 4, 5, and 6). Group A Paks have an Autoinhibitory Domain (AID) overlapping the GBD (GTPase Binding Domain), while the Group B Paks have a related sequence adjacent to the GBD.

on the A-loop and several other sites, and activated[16,17,19-22] (see **Fig. 2**).

The group B Paks were at first not thought to have autoinhibitory domains, but later work revealed AID-like domains in Paks 4, 5, and 6.[15,23,24] Nevertheless, group B Paks are activated by completely different mechanisms than the group A Paks, and they function as monomers rather than as dimers.[15] In fact, the group B Paks are usually found to be constitutively autophosphorylated at the A-loop, even in quiescent cells.[15] Instead of regulating A-loop autophosphorylation, the group B Pak AID is thought to allosterically modify the constitutively phosphorylated kinase so that it becomes active.[15] Exactly how the AID like domains work in the group B Paks is controversial, but one prevailing model is that the AID binds to the Pak catalytic domain in cis, which keeps the kinase in an inactive conformation, even though it is constitutively phosphorylated. When GTP bound Cdc42 binds the GBD, this interaction is disrupted, leading to a conformational change which results in Pak activation[15] (see **Fig. 2**). Recently, a second model for group B Pak activation has also been proposed for Pak4,[24] though Pak5 and Pak6 could operate by a similar mechanism. The model involves an autoinhibitory pseudosubstrate (PS), which like the AID, is adjacent to the GBD. The PS is a proline rich region, and thus has the potential to interact with proteins that contain SH3 domains. According to the model, the PS is recognized by the Pak kinase domain, leading to an interaction between the 2 domains. This interaction is disrupted when the PS domain binds to proteins that contain SH3 domains, resulting in relief of autoinhibition. In fact, Pak4 was shown to be activated by the Src SH3 domain, thus supporting this model. This model relies on a 2-step activation process. First, Cdc42 or Rac bind to the Pak4 GBD. This would affect the localization of Pak, presumably bringing it to cellular regions containing other activating proteins and substrates. Upon relocalization, the second signal would come into play. This second signal involves binding by SH3 domain containing proteins, which results in relief of autoinhibition and activation of the kinase (see **Fig. 2**). Interestingly, upon a screen for mutations, mutations in the autoinhibitory PS region were found in both Pak5 and Pak6 in human cancer cells, including lung cancer and melanoma,[24] suggesting that disruption of this regulatory mechanism may be associated with cancer.

While the Pak kinases are known for their regulation by Rho GTPases, a number of other signaling proteins can also play key roles in Pak activation. Pak1 can in fact even lie upstream to Rac, by binding to the Pak interacting exchange factor (PIX), which is a guanine nucleotide exchange factor (gef) for Rac.[18,25,26] Binding of Pak1 to Pix causes Pak1 to localize to focal complexes.[25] In PC12 cells, this interaction causes an increase in neurite outgrowth.[26] PIX also interacts with the G protein coupled receptor kinase interacting target (GIT1), and Pak1 is found in a trimeric complex with PIX/GIT1. Both PIX and GIT1 have key roles in targeting Pak1 to focal complexes.[27] GIT1 activates PAK in this complex, and while PIX appears to play an important role in this activation process, activation can occur independently of

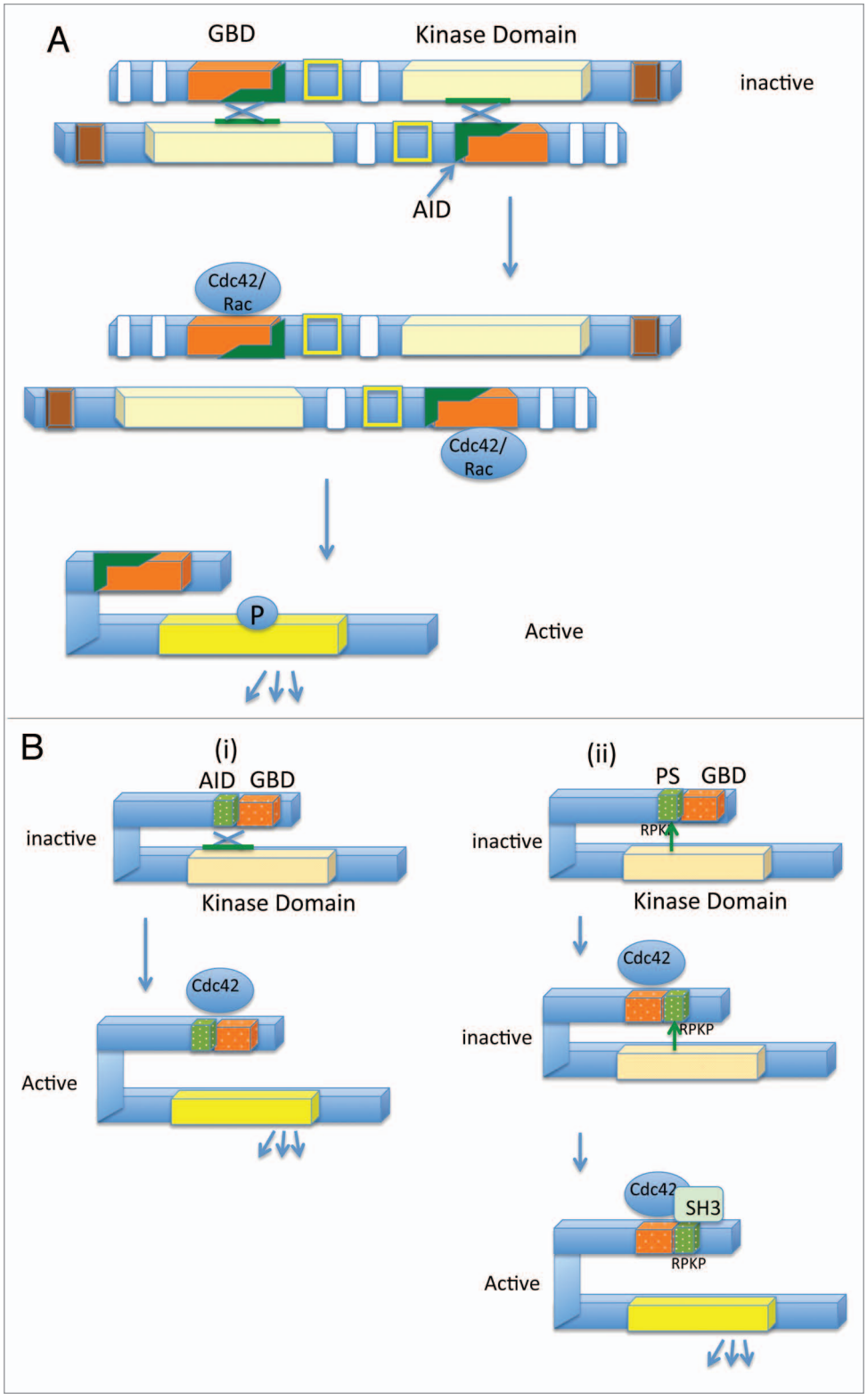

Figure 2. Models representing the activation mechanism of the group A and group B Paks. (**A**) Activation of the group A Paks: Inactivated group A Paks form dimers, where the AID of one Pak binds the kinase domain of the dimerizing Pak, and inactivates it. Binding to Cdc42 or Rac can relieve this inhibition, resulting in autophosphorylation and kinase activation. (**B**) Two different models for regulation of the group B Paks: In the first model (i), the AID binds to the kinase domain of the monomeric Pak, in trans, resulting in an inactive conformation. Binding of Cdc42 relieves the inhibition and leads to Pak activation. Unlike the group A Paks, the group B Paks are constitutively phosphorylated, but the kinase takes on an active conformation upon Cdc42 binding. In the second model (ii), the autoinhibitory pseudosubstrate (PS) containing the sequence RPKP is recognized by the kinase domain. This interaction inhibits Pak kinase activity. Cdc42 binding relocalizes Pak within the cell, and proteins containing SH3 domains, such as Src, subsequently activate Pak by competing with the Pak kinase domain, for interacting with the pseudosubstrate domain (modified from refs. 15, 16, and 24)

Cdc42/Rac binding.[27] In addition to its role in focal complexes, the PAK/PIX/GIT complex also has a role at the centrosome.[28] As cells near M phase, Pak1 is recruited to the centrosomes where it interacts with GIT1/PIX complex. Similar to what is seen at focal complexes, recruiting Pak1 to the centrosome activates Pak, and this is independent of Cdc42 or Rac, but is dependent on centrosome integrity.[28]

Group B Paks can also be regulated by complex mechanisms. In MDCK epithelial cells, PAK4 is activated by Hepatocyte Growth Factor (HGF). HGF activation of PAK4 is dependent on PI3 Kinase. Once cells are stimulated with HGF, PAK4 localizes to the cell periphery. In turn, PAK4 modulates cell adhesion and the organization of the cytoskeleton.[29] PAK4 also binds to the cytoplasmic domain of the keratinocyte growth factor (KGF) receptor in a transformed kidney cell line, and it has important roles in cell survival pathways downstream to KGF.[30] Finally, Pak6 plays a role in androgen receptor signaling,[11,13,14] in a pathway that is independent of the Rho GTPases.

Pak Kinases and Cytoskeletal Organization

The Paks are effectors of Cdc42 and Rac, which are key cytoskeletal regulatory proteins. Likewise, major functions of the Paks include their roles in regulating the cytoskeleton, consequently affecting cell shape, motility, and adhesion. The Paks control the cytoskeleton primarily through the regulation of polymerized actin structures, particularly the formation of filopodia and lamellipodia, but they can also act upon microtubule organization. The major role for the group B Paks in cytoskeletal organization are their roles in the formation of filopodia in response to Cdc42.[6] Activated Pak5 was shown to lead to cytoskeletal changes that are associated with neuronal structure. These include induction of filopodia and the formation of neurite like extensions in neuroblastoma cells.[9] Not surprisingly, many Pak substrates are known for their roles in cytoskeletal organization. Group A Paks, for example, phosphorylate a serine residue on the regulatory myosin light chain (MLC)[31-34] in neural cells. Myosins are important cytoskeletal regulatory proteins that interact with actin. They fall into a family of proteins found in muscle cells, smooth muscle cells, and non-muscle cells. Phosphorylation of MLC by Pak stabilizes polymerized actin, and in neuronal cells it contributes to the regulation of dendritic spine formation.[35] Group A Paks also phosphorylate the regulatory MLC of myosin VI, a nonconventional myosin involved in membrane trafficking and cell migration[36].

In other types of cells Pak can have the opposite effect and lead to decreased MLC phosphorylation, and this is associated with stress fiber dissolution. Pak proteins lead to formation of polymerized actin structures such as lamellipodia and filopodia, but they also lead to the dissolution of stress fibers.[8] Stress fibers are polymerized actin structures that exert tension on the cell and that are directly linked to focal adhesions. Dissolution of stress fibers thus also leads to loss of focal adhesions.[8] In the case of Pak1 and Pak2, this has been shown to be mediated by phosphorylation of Myosin Light Chain Kinase (MLCK) in fibroblasts, epithelial cells, and endothelial cells.[37,38] Phosphorylation of MLCK decreases MLCK's kinase activity, leading to decreased MLC phosphorylation and subsequent stress fiber dissolution.[37] MLCK is not a direct substrate for PAK4, and yet PAK4 also causes stress fiber dissolution.[8] Instead, PAK4 phosphorylates GefH1, which in turn inhibits Rho activation. Since Rho leads to stress fiber formation, its inhibition leads to a decrease in stress fibers.[39] Pak1 also phosphorylates GefH1 although on different sites.[33,40] This phosphorylation affects the crosstalk between actin and microtubule networks[39]

Another substrate for both group A and group B Paks is LIM Kinase 1 (LIMK1).[41] When it is phosphorylated, LIMK phosphorylates the actin depolymerization protein cofilin, thereby inhibiting actin depolymerization.[42,43] The phosphorylation of LIMK1 by Pak kinases therefore represents another mechanism by which the Paks promote the formation or stability of polymerized actin structures such as filopodia and lamellipodia. In addition to direct phosphorylation of LIMK1, PAK4 also activates LIMK1 indirectly, via inhibition of SlingShot Phosphatase (SSH1), a LIM Kinase phosphatase.[44] LIMK1 phosphorylation by PAK4 is also regulated by DGCR6L. DGCR6L binds to PAK4, and this interaction enhances LIMK phosphorylation, leading to increased migration of gastric cells.[45]

Filamin A (FLNa) is another cytoskeletal regulatory protein that is targeted by the Paks.[46] FLNa is an actin binding protein which connects actin filaments to the cell membrane. FLNa is phosphorylated by Pak1, which in turn controls actin stability. Interestingly, FLNa can also bind to the Pak1 GBD and stimulate PAK1 kinase activity, indicating the presence of a positive feedback loop.[33,46] Another mechanism by which Paks control actin-cytoskeletal organization is via its actions on the ARP 2/3 complex. The ARP 2/3 complex controls actin nucleation and branching. Phosphorylation of the p41-ARC subunit of ARP 2/3 by Pak1 stimulates the assembly of the complex at the cellular cortex of migrating cells. This plays an important role in constitutive and growth-factor induced cell motility.[47]

In addition to their affects on the actin cytoskeleton, the Paks can also affect microtubules. One outcome of this is the regulation of mitotic spindles. Pak1 affects mitotic spindle function and organization by interacting with Tubulin Cofactor B (TCoB), a cofactor in the assembly of α/β-tubulin. It phosphorylates TCoB on Ser65 and Ser128 and co-localizes with TCoB on newly polymerized microtubules and centrosomes.[47] Phosphorylation of TCoB by Pak1 is essential for microtubule polymerization. Coordinate dysregulation of Pak1 and TCoB can promote multiple spindle formation, as seen in human breast tumors. Pak1 thus plays an important role in maintaining microtubule dynamics, which is crucial for mitotic spindle function. Another mechanism by which Pak1 regulates mitotic spindle function during mitosis is by phosphorylating centrosome-located Polo-like kinase1 and Aurora-A kinase, 2 important mitotic regulators The Git-Pix complex activates Pak1 by recruiting it to the centrosome. Activated Pak1 then phosphorylates Plk1 and Aurora A kinase. Pak1 phosphorylates Plk1 at Ser49 following which both PlK1 and Aurora A Kinase co-localize on spindle poles, the central spindle, and the

midbody. This is important for establishing a functional bipolar spindle.[48] Activated Pak1 activates Aurora-A by phosphorylating 2 sites, Thr288 and Ser342.[28] Enhanced Aurora-A activity can result in abnormal mitotic spindle organization and anchorage-independent growth in human breast epithelial cells.[49] Paks also play important roles in cell motility by regulating leading edge microtubule dynamics. Microtubules in the protruding edge of migrating cells exhibit decreased catastrophic frequency and increased net growth. Pak1 has been shown to phosphorylate stathmin at Ser16 in vitro in Hep-2 cells. This leads to downregulation of stathmin, which is a protein that normally inhibits tubulin polymerization. Consequently, stathmin phosphorylation enhances cell motility.[50] Xenopus and mammalian PAK4 were shown to phosphorylate the GTPase Ran, and PAK4 mediated phosphorylation of Ran regulates the assembly of Ran-dependent complexes on the mitotic spindle, pointing to a role for this complex in mitosis.[51]

The group A and group B Paks share some substrates in common, but also have some non-overlapping substrates. Several of the group B Pak substrates are linked to cell adhesion. Pak5 binds to p120-catenin in vitro, resulting in p120 phosphorylation. A constitutively active PAK4 mutant also leads to phosphorylation of p120.[52] p120 catenin has important roles in controlling cell shape and adhesion. It also has a role in anchorage independent cell growth,[53] an important hallmark of cancer. PAK4 phosphorylates the cytoplasmic tail of β-5 integrin, which subsequently has important implications in cell adhesion and migration.[54] Phosphorylation of p120 and β-5 integrin by the group A Paks has not yet been reported. Group A Paks are also implicated in cell adhesion, however, and one important mediator is GIT1. GIT1, a Pak1 substrate, is a GAP protein for the Arf GTPase. PAK1 phosphorylates GIT1, and this increases GIT1 binding to paxillin, a focal adhesion adaptor protein. This entire pathway is important for regulating focal adhesion turnover.[28,55]

Biological Roles of the Pak Kinases

The Paks roles in cytoskeletal organization, along with their expression in the nervous system, has led many researchers to study their roles in neurons. Cytoskeletal organization and cell shape changes are important in neuronal cells, especially during development when neurons extend axons and dendrites and connections are made with target cells.[56-58] During growth cone guidance and elongation, for example, filopodia and lamellipodia move toward attractive cues and away from repulsive cues, and neurite extension occurs when these structures are stabilized.[37,41] Because the formation of filopodia and lamellipodia is so important for neurite development, there has been considerable interest in the roles for the Rho GTPases and Paks in neurons. Rho GTPases are expressed in the nervous system, and they have been shown to have important roles in the regulation of neuronal development and neurite outgrowth in several systems,[57-65] as well as in the formation of dendritic spines.[35]

As targets of the Rho GTPases, Paks also have important roles in regulating neuronal development and morphology. The 2 PAK related proteins that have been isolated from *Drosophila* both have roles in neuronal development. *Drosophila* PAK (DPAK) (56), which falls into group A, is expressed at relatively high levels in axons and growth cones of the developing visual system, and it has been shown to be necessary for photoreceptor axon guidance.[66] Mushroom Body Tiny (MBT) is a *Drosophila* Pak that falls into group B.[67] Mutations in the mbt gene result in a decreased number of Kenyon cells in the *Drosophila* mushroom body, a structure of *Drosophila* brain, indicating that it is important for the regulation of neuronal survival or proliferation.[67]

In humans, Pak3 is so far the most clinically relevant Pak with respect to the nervous system. This is because mutations in PAK3 have been linked to nonsyndromatic X-linked mental retardation (MRX).[68] A subset of individuals with this disorder (referred to as (MRX30) have point mutations in Pak3, resulting in a truncated protein. The mutation truncates the kinase domain, but Pak3 is still capable of binding Cdc42 and Rac. A mutated Pak3 can result in aberrant or absent neuronal connections, leading to decreased neuronal connectivity, as seen in MRX30 patients. Furthermore, Pak3 kinase activity is required for activation of JNK1 and p38 MAP kinase pathways, and these pathways play important roles in transcriptional activation in post-synaptic neurons.[69] Other Paks and Pak targets are also important in the nervous system. A defect in the PAK target LIMK1, for example, is linked to the neurological disorder Williams Syndrome, and prevents neurons from making their proper connections.[70] Consistent with this, overexpression of the LIMK target cofilin promotes neurite outgrowth in cell lines.[71] Expression of PAK1 triggers neurite outgrowth in PC12 cells,[61] and an activated PAK5 promotes filopodia formation and neurite outgrowth in N1E-115 neuroblastoma cells.

One interesting function of the Pak proteins is their role in dendritic spine morphology.. Dendritic spines are long, thin, actin-rich protrusions that are the main sites of excitatory synaptic input for most of neurons. Formation and maintenance of synaptic plasticity is mediated by Rac, and this is important for cognitive functions such as learning and memory. Locally regulated activation of Rac by synaptic targeting of the GIT1-PIX complex plays an important role in synapse formation.[28,35] Once activated, Rac binds to Pak1 and Pak3. Pak1 and Pak3 activates the downstream effector MLC by phosphorylating it on Ser19. Active MLC localizes to dendritic spines where it stabilizes the actin network which leads to increased actomyosin contractility, which is essential for formation of dendritic spines and excitatory synapses. Mislocalization of GIT1-PIX complex away from the synapses can cause mislocalized activation of Rac. This can lead to formation of multiple dendritic protrusions, a common spine morphology in patients with mental retardation. Thus, the GIT1-Pak Pix complex plays a key role in spine morphogenesis. Alterations in this complex are associated with decreased neuronal connectivity and cognitive defects such as those seen in nonsyndromic mental retardation.[28,35]

All of the Paks are expressed in the nervous system, and mouse knockouts have given important clues as to their functions. Pak1 knockouts have abnormalities in neuronal function. Specifically,

synaptic plasticity is altered in such a way that the knockout mice are deficient in long-term depression (LTD), indicating that Pak1 is important for synapse function. Dendritic spines of the Pak1 knockout mice are also abnormal, having low levels of polymerized actin compared with those of control mice.

Pak3 knockout mice appear normal,[72] including the brain and nervous system. However late phase LTP (L-LTP) is reduced in Pak3 knockout mice, and this is associated with a decrease in phosphorylation of the transcription factor CREB. CREB is required for L-LTP and memory formation, and interestingly, the knockout mice also have deficits in memory retention. Pak1 knockout mice show many similarities to Pak3 knockout mice. Pak1/Pak3 double knockout mice have learning and memory deficits, and display hyperactive behavior. They also exhibit less complex neuronal morphology including reduced dendrite length and reduction in the amount of dendritic tips, indicating that these Paks are important for branch formation.

PAK4 has been conditionally deleted in the nervous system.[73] These conditional knockout mice are born normally, but display growth retardation and die prematurely. The brains show loss of neuroepithelial adherens junctions, and a dramatic decrease in proliferation of cortical and striatal neuronal progenitor cells. In vitro analyses also revealed deficits in proliferation and self-renewal of neural progenitor cells. The knockout mice only live until approximately 4 wk after birth, and after this point severe hydrocephalus is evident. These studies indicate that PAK4 plays important roles in the development of the brain, especially in the control of neural progenitor cell proliferation.

Unlike PAK4, which is ubiquitously expressed during development, Pak5 and Pak6 have more restrictive tissue specific expression patterns, and are particularly high in the nervous system. Interestingly, Pak5 and Pak6 knockout mice appear phenotypically normal and have normal life spans. When Pak5/Pak6 double knockouts were observed in depth, however, noticeable abnormalities were detectable. Specifically, the double knockouts have lower activity levels compared with the wild-type mice, and they display less aggressive behavior. When learning tests were performed, the mice also displayed significant deficits in both learning and memory.[74] Pak5 and Pak6 levels are high in the cortex, hippocampus, and striatum, and interestingly these are structures with highly important roles in cognitive functions. While the Pak5/Pak6 knockout brains appear phenotypically normal as assessed by histology, differences are seen when neurons are cultured in vitro. Specifically, cultured neurons from the knockouts have abnormally small growth cones and a reduction in neurite outgrowth.

It is interesting that there are such big differences between the phenotypes of the PAK4 knockouts and the Pak5/Pak6 knockout mice. These differences are most likely due to the different expression patterns of these proteins. Specifically, while PAK4 levels are highest in embryogenesis, Pak5 and Pak6 levels are usually higher after birth, particularly in the brain. This can explain why PAK4 appears to function mostly in early neuronal differentiation, while Pak5 and Pak6 have roles later in neuronal development or maintenance.

Developmental Functions of Pak Kinases as Assessed by Studying Knockout Mice

The biological functions of the Pak kinases have been studied in part by developing the knockout mice described above. Both Pak2 and PAK4 are embryonic lethal when they are knocked out, indicating an essential role for these proteins in embryogenesis. The embryos have abnormalities in multiple organs, including the heart and brain.[75,76] Pak1 knockout mice are viable and appear normal. They do, however, have subtle abnormalities in the immune system.[72] Wild-type bone marrow derived mast cells (BMMCs), when antigen sensitized and challenged with IgE, trigger rapid release of histamine containing granules. In contrast, BMMCs derived from Pak1 knockout mice failed to be stimulated with IgE. They were also incompetent in F-actin disassembly following allergen stimulation, resulting in defective mast cell degranulation.[77] The exact mechanism is unknown, but likely involves lowered activation of ERK, JNK and p38 pathways in Pak1 knockout mice, which play important roles in mast cell function. Another abnormality in Pak1 knockout mice is a deficit in glucose homeostasis. This includes inefficient insulin secretion, and abnormal glucose clearance. This is reminiscent of the biological role for Pak1 in humans, where Pak1 levels are reduced in type 2 diabetic islets.[78] Pak1 is thus directly implicated in the control of glucose metabolism.

Both groups of Paks have significant roles in the heart. When Pak1 is conditionally deleted in the heart, the mice appear normal. However, when Pak1 was conditionally deleted in cardiomyocytes and mice were subjected to pressure overload, significant increases were observed in heart weight/tibia length ratio and cross-sectional cardiomyocyte area. This is due to attenuated JNK pathway stimulation in the knockout mice, which is usually induced in wild-type mice when subjected to pressure overload. Thus, Pak1 acts as an anti-hypertrophic agent, revealing a link between Pak1 and ventricular hypertrophy, and indicating that Pak1 knockouts are more susceptible to heart failure when the heart is subjected to pressure overload.[72,79] Deletion of PAK4 leads to embryonic lethality, but among the different abnormalities in the PAK4 knockout embryos, is a dramatic abnormality in heart development.[76] These include thinning of the myocardial walls of the bulbus cordis and the ventricle, and severe dilation and distortion of the sinus venosus region.[76] PAK4's role in cytoskeletal organization is in many ways consistent with a role in cardiac development. Heart muscle cells, like all muscle cells, have a complex and unique cytoskeletal organization. The major cytoskeletal component of the heart cell is the sarcomere. The sarcomere is a complex of actin and myosin and a network of associated proteins, and it is absolutely required for proper heart cell structure and for contractility. Conditional deletion of PAK4 in the heart leads to severely disorganized sarcomeric structures. The abnormalities include a dramatic reduction in the level of F-actin, while the normally repetitive pattern of α-actinin appears less organized. Disruption of sarcomeric structure in the PAK4 knockout cardiomyocytes also affects beating (contractility) of the cells. In PAK4 knockout cardiomyocytes and embryos,

the levels of LIMK1 and phospho-LIMK1 are reduced. Likewise, the levels of phospho-cofilin, the LIMK substrate, are also reduced.[80] These studies indicate that PAK4 has an important role in cardiomyocyte development, with a specific role in the sarcomere. Interestingly, Pak1 is also associated with the sarcomere. In fact, Pak1 has been shown to localize to the sarcomeric Z disc,[81] though a precise role for Pak proteins in sarcomeric structure has not been defined.

PAK4 null embryos have a marked reduction in blood vessels, throughout the embryo and extraembryonic tissue.[82] Although some early vessels form, there is almost a complete lack of branching. Blood vessel development and branching require complex cytoskeletal changes. The role for PAK4 in cytoskeletal organization could help explain its role in blood vessel formation, since formation of vessels requires precise control of the cytoskeleton.

Paks and Cancer

As described above, the Pak kinases and Rho GTPases have important roles in normal development. When they are improperly expressed, however, they are frequently associated with cancer. This is consistent with their roles in controlling cell survival, proliferation, cytoskeletal organization, and migration.[74,83-89] Neither the group A nor the group B Paks are frequently mutated in human cancer. Instead, overexpression and gene amplification of the Pak kinases, however, are commonly seen in cancer. Pak1 and PAK4 are the Paks that are most strongly associated with cancer. Both Pak1 and PAK4 genes are found on chromosomal regions that are frequently amplified in cancer.[90] While gene amplification is one way that Pak proteins become overproduced in cancer, there are also other mechanisms leading to Pak overexpression. Pak1, for example, has been shown to be overexpressed via a mechanism that involves microRNA downregulation.[90,91]

Paks and cell growth control

Among the group B Paks, PAK4 is involved in many cellular activities that are associated with the control of cell growth, which are important for the oncogenic process. For example, expression of PAK4 is associated with increased cell survival and prevention of apoptosis,[74,84] Conversely, cells lacking PAK4 have an increased susceptibility toward apoptosis.[85] This is important, because increased survival is an important part of tumor formation and growth. PAK4 promotes cell survival by different mechanisms in different situations. When serum is removed from cells, PAK4 can protect the cells from apoptosis by phosphorylating the pro-apoptotic protein Bad.[84] When cells are exposed to ligands that bind to death domain receptors, however, such as TNF and Fas receptors, PAK4 protects cells by a different mechanism. In this case, PAK4 inhibits recruitment of caspase-8 to the DISC complex that forms on the cytoplasmic side of the receptor.[74,84] This leads to inhibition of the caspase cascade, and the entire process is independent of the kinase activity of PAK4.[74,84] The mechanisms of PAK4 induced survival in this case involve activation of cell survival pathways, leading to NFKB and ERK activation.[85] Pak5 and Pak1 also protect cells from apoptosis,[92,93] via a pathway involving Raf and Bad. Both Pak1 and Pak5 phosphorylate Ser338 on Raf, stimulating translocation of Raf1 to the mitochondria. Phosphorylated Raf-1 forms a complex with the Bcl-2 proto-oncogene. This complex leads to phosphorylation of the pro-apoptotic protein Bad at Ser112, which prevents its binding to Bcl-2. This prevents the release of pro-apoptotic factors from the mitochondria, thereby inhibiting apoptosis.[92,94] Another mechanism by which Pak1 can prevent cells from undergoing apoptosis is by stimulation of transcription factor NFkappaB, which can promote cell survival, proliferation and angiogenesis,[95,96] and which inhibits the pro-apoptotic factor FKHR.[97] Pak5 overexpression also inhibits camptothecin induced apoptosis in colorectal cancer cells, and this is mediated by inhibition of caspase-8.[93]

Like activated Cdc42,[98-100] activated PAK4 promotes anchorage independent growth in immortalized fibroblasts,[8,39] an important hallmark of oncogenic transformation. In fact, activated PAK4 is as efficient as oncogenic Ras, a very strong oncogene, in promoting foci in soft agar.[8] Consistent with this effect, dominant negative PAK4 partially inhibits focus formation in response to oncogenic Dbl in fibroblasts,[8] and in some cells it also inhibits transformation by oncogenic Ras.[12]

Metastasis, which is one of the most challenging aspects of cancer treatment, is tightly linked with cell migration and invasiveness. There is evidence that PAK4 has a direct role in this process. When activated PAK4 is overexpressed in pancreatic ductal cells, it leads to increased migration and increased invasiveness in in vitro assays. In contrast, blocking PAK4 reduces invasiveness in a pancreatic tumor cell line.[101] PAK4 overexpression was also shown to promote invasion and migration, as well as proliferation, of choriocarcinoma cells. Here again, inhibition of PAK4 has the opposite effect and blocks migration and proliferation.[102] Reducing PAK4 levels in prostate cancer cells also decreases cell migration and leads to reduced cell-adhesion turnover rates, indicating that PAK4 has a role in prostate cancer cell migration and adhesion.

Paks are overexpressed in human cancers

PAK4 is associated with a number of different types of cancer.[12,103-106] Occasionally, point mutations have been found in PAK4, as is the case in some colorectal cancers,[107] but most often overexpression of wild-type PAK4 is sufficient for oncogenesis. The mechanism of PAK4 overexpression in cancer is an important area of investigation. In some cases, PAK4 gene amplification is linked to cancer. The PAK4 gene is located on a chromosomal region (19q13.2) that is often found to be amplified in cancer. For example, the PAK4 gene was shown to be amplified in a series of pancreatic cancer samples, including pancreas ductal adenocarcinomas (PDACs),[101,108,109] and squamous cell carcinomas,[110] The 19q13.2 region is also often highly overexpressed in aggressive breast cancers with basal-like features.[111]

PAK4 mRNA levels were found to be elevated in almost all members of a panel of 60 tumor cell lines, which represent a range of different types of cancers.[12] In contrast, PAK4 levels are low in most normal tissues. PAK4 is overexpressed in a subset of gastric tumors, and overexpression of PAK4 is associated with poor survival rates in patients with these types of tumors.[105] Liver cancer is also linked to high PAK4 expression. In human hepatocellular

cancer carcinomas (HCC),[105] PAK4 is often found to be overexpressed and activated, and this correlates with overexpression of the CDK5 Kinase Associated Protein CDK5RAP3. CDK5RAP3 in turn, is linked with tumor aggressiveness and invasiveness.[112] Further evidence for the role for PAK4 in liver cancer comes from studying microRNAs.[113] The microRNA miR-199a/b-3p is highly expressed in liver, but consistently decreased in HCC. This microRNA, which has an antitumor effect in cells, inhibits PAK4 expression, as well as downstream ERK activation.[113] This evidence strongly links PAK4 to liver cancer.

Ovarian cancer is also frequently associated with high PAK4 levels, as well as an increase in phosphorylated PAK4. High PAK4 levels in these types of tumors are highly linked with metastasis, poor survival, and reduced chemosensitivity.[106] Reduction of PAK4 in ovarian cancer cells reduces cell migration, invasion, and proliferation, and abrogates a number of signaling pathways associated with cell proliferation. It also blocks the ability of the cells to form tumors in mice. In contrast, overexpression of PAK4 in ovarian cancer cells increases cell migration and invasion.[106]

Pak5 overexpression has been seen in some colorectal cancers, and Pak5 plays a role in invasiveness of colorectal cancer cells.[114] Pak6 protein levels are elevated in some prostate and breast cancer cell lines,[115] and Pak6 mRNA levels are also overproduced in some cancer cells.[12] Pak6 levels are elevated in prostate tumors that relapsed after androgen deprivation therapy, and it plays a role in motility and stress responses of tumor cells.[115] Inhibition of Pak6, combined with irradiation, decreases survival of prostate cancer cells.[116] This indicates that Pak6 is linked to radiosensitivity in prostate cancer cells. The role for Pak6 in prostate cancer is complex though, because it was also identified as a gene that is sometimes hypermethylated in prostate cancer, which is more often associated with suppression of tumorigenesis.[117] The exact role for Pak6 in prostate cancer thus remains to be fully clarified.

Pak1 is overexpressed in a number of different types of tumors, including those of the breast, kidney, colon.[47,118] Although in some cases Pak1 kinase activity is high in tumors, it is almost always found in its wild-type form, without known activating mutations. High Pak1 levels were seen in invasive prostate cancer cells, compared with the non-invasive ones. Pak6 levels are also high in prostate cancer cells, but unlike Pak1, it is not correlated with the metastatic potential of the cells. Pak1 was shown to play a predominant role in microinvasion of the cells, and is necessary for prostate tumor growth and micrometastasis. Pak1 stimulates invasiveness of prostate cancer cells via its actions on the cytoskeletal network that enhance the directional migration of these cells. It also stimulates prostate tumor growth through enhanced expression of various tumor-promoting factors such as MMP9 and reduced expression of TGFβ, a factor that inhibits tumor growth.[119]

Pak1 DNA copy number, mRNA and protein expression are upregulated in human melanoma. Dysregulated Pak1 expression, however, had a negative correlation with BRAF mutation. While BRaf mutation is associated with a subset of melanoma, Pak1 upregulation is associated primarily with melanomas that lack the BRAF oncogenic mutation. This is significant, because wild-type BRaf melanoma has no effective targeted therapy. These observations raise the possibility that targeted Pak1 inhibition could serve as a pharmacologically effective treatment for wild-type BRAF melanomas.[120] PAK4 and Pak5 have been implicated in colon cancer cell transformation, but it is Pak1 that is most commonly associated with colon cancer. Pak1 mediates cell proliferation, migration and survival of colon cancer cells by regulating the Wnt, Erk and Akt Pathways.[121]

Pak kinases and breast cancer

Pak1 is frequently overexpressed in breast cancer, and expression levels of endogenous Pak1 correlate with invasiveness and increased survival.[47] PAK4 is also frequently overexpressed in breast cancer.[12,103,104] Like Pak1, PAK4 is often found in its wild-type form in tumors.[101,108]

Transgenic mice have been generated that express a constitutively active Pak1 mutant (Pak1T423E) in the mammary gland. These mice develop mammary tumors, but at low penetrance and with a long latency period, suggesting that other genetic events are required in the transformation process.[122] The breast cancer cell line (MDA-MB-631) forms tumors when injected into the flanks of severe combined immune deficiency (SCID) mice. Dominant negative Pak1 (Pak1K299R) and a Pak1 inhibitor (the Pak1 inhibitory domain: PID) lead to a significant reduction in the sizes of tumors formed by these cells. These results indicate that Pak1 kinase activity is required for tumor formation in this breast cancer cell line.[123] The MCF10A progression cell line series is useful as an in vitro model of breast cancer. This panel of cells consists of MCF10A, neoT, ATI, and DCIS cells, which are all derived from MCF10A cells. MCF10A represent normal breast epithelium,[124] and the other cells are models for increasing levels of oncogenic transformation.[125-127] When grown in 3D culture, the cell lines in the series develop into increasingly disorganized acinar structures. PAK4 levels increase in the more malignant versions of the cells.[128] Likewise, Pak1 expression and phosphorylation increase in the more malignant versions of the cells,[129] Dominant negative Pak1 can partially reverse the abnormal morphologies of the malignant cells, and it also inhibits proliferation and migration of all cells in the series. Overexpression of exogenous wild-type or activated Pak1, however, has no effects on cell proliferation, invasion, or acinar structure.[129] Overexpression of activated Pak1 in MCF7 breast cancer cells, however, leads to abnormal centrosome number and abnormal spindle organization. This leads to aneuploidy, which can result in loss of tumor suppressor genes and accumulation of oncogenes.[47]

Her2/neu/ErbB2 is a growth factor receptor that is frequently overexpressed in breast cancer, and recent evidence points to a role for Pak1 in ErbB2 positive breast cancer.[123] Activation of ErbB2 in MCF10A cells led to a decrease in apoptosis and an increase in proliferation in 3D cultures, and this corresponded with increased Pak1 kinase activity.[123] Expression of a constitutively active mutant of Pak1 (Pak1 L107F) has similar effects, and inhibition of Pak1 kinase activity blocks the effects of ErbB2. Pak1 is activated in breast cancer cells that are estrogen receptor (ER) negative and that overexpress oncogenic Erb-B2.[123] When Pak1 activity is blocked, ErbB2 induced transformation of MCF10A is abrogated. Blocking Pak1 also inhibits the ability of ErbB2 positive breast cancer cell lines to form tumors in mice.

Finally, an activated Pak1 mutant can bypass the requirement for ErbB2 activity in transformation.[123]

The mouse mammary epithelial cell line iMMEC has been used as a model to study the role for PAK4 in breast cancer.[130] iMMECs can be grown in 3D culture conditions where they grow into spherical acini that resemble the acinar structures that form in normal non-cancerous breast epithelia.[131] As is the case in normal mammary epithelium, wild-type iMMECs have nearly undetectable levels of PAK4. When wild-type iMMECs are stably transfected with wild-type PAK4, however, the cells grow into acini that have features usually associated with oncogenesis. The acini become abnormally large, and their lumens never become completely empty. They have a larger layer of epithelial cells surrounding the lumens, and the cells within the structures have increased levels of cell proliferation and decreased levels of apoptosis,[130] Many of these abnormalities are reminiscent of changes that occur during pre-cancerous conditions and early tumorigenesis. Some of the changes, such as the partial filling of the luminal space with cells, are reminiscent of atypical hyperplasia and DCIS.[132] When iMMECs transfected with wild-type PAK4 are implanted into the mammary fat pads of mice, the mice develop mammary tumors at a high frequency,[130] indicating that wild-type PAK4 can be a driving force in oncogenic transformation of mammary cells. Oncogenes such as ErbB2 and oncogenic Ras also cause oncogenic transformation of iMMECs.[131,133] Interestingly, these oncogenes also lead to high levels of PAK4. These studies all point to an important role for PAK4 in the signaling pathways leading to mammary tumorigenesis.[130]

Paks and Neurofibromatosis

Neurofibromatosis types 1 and 2 (NF1 and NF2) are caused by loss of function of the NF1 or NF2 tumor suppressor genes. The disease is associated with tumors that form in the nervous system, particularly in Schwann cells. Interestingly, loss of these genes are associated with improper activation of Pak1, and Pak1 appears to be important for growth of NF tumors.[90,134] The NF1 gene product, neurofibromin, is a GAP protein. Its loss leads to activation of the Ras pathway. Ras normally leads to activation of several different pathways, including the PI3 Kinase pathway. Since PI3 Kinase can also lead to Cdc42 and Rac activation and ultimately to Pak activation, Pak is a good candidate for a target operating downstream to NF1 inactivation. In fact, dominant negative Pak is strong inhibitor of Ras transformation in Schwann cells and peripheral nerve sheet tumor cells from an NF1 patient.[90,134] NF2 functions differently from NF1. The NF2 gene product is Merlin, which is a tumor suppressor that is associated with the cytoskeleton. Merlin is highly expressed in Schwann cells and cells of the nervous system. It has a growth suppressive role and mediates contact dependent growth inhibition. Pak1 phosphorylates Merlin, and this phosphorylation inhibits its growth suppressive activity. Pak2 and Pak6 can also phosphorylate Merlin.[135,136] There is also an inhibitory feedback mechanism from Merlin to Pak. Merlin can bind to Pak and prevent its activation, but once Merlin gets phosphorylated it undergoes a conformational change and disrupts its interaction with Pak1, which then becomes activated. Consequently, inactivation of Merlin corresponds to dysregulated Pak1 activity and cytoskeletal changes associated with cancer. Overall Pak1 is considered an important target for both NF1 and NF2, particularly because of these roles in Schwann cells.[135,136] Similarly, both Pak2 and Pak6 have also been shown to phosphorylate Merlin.[135,136]

Conclusions and Future Directions

The Pak kinases are effector proteins for the Rho GTPases Cdc42 and Rac. They have a wide range of cellular functions, including the control of cytoskeletal organization, cell growth, and cell survival. In animal models they have been shown to have important roles in development, including development of the heart, and the growth and development of the nervous system. The Paks are implicated in human disorders, and as discussed in this review, mutations in of Pak3 are associated with mental retardation. Several Pak family members, especially Pak1 and Pak4, are frequently found to be overexpressed in human cancer, and have been implicated in oncogenic transformation in cells and in mice. Because of this strong link between Pak proteins and cancer, there has been considerable interest in developing Pak inhibitors. Development of Pak inhibitors has challenges, because the different Paks share strong sequence similarity, and because of similarities between the kinase domains of many different protein kinases. Several different inhibitors, however, have been generated.[137] IPA-3 (p21-activated kinase inhibitor 3), for example, is an allosteric inhibitor of Pak1, and is specific for the group A Paks.[137,138] Peptide inhibitors have also been generated, including the PAK1 AID,[139] and TAT-PAK18,[140,141] the latter of which blocks the growth of ovarian cancer cell lines. Pak1 shRNA inhibits cell proliferation,[142] suggesting that shRNA may be another strategy for blocking the Paks. FL172[143] and OS-2[144] are examples of kinase inhibitors with some specificity toward the group A Paks. Pfizer has developed the inhibitor, PF-3758309, which was designed specifically to block the Pak4 kinase activity, but which turned out to be broadly inhibitory toward both group A and group B Paks, and also other kinases, including AMPK (AMP-dependent kinase) and RSK (ribosomal S6 kinase).[145,146] In vitro results for this inhibitor were initially encouraging, because when it was tested on a panel of 92 tumor cell lines, half of them exhibited IC_{50} (inhibitory concentration for 10% maximal effects) values of less than 10 nM. The compound also inhibits tumor growth in mouse xenograft models, and it reduces proliferation and inhibits apoptosis in HCT116 colon tumor cells.[146] Results from clinical trials on human patients, however, have been hampered by a lack of detectable tumor responses, as well as undesirable PK characteristics of the drug, and adverse side effects.[147,148] A second Pak4 inhibitor, LCH-779944 has also been developed.[149] LCH-779944 inhibits Pak4 activity more modestly than PF-3758309, and also has some inhibitory activity toward Paks 1, 5, and 6. EGFR phosphorylation and c-Src phosphorylation were also abrogated in cells treated with the inhibitor. LCH-779944 reduces proliferation and invasion of gastric cancer cells in vitro, as well as filopodia formation and cell elongation, but it has not been tested in tumors in animals or clinically in cancer patients. Genentech has recently generated a highly specific

group B Pak small molecule inhibitor called compound 17,[150] which leads to decreased migration and invasiveness in 2 breast cancer cell lines. These types of inhibitors are promising for the future treatment of disease, but important challenges lie ahead. These include development of the most specific inhibitors possible, and development of a better understanding of the signaling pathways linked to the Pak proteins, so that these pathways can be most effectively blocked or modified for the treatment of human disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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