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Pak protein kinases and their role in cancer

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Abstract

Some of the characteristics of cancer cells are high rates of cell proliferation, cell survival, and the ability to invade surrounding tissue. The cytoskeleton has an essential role in these processes. Dynamic changes in the cytoskeleton are necessary for cell motility and cancer cells are dependent on motility for invasion and metastasis. The signaling pathways behind the reshaping and migrating properties of the cytoskeleton in cancer cells involve a group of Ras-related small GTPases and their effectors, including the p21-activated kinases (Paks). Paks are a family of serine/threonine protein kinases comprised of six isoforms (Pak 1–6), all of which are direct targets of the small GTPases Rac and Cdc42. Besides their role in cytoskeletal dynamics, Paks have recently been shown to regulate various other cellular activities, including cell survival, mitosis, and transcription. Paks are overexpressed and/or hyperactivated in several human tumors and their role in cell transformation makes them attractive therapeutic targets. Pak-targeted therapeutics may efficiently inhibit certain types of tumors and efforts to identify selective Pak-inhibitors are underway.

Keywords

p21-activated kinase; Cell transformation; Cytoskeleton; Rac; Cdc42

1 Pak kinase family

The vast majority of cancers are controlled by oncogenes and their signaling pathways. The dissection of these pathways has led to a wealth of targets for therapeutic intervention and several new drugs that are already on the market to treat tumors. Some of the most successful drugs have been targeted against protein kinases. Protein kinases that have key roles in cell survival, cell proliferation, and cell migration, are important targets because of

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their roles in cancer cell growth and tumor invasion. One group of such kinases, the Pak kinases, show promise as a potential target.

1.1 Pak isoforms and structure

Pak kinases were first identified in screens for Rac and Cdc42 effectors and independently as a proteinase-activated kinase [1, 2]. They are widely conserved and found in yeast as well as Drosophila [3]. In mammals, six isoforms of Pak kinase have been found (Pak1–6), and these are subdivided into two groups (Fig. 1). Group I consists of Pak1 (αPak), Pak2 (γPak), and Pak3 (βPak), while Group II consists of Pak4, Pak5 and Pak6 [4].

All Paks are characterized by an N-terminal regulatory domain and a highly conserved C-terminal kinase domain. The kinase domains of Group I Paks are at least 93% homologous and about 54% homologous to members of the other group. The regulatory domains of all Paks consist of GTPase-binding domain (PBD) and several proline-rich regions that serve as docking sites for SH3 domain containing proteins. Group I Paks additionally possess an autoinhibitory domain (PID) overlapping with the PBD [5]. Group I Paks appear to form homodimers in cells, adopting a trans-inhibited conformation where the N-terminal PID of one molecule binds and inhibits the catalytic domain of the other [6]. Binding of activated Cdc42 or Rac to the PBD disrupts dimerization and activates Group I Paks, by releasing the PID-mediated inhibition and allowing autophosphorylation of the activation loop (Thr423 for Pak1). Phosphorylation of this site prevents refolding and consequent inhibition, even in absence of Rac and Cdc42. An acidic substitution at this site (T423E) renders Pak1 constitutively active. Autophosphorylation of other sites, mostly found in the N-terminus, also contributes to activation [7, 8].

Group II Paks do not possess an identifiable PID and have a higher basal kinase activity than Group I Paks. Although Group II Paks are still able to bind GTP-Rac and GTP-Cdc42, this does not enhance their kinase activities [9, 10]. However, binding of Rac and Cdc42 may regulate localization of Group II Paks and/or their interaction with other proteins.

1.2 Pak activation mechanisms

Signals from several growth factor receptor tyrosine kinases (e.g. insulin, EGF, PDGF, and VEGF receptors) and G protein-coupled receptors lead to activation of Pak [11–18]. These pathways generally activate Pak through sequential activation of PI-3 kinase (PI3K) and a guanine nucleotide exchange factor (GEF) from the Dbl family, which then activates the small GTPases Rac and Cdc42 (Fig. 2).

While activation of Pak via Rac and Cdc42 is well characterized, a number of GTPase-independent mechanisms also modify Pak activity and function. For instance, the cell cycle regulated kinases Cdc2 and Cyclin-dependent kinase 5 (Cdk5) phosphorylate Pak1 at Thr212. Phosphorylation at this site alters the association of Pak1 with binding partners and/or substrates, affecting morphological changes such as postmitotic cell spreading in fibroblasts and microtubule dynamics in neurite outgrowth [19, 20]. PDK1 is able to activate Pak1 by direct phosphorylation of Thr423 [21], and the adaptor proteins Nck and Grb2 bind proline-rich regions near the N-terminus of Pak and can activate Pak by directing it to receptor tyrosine kinases at the cell membrane [22–24]. A family of guanine nucleotide exchange factors, collectively known as PIX or COOL, bind a different proline region on Pak to activate it through both a GTPase-dependent and a GTPase-independent mechanism [25, 26]. Paks can also be activated by sphingolipids [21]. Most of these GTPase-independent mechanisms have only been documented for Group I Paks.

In cancers, Pak activation frequently occurs via mutated Ras. Ras is one of the most commonly mutated oncogenes and activates the MAP kinase pathway as well as PI3 kinase (see Fig. 2) [27].

Several proteins have been identified, which negatively regulate Pak activity. Pak-interacting protein (PIP) abolishes kinase activity by binding to the regulatory domain of Pak, and inhibits Pak-mediated Jun kinase and NFkappaB signaling [28]. Nischarin interacts with the kinase domain of active Pak and inhibits the ability of Paks to phosphorylate substrates [29]. Nischarin may be important for local limitation of Pak activity in migrating cells. Merlin binds Pak1 through its PBD domain and is discussed in detail in Section 3.3 [30]. Partner of PIX1 and 2 (POPX1 and POPX2) are two phosphatases that dephosphorylate the Pak activation loop [31].

1.3 Pak substrate recognition

As for most protein kinases, there is some flexibility in the recognition sequences phosphorylated by Pak. One study used Pak2 and compared a limited number of peptides derived from the substrate KKRKSGL. This yielded a recognition sequence for Pak2 that is characterized by two basic amino acids in the −2 and −3 positions. For example, the peptide (K/R)RXS, in which the −2 position is an arginine, the −3 position is an arginine or a lysine is efficiently phosphorylated at the serine residue (X can be an acidic, basic, or neutral amino acid) [32]. A more recent study used a wider array of peptides and found that Pak1 and Pak2 preferred large hydrophobic residues in positions from +1 to +3, in addition to their preference for basic amino acids at the −2 and −3 positions [33]. While Pak1 and Pak2 shared nearly identical substrate specificities in this study, the substrate specificity of Pak4 was significantly different. Pak4 had a strong preference and for alanine at the +2 and serine at the +3 position. It should be noted that although there are differences in the preferred consensus sequences for Group I and Group II Paks, most known substrates are phosphorylated by both groups. Additionally, both groups strongly prefer serine over threonine as a phospho-acceptor site and do not phosphorylate tyrosine at all. Although the later study was able to identify a new Pak substrate by scanning databases, there are limitations to identifying substrates by sequence searches. The study found that none of the known Pak substrates fell into the top 2% of the predicted substrates, suggesting that other factors such as protein/protein interactions facilitate phosphorylation of what are otherwise less-ideal substrates.

2 Pak functions

Up to date, over 40 proteins have been identified as substrates for Paks (see Table 1), reflecting the significant roles the Pak kinase family plays in a range of biological activities. The most prominent functions are discussed below, and include stimulation of cell proliferation, cell survival, and cell motility. Deregulation of these cellular processes promotes carcinogenesis and Pak signaling can thus play a mechanistic role in cell transformation. Of note, Paks have also been implicated in other cellular processes that are relevant in tumorigenesis, such as angiogenesis [34], epithelial-mesenchymal transition [35], anchorage-independent growth [36, 37], and metabolism [38, 39].

2.1 Cell cycle and aneuploidy

Pak appears to have a critical role during cell cycle progression, its kinase activity peaks at mitosis entry and remains sustained during mitotic progression. Pak1 localizes to specific structures during mitosis, including chromosomes, centrosomes, mitotic spindles, and the contraction ring during cytokinesis [40]. Overexpression of activated Pak1 in MCF-7 breast cancer cells leads to abnormal centrosome number and spindle organization, and

consequently to aneuploidy [37]. The genomic plasticity afforded by aneuploidy facilitates the loss of tumor-suppressor genes and the accumulation of oncogenes.

Mitotic spindle function is inextricably linked with microtubule dynamics. Tubulin cofactor B (TCoB), a cofactor in the assembly of alpha/beta-tubulin, was recently identified as an interacting substrate of Pak1 [41]. Pak1 phosphorylates TCoB on Ser65 and Ser128 and co-localizes with TCoB on newly polymerized microtubules and centrosomes. Coordinate deregulation of TCoB and Pak1 may contribute to the multiple-spindle phenotype seen in human breast cancer cells and other tumors.

In the early phase of mitosis Pak1 may have a role in chromosome condensation. Pak1 co-localizes with Histone H3 on condensing chromosomes and phosphorylates Histone H3 on Ser10, an event that is required for the initiation of chromosome condensation [40, 42]. Interestingly, Histone H3 appears to interact specifically with Pak1 but not Pak2 or Pak3.

The protein kinases Aurora-A and Polo-like kinase 1 (Plk1) are two other important regulators of mitotic events that are phosphorylated by Pak. As cells near the M phase, Pak1 is recruited to the centrosomes where it interacts with a GIT1-PIX complex. GIT1 and PIX are two Pak-binding proteins that also interact with Pak1 during focal adhesion turnover in cells. They are present at the centrosome throughout all phases of the cell cycle. Interaction with GIT1-PIX activates Pak1 independently of the small GTPases Cdc42 or Rac. Activated Pak subsequently activates Aurora-A via phosphorylation on Thr288 and Ser342 [43]. Maroto et al. showed that Pak1 regulates Plk1 activity by phosphorylation of Plk1 at Ser49 [44]. Pak1 and Plk1 co-localize on the spindle poles, the central spindle, and the midbody. Pak1-mediated phosphorylation of Plk1 is important in establishing a functional bipolar spindle.

Regulation of cyclin D1 expression may be another mechanism, by which Pak promotes cell cycle progression. Several studies have shown that constitutively active PAK1 induces transcription of cyclin D1 via activation of the transcription factor NFkappaB [12, 45].

The ability of Pak to regulate the MAP kinase pathway may also contribute to cell proliferation. Pak phosphorylates two mediators of the MAP kinase pathway, MEK1 and Raf1, at Ser298 and at Ser338, respectively [46–50]. While phosphorylation of these sites by Pak is not sufficient to activate Raf1 or MEK1, it significantly facilitates the activation of these kinases by their upstream activators Ras and Raf1, respectively.

2.2 Cell motility and cancer metastasis

Dynamic changes of the cytoskeleton are critical for normal cell motility, neurogenesis, and angiogenesis. During malignant transformation, the signaling pathways controlling these cytoskeletal dynamics are altered, and an increase in cell motility allows cancer cells to invade surrounding tissues and metastasize. Pak functions as a downstream effector of Rac in the regulation of the actin cytoskeleton and stimulates cell motility and invasion.

PDGF, insulin, and certain other cell stimuli cause the redistribution of Pak1 from the cytosol into cortical actin structures, such as lamellae at the leading edge, circular dorsal ruffles, and peripheral dorsal ruffles [13, 51]. In addition, Pak1 localizes to focal adhesions. Expression of a constitutively active form of Pak1 induces the rapid formation of lamellipodia, filopodia, and dorsal ruffles, as well as an in increase in focal adhesion turnover and the disassembly of stress fibers [52–55]. There are several reported substrates of Pak that are involved in cytoskeletal reorganization, including LIM kinase, myosin light chain kinase, merlin, filamin A, p41-ARC, and Op18/stathmin.

Pak1 activates Lim kinase (LIMK) by phosphorylating it on Thr508 in the kinase activation loop. Active LIMK then phosphorylates the actin binding protein cofilin and inhibits its activity. Active cofilin promotes actin filament cycling and retrograde flow, while inhibition of cofilin through Pak1/LIMK signaling promotes integrity of the actin filament network in the lamellipodium and cell protrusion efficiency [56]. Pak1 also phosphorylates the p41-ARC subunit of the ARP2/3, a protein complex controlling actin nucleation and branching. Phosphorylation of p41-ARC by Pak1 stimulates complex assembly at the cellular cortex of migrating cells and is required for both constitutive and growth factor-induced cell motility [57].

Leading edge microtubule dynamics also plays a role in cell motility. In the protruding edge of migrating cells, microtubules exhibit decreased catastrophe frequency and increased net growth. Local regulation of Op18/stathmin, a protein that inhibits tubulin polymerization, could account for this. Rac1/Pak1 signaling appears to negatively regulate Op18/stathmin [58, 59]. Expression of constitutively active Rac1 leads to phosphorylation of stathmin at Ser16, a site which downregulates its inhibitory activity on tubulin polymerization, and Pak1 readily phosphorylates stathmin at Ser16 *in vitro*.

A third component of the cytoskeleton that is involved in cytoskeletal dynamics is myosin. In nonmuscle cells, the activity of myosin II is regulated by phosphorylation of the myosin light chains. Phosphorylation of the light chains promotes the assembly of myosin into bipolar filaments that generate tension on the actin and bundle actin filaments into stress fibers. While protrusion at the leading edge is critical for cell movement, it is equally important to stimulate disassembly and turnover of focal adhesions and stress fibers. Myosin light chain kinase (MLCK), the kinase phosphorylating myosin light chains, is a substrate for Pak [60]. Phosphorylation of MLCK by Pak decreases its activity, which in turn results in decreased myosin light chain phosphorylation and a decrease in actin-myosin filament assembly. Pak's ability to inhibit myosin light chain phosphorylation is likely to account for the disassembly of stress fibers and focal adhesions observed in cells overexpressing activated Pak.

2.3 Cell survival and apoptosis

Apoptosis, or programmed cell death, is a fundamental process in the development of multicellular organisms. Apoptosis enables an organism to eliminate unwanted or defective cells through an organized process of cellular disintegration. Apoptosis is also a prominent tumor-suppression mechanism and cancer cells require inactivation of pro-apoptotic pathways for tumor formation and progression. Pak activity has been shown to downregulate several important pro-apoptotic pathways.

Pak1 protects cells from intrinsic apoptotic signals via a Pak-Raf1-BAD pathway. Pak1, and as well Pak5, induce phosphorylation of Raf1 at Ser338 and stimulate translocation of a subpopulation of Raf1 to the mitochondria [61–63]. At the mitochondria, Raf-1 forms a protective complex with Bcl-2 and phosphorylates the pro-apoptotic protein BAD at Ser112. Bcl-2 is a proto-oncogene that maintains the integrity of the mitochondrial barrier if bound in protective complexes, whereas binding of Bcl-2 to the pro-apoptotic protein BAD induces release of pro-apoptotic factors from the mitochondria and leads to apoptosis. Phosphorylation of BAD at specific sites, including Ser112, renders it unable to bind Bcl-2. The phenotype of Raf-1 knock out cells supports a protective role of Raf-1 in apoptosis, as these cells have high rates of apoptosis while exhibiting normal proliferative rates and ERK activation [64].

Other protective signals transduced through Pak include stimulation of the transcription factor NFkappaB and inhibition of the pro-apoptotic transcription factor FKHR. NFkappaB

has been shown to regulate genes involved in cell survival, proliferation, and angiogenesis. In tumor cells NFkappaB is commonly activated. Several studies have shown that Pak1 can activate NFkappaB but the exact mechanism is still elusive [12, 45, 65, 66]. FKHR promotes the expression of pro-apoptotic genes and is important for the execution of apoptosis. Pak1 appears to directly phosphorylate and inactivate FKHR by regulating its subcellular distribution [67]. Phosphorylated FKHR is maintained in the cytosol and is therefore unable to activate transcription of its target genes.

Pak1 also promotes cell survival by phosphorylating dynein light chain 1 (DLC1) and BimL [68]. BimL is a proapoptotic protein that inhibits Bcl-2 in a similar manner as BAD. Following apoptotic stimuli, DLC1-BimL dimers are released from the dynein motor complex, allowing BimL to interact with Bcl-2 at the mitochondria. Phosphorylation of BimL by Pak1 prevents it from binding and inhibiting Bcl-2.

Pak2 is unique among the Pak isoforms in that it has both pro- and anti-apoptotic functions. During the late events of apoptosis, Pak2 is cleaved by caspase-3, which removes the N-terminal regulatory domain. This generates a constitutively active 34 kDa Pak2 kinase fragment [69–71]. This fragment is subsequently myristoylated and accumulates at plasma membrane ruffles and internal membranes, where it promotes cell death. Interestingly, the Pak2 fragment promotes cell death without compromising mitochondrial integrity. Instead, changes in the cytoskeleton mediated by the Pak2 kinase fragment may trigger cell death due to mechanical stress. Of note, while the 34 kDa Pak2 fragment promotes cell death, the full length activated Pak2 protects cells through mechanisms similar to those seen with Pak1 [72].

2.4 Isoform-specific functions of Pak

Although many identified Pak substrates are phosphorylated to a similar extent by all Pak isoforms, differences in preferred consensus sequences have been found (see Section 1.3. for Pak substrate recognition), and there is evidence that substrate specificities differ under physiological circumstances [73].

Individual Pak isoforms show differences in tissue distribution and subcellular localization, which may in part account for individual substrate specificities. In mice, Pak2 and Pak4 are expressed ubiquitously, whereas Pak1, Pak3, Pak5, and Pak6 have more restricted tissue-specific expression patterns. Pak1, Pak3 and Pak5 are all highly expressed in neuronal tissues [10, 74, 75]. Pak6 is highly expressed in prostate and appears to have a unique role in hormone signaling, as it binds to the androgen receptor and represses androgen receptor-mediated transcription [76]. A substantial portion of Pak5 localizes to the mitochondria, mediating Raf-1 translocation and BAD phosphorylation in survival signaling [63].

Targeted deletions of Pak isoforms in mice have further elucidated biological processes controlled by individual Pak isoforms. Individual Pak1, Pak3, and Pak5 null mice are viable, whereas Pak2 or Pak4 gene deletion results in embryonic lethality [74, 77, 78]. In humans, loss-of-function mutations in the Pak3 gene have been identified, which were associated with X-linked nonsyndromic mental retardation [79, 80]. In accordance with this, mice lacking Pak3 exhibit abnormalities in synaptic plasticity and cognition [74]. Analysis of Pak4-null embryos revealed abnormalities in the heart and nervous system, indicating an essential role for Pak4 in the development of the heart and neural tube, as well as in differentiation and migration of neurons. Mice lacking Pak5 appear not to have any defects and there may be functional redundancy between Pak5 and other Pak kinases. A recent study by Coniglio et al. used siRNAs in breast carcinoma cells to identify functional differences between Pak1 and Pak2. In this study, both Pak1 and Pak2 contributed to promote cell

motility but had distinct roles in the regulation of cofilin, myosin light chain, lamellipodia formation, and focal adhesions.

3 Pak functions in human cancers

Accumulating evidence implicates Pak kinases in oncogenic growth. In cultured cells, ectopic expression of a constitutively active form of Pak induces many phenotypic hallmarks of transformation, such as increased cell motility, anchorage-independent growth, and resistance to apoptosis. Transgenic mice that overexpress a constitutively active Pak1 under a beta-lactoglobulin promoter develop malignant mammary gland tumors, although with a relatively long latency period and low penetrance [81]. Inhibition of Pak on the other hand, efficiently blocks the ability of oncogenic Ras to cause cell transformation [36, 47, 82].

3.1 Pak expression and activation in cancer

Overexpression and/or hyperactivation of Pak family members have been detected in various human tumors. Most frequently, the Pak1 isoform is overexpressed but other Pak family members have also been found overexpressed in specific cancers (Table 2). Pak4, for example, is overexpressed in 75% of the NCI 60 cell line panel and a dominant negative mutant will block anchorage-independent growth of a colon cancer cell line [36].

Several distinct molecular mechanisms have been identified that cause aberrant Pak signaling in cancer, including gene amplification and alteration of upstream regulators. Both Pak1 and Pak4 are localized to genomic regions, which are frequently amplified in cancer cells. The Pak1 gene is localized within the 11q13 region, and 11q13. q14 amplifications involving the Pak1 locus have been recently reported in bladder, ovary, and breast cancer [83–85]. Pak4 localizes to another amplicon, 19q13.2, and Pak4 gene amplification has been found in colorectal and pancreatic cancers [86, 87].

Pak gene amplifications are not frequent enough to account as the only molecular mechanism leading to Pak overexpression/hyperactivation in cancer. A recent report identified a novel mechanism for the overexpression of Pak1 through microRNA downregulation. Reddy et al. found that the levels of endogenous microRNA miR-7 inversely correlated with Pak1 expression in a variety of cancer cell lines [88]. Moreover, transfection of miR-7 downregulated Pak1 expression in breast cancer cells, and suppressed motility and invasiveness of these cells.

There is little evidence for cancer cells having activating mutations in Pak genes. In a colorectal tumor sample a mutation was identified in the Pak4 kinase domain (E329 K) but whether it affected kinase activity was not investigated [86]. There are no reports of Pak involvement in lung cancers; however, a mouse model for Ras-induced lung cancers is highly sensitive to Rac inhibition, suggesting that lung cancers may also be dependent on Pak [89]. Activation of the Ras pathway could be a general mechanism of Pak activation in cancers.

There are two types of cancers, Neurofibromatosis and breast cancers, in which the biological consequences of increased Pak signaling have been explored extensively. These are discussed below.

3.2 Pak in breast cancer

In breast cancer, deregulation of Pak1 is well documented and correlates with increased invasiveness and survival of these cancer cells. More than 50% of human breast cancers display overexpression and/or hyperactivation of Pak1 [45]. The molecular mechanisms by

which Pak1 promotes mammary epithelial cell transformation have been extensively studied in 3-dimensional culture model systems.

A recent study examined Pak1 activity in a pre-malignant progression series of MCF10A mammary epithelial cell variants. Pak1 expression levels increased in correlation with the progression stages in this series, indicating a role for Pak1 in the early stages of cell transformation [90]. Activation of the transcription factor NFkappaB appears to be a prominent mechanism by which Pak1 regulates survival of breast cancer cells. Friedland et al. showed a functional link between the resistance of mammary epithelial cells to apoptosis in 3-dimensional cultures and Pak1-mediated activation of NFkappaB [66]. Notably, NFkappaB also promotes cell proliferation via cyclin D1 transcription in breast cancer cells [45]. Phosphoylation of the pro-apoptotic proteins BAD and FKHR, and phosphorylation of DLC1 are other mechanisms by which Pak1 may promote breast cancer cell survival.

Pak substrates that control different aspects of cytoskeletal dynamics, such as LIM kinase, p41-ARC, filamin A, Op18/stathmin, and TCoB, are likely to promote the invasiveness of breast cancer cells (see chapter 2 for discussion of Pak substrates). In addition, a recent study showed that the multimodular protein Scrib positively regulates activation of Pak1 and participates in lamellipodia formation at the leading edge of migratory breast cancer cells [91].

Holm et al. showed a mechanistic link between increased nuclear levels of active Pak1 and tamoxifen resistance in breast cancer [92]. Approximately 70% of all breast cancers express estrogen receptor, and tamoxifen is a selective antiestrogen, which is widely used for treatment of this group of breast cancers. Pak1 is one of many kinases that phosphorylate estrogen receptor alpha (ERalpha). Deregulated activation of Pak1 produces multiple or inappropriate phosphorylation of ERalpha, creating a promiscuous receptor that is resistant to tamoxifen treatment and activates growth mechanisms in absence of estrogen. The link between Pak1 and ERalpha raises the possibility that tamoxifen resistance might be prevented or reversed by Pak1 inhibition.

3.3 Neurofibromatosis

Neurofibromatosis types 1 and 2 (NF1 and NF2) are dominantly inherited autosomal diseases caused by the loss-of-function mutations of the tumor suppressor genes NF1 and NF2, respectively. NF1 is a common disease, having a birth incidence of about 1 in 3,000, while NF2 is a relatively rare disorder with an incidence of about 1 in 25,000. Neurofibromatosis patients are predisposed to the development of multiple tumors of the central and peripheral nervous system. Schwann cells, the cells that comprise the myelin sheath around nerves, are predominantly affected in both tumors. Patients carry heterozygous mutations in either the NF1 or NF2 gene but their tumors typically display loss of the residual wild-type allele, conforming to the classic two hit Knudsen paradigm seen with most tumor suppressors. Although NF1 and NF2 are genetically and clinically distinct diseases, loss of each gene product leads to abnormal activation of Pak1, albeit through different mechanisms. Experimental results suggest that Pak1 is important for the malignant growth in both types of neurofibromatosis.

The mechanism of Pak1 activation through NF1 proceeds through the Ras pathway. The product of the NF1 gene is a cytoplasmic protein called Neurofibromin. It is widely expressed across a range of tissues but with high concentrations in the nervous system. Neurofibromin is a GTPase activating protein (GAP) and acts by accelerating the intrinsic GTPase activity of Ras. Consequently, loss of Neurofibromin is associated with increased levels of activated GTP-bound Ras, which activates oncogenic pathways, including the MAP kinase cascade and PI3 kinase. Downstream signals of PI3 kinase activate Pak via Rac

and Cdc42. Dominant negative Pak mutants were shown to be potent inhibitors of Ras transformation in both rat Schwann cells and a malignant peripheral nerve sheath tumor (MPNST or neurofibrosarcoma) cell line from an NF1 patient [93].

While NF1 activates Pak through effector pathways, NF2 interacts directly with Pak1. The NF2 gene product is a cytoskeleton-associated tumor suppressor named Merlin (also called Schwannomin). Merlin is structurally related to the moesin/ezrin/radixin proteins, which link the actin cytoskeleton to cell surface glycoproteins that control growth and cellular remodeling. Merlin is widely expressed in Schwann cells, meningeal cells, peripheral nerves, and the lens. In non-neoplastic cells, merlin mediates contact-dependent growth inhibition. The growth suppressive function of Merlin depends on its phosphorylation status at Ser518 [94]. Under growth restrictive conditions, Merlin is unphosphorylated and inhibits cell proliferation, while under growth permissive conditions, Merlin is phosphorylated. Both cAMP-dependent protein kinase A (PKA) and Pak1 are able to phosphorylate Merlin at Ser518 and thereby inhibit its growth suppressive activity [95–97]. Phosphorylation of Merlin at Ser518 was also demonstrated by Pak2 and Pak6, however they may not be highly expressed in Schwann cells and therefore are of uncertain relevance to NF2 [96].

While Pak phosphorylates and inhibits Merlin, there is also an important inhibitory feedback mechanism from Merlin to Pak. Merlin associates with inactive Pak and prevents its activation, perhaps by competing with Rac [30, 98]. Phosphorylation at Ser518 induces a conformation change in Merlin and consequently disrupts interaction with Pak1, allowing Pak1 to be activated. Thus, in NF2 patients, loss of Merlin is associated with abnormal Pak1 activity, which also leads to elevated levels of Rac as well as pronounced cell ruffling [99, 100]. In cell culture experiments, the Pak1 inhibitors CEP-1347 and WR-PAK18 were able to inhibit the growth of merlin-deficient tumor cells, but not merlin-positive cells [98].

Together, these studies suggest that Pak1 is a major driver underlying Schwann cell transformation and an attractive target for therapeutics.

4 Pak as drug target

Protein kinases currently constitute a major focus for drug discovery with most major pharmaceutical companies developing inhibitors. Small molecular weight inhibitors typically target the highly conserved ATP-binding pockets of the kinase domain and compete with ATP binding. Because of similarities in the active sites of many kinases, specificity issues are common for inhibitors targeting the ATP-binding pocket, and cross-reactivity may cause unwanted toxicities. However, for several kinases this approach has been successful and in recent years a number of protein kinase inhibitors have successfully been taken through clinical trials to enter clinical practice. Sorafenib (Nexavar®), imatinib mesylate (Gleevec®), temsirolimus (Torisel®), erlotinib (Tarceva®), sunitinib (Sutent®), and gefitinib (Iressa®) are examples of such small molecule kinase inhibitors. The targets for these drugs include Raf-1, Abl, mTOR, and the receptor tyrosine kinases EGFR and VEGFR.

The importance of Pak in cell and animal models of tumorigenesis and metastasis provides a rationale for the development of Pak inhibitors as anti-cancer therapeutics. Several compounds have been identified that can act as ATP-competitive Pak inhibitors but they lack selectivity and inhibit too many other kinases to be of therapeutic use. The most potent are CEP-1347, a derivative of staurosporine, and OSU- 03012, a derivative of the Cox-2 inhibitor celecoxib [101, 102]. Nevertheless, ATP competitive compounds with enough specificity to be useful may eventually be developed.

Another approach has been to use non-ATP competitive inhibitors. Most of these derive from Pak itself. Recombinant pepide fragments of the Pak autoinhibitory domain (Pak PID) efficiently inhibit the activity of endogenous Pak protein. However, the need to introduce the autoinhibitory peptide into cells makes this approach less suitable for therapeutic use. Moreover, a recent study reported unintended side-effects with the use of the Pak-PID fragment [103]. Isolated Pak-PID induced cell cycle arrest and inhibition of cyclin D1 and D2 expression, independently of Pak1 kinase activity. This indicates that the PID may bind to and inhibit other targets and experiments using this fragment should be interpreted with caution.

The tight regulation of Group I Paks by autoinhibition presents an opportunity to develop allosteric inhibitors. Allostery refers to the phenomenon whereby the binding of a small molecule to one site regulates the activity of another site on the protein. This approach of targeting allosteric transition states during kinase activation has led to the identification of very selective inhibitors for Akt isoforms and other kinases, as the inhibitor binding site is usually more unique than the ATP-binding pocket. An elegant proof of principle for allosteric inhibition of Pak was recently shown by Peterson and his colleagues [104]. Peterson's group identified a small molecule inhibitor that they named IPA-3, by screening for inhibitors that prevented Cdc42 activation of Pak. While all three Group I Paks were inhibited by IPA-3, Group II Paks were insensitive to the compound. This is to be expected, as Group II Paks are not regulated by an autoinhibitory domain. IPA-3 targets the distinct autoregulatory mechanism used by the Group I Paks and does not inhibit Pak after it is already activated. Although IPA-3 is not stable or potent enough to be used *in vivo*, it is a promising lead to develop effective inhibitors for Group I Paks.

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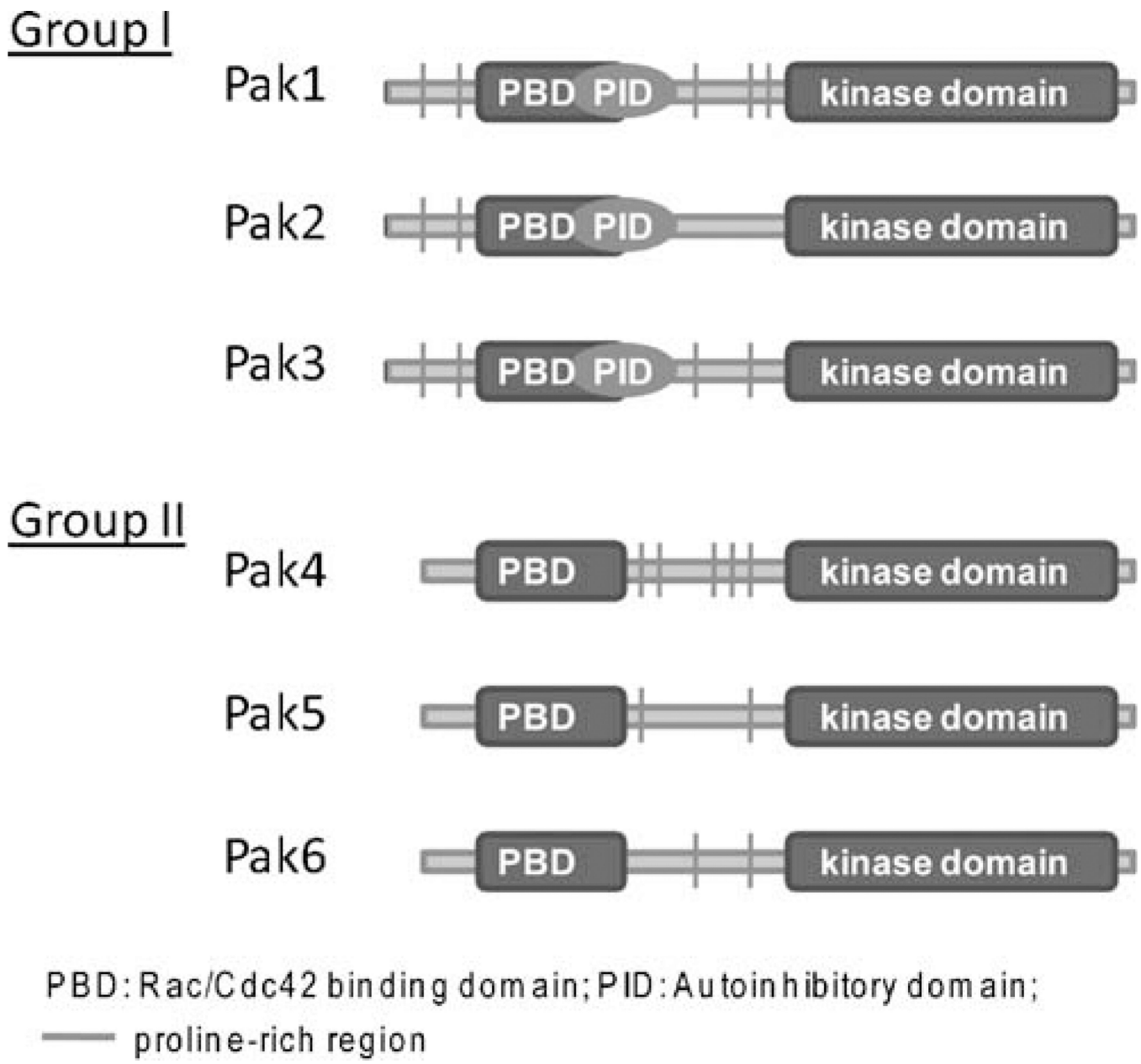

Fig. 1.
Domain structure of Pak isoforms. The p21 (Rac/Cd42)-binding domain (PBD) and kinase domain are shown, as well as proline-rich putative SH3-binding motifs. Groups I Paks additionally contain an autoinhibitory domain (PID) that is overlapping with the PBD

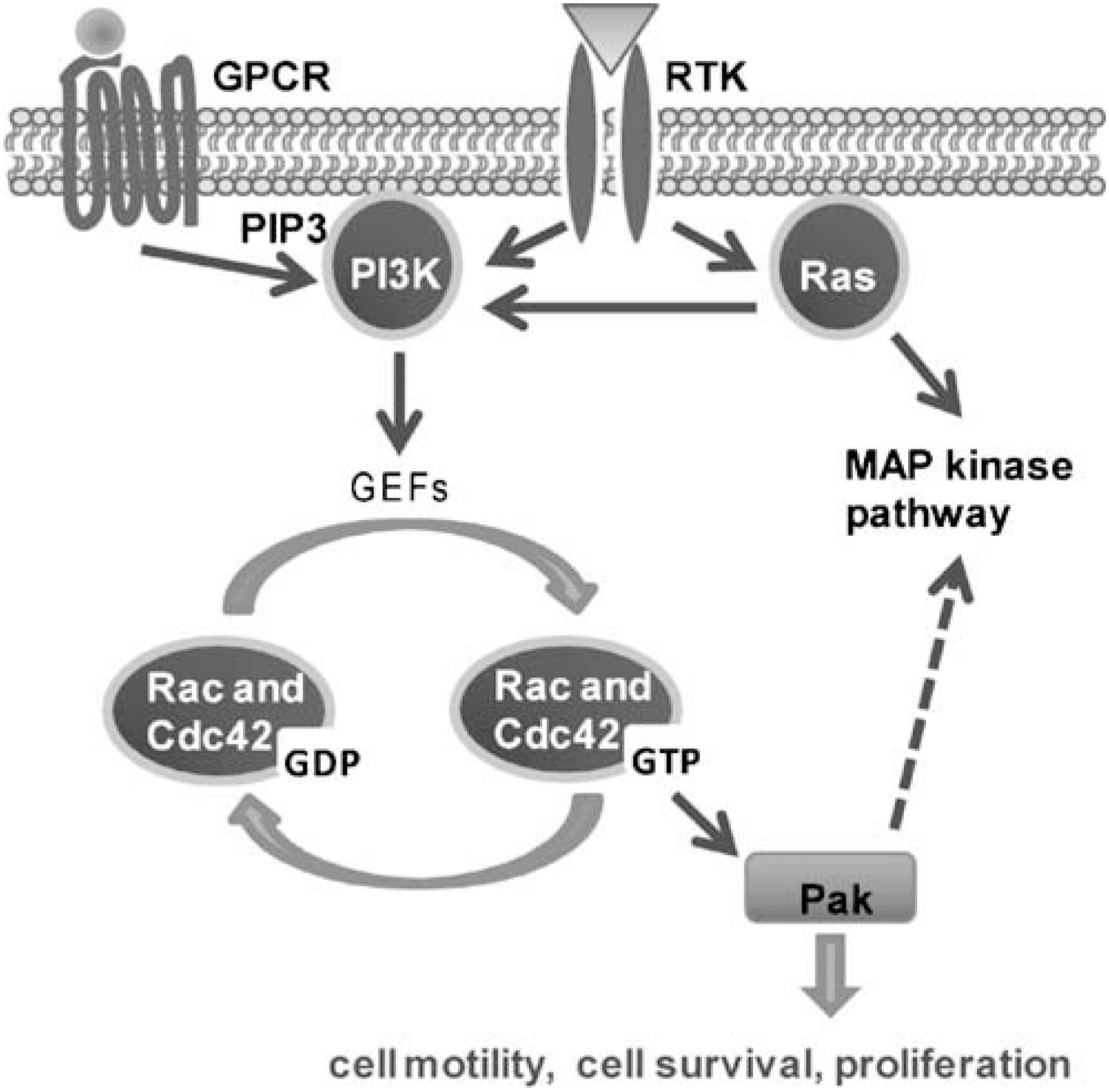

Fig. 2.

Schematic diagram of Pak activation by the small GTPases Rac and Cdc42. Signals from receptor tyrosine kinases, (e.g. insulin, EGF, PDGF, and VEGF receptors) and G protein-coupled receptors lead to activation of Pak via GTP-bound Rac and Cdc42. Activated Pak in turn initiates signaling cascades that culminate in the cellular response. In addition, activated Pak potentiates activation of the MAP kinase pathway. Of note, while activation of Pak via Rac and Cdc42 is well characterized, a number of GTPase-independent mechanisms for Pak activation have also been identified. GPCR, G protein-coupled receptors; RTK, receptor tyrosine kinase; PI3 K, phosphatidylinositol-3 kinase; PIP3, phosphatidylinositol (3, 4, 5) trisphosphate

Table 1

Reported Pak substrates

Process	Substrate	Sites	Isoform	Reference
Cytoskeleton remodelling	alpha-PIX	S488	Pak1	[33]
	beta-PIX	S340, S525 (S497, S682)*	Pak1, Pak2	[33, 105]
	Caldesmon	S657, S687	Pak1, Pak3	[106, 107, 108]
	CPI17	T38	Pak1	[109]
	Desmin		Pak1	[110]
	Filamin A	S2152	Pak1	[111]
	GEF-H1	S885	Pak1	[112]
	GIT1	S517	Pak1	[43]
	LIM kinase	T508	Pak1, Pak4	[113, 114]
	MBS	T641	Pak1	[109]
	MLCK	S439, S991	Pak1, Pak2	[60, 115]
	NET1	S152, S153	Pak1	[116]
	Op18/stathmin	S16	Pak1	[59]
	p41-ARC	T21	Pak1	[57]
	Rho GDI	S101, S174	Pak1	[117]
	R-MLC	S19	Pak2	[118, 119]
	TCoB	S65, S128	Pak1	[41]
	Vimentin	S25, S38, S50, S56, S65, S72	Pak1	[120–124]
Cell growth	Abl1	S637, S638	Pak2	[125, 126]
	Aurora A	T288, S342	Pak1	[43]
	B-Raf	S446	Pak1	[127]
	c-Myc	T358, S373, T400	Pak2	[128]
	C-Raf1	S338; S339	Pak1, Pak2 Pak3	[46, 50, 61, 129–131]
	ER alpha	S305	Pak1	[132, 133]
	Histone H3	S10	Pak1	[40]
	MEK1	S298	Pak1	[46, 134–137]
	MEKK1	S67	Pak1	[138]
	Merlin	S518	Pak1	[95, 96, 100]
	MNK1	S39	Pak2	[139]
	Plk1	S49	Pak1	[44]
	Prolactin	S179	Pak2	[140]
Cell survival	BAD	S111 (S136 through Raf-1)	Pak1, Pak2	[61, 72, 141]
	DLC1	S88	Pak1	[68]
	FKHR	S256	Pak1	[67]
Miscellaneous	CtBP1	S158	Pak1	[142]
	ESE1	S207	Pak1	[143]
	G alpha z	S16	Pak1	[144]
	p47 phox	S303, S304, S320, S328	Pak1	[14, 145]
	p67 phox	Not mapped	Pak1	[146]

Process	Substrate	Sites	Isoform	Reference
	PGAM–B	S23, S118	Pak1	[38]
	PGM	T466	Pak1	[39]
	SHARP	S3486, T3568	Pak1	[147]
	SNAI1	S246	Pak1	[35]
	STAT5a	S779	Pak1	[148]
	Syk	Not mapped	Pak2	[149]
	Synapsin I	S603	Pak1	[150]
	Troponin I	S149	Pak1	[151]
Pak auto-phosphorylation	Pak1	S21, S57, S144, S149, S199, S204	Pak1	[8]
	Pak2	S19, S20, S55, S141, S165, S192, S197	Pak2	[7, 8]
	Pak3	S50, S139	Pak3	[8]

Abl1 Abelson murine leukemia viral oncogene homolog 1, *BAD* Bcl-2 antagonist of cell death, *CPI17* 17-kDa PKC-potentiated inhibitory protein of PP1, *CtBP1* C-terminal-binding protein 1, *DLC1* dynein light chain 1, *ER* estrogen receptor, *ESE1* epithelium-specific Ets transcription factor 1, *FKHR* Forkhead box protein O1, *G alpha z* guanine nucleotide binding protein (G protein), alpha z, *GEF-H1* guanine nucleotide exchange factor H1, *GIT1* G protein-coupled receptor kinase-interactor 1, *MBS* myosin binding subunit of type 1 protein phosphatase, *MEK1* mitogen-activated protein kinase kinase 1, *MEKK1* mitogen-activated protein kinase kinase kinase 1, *MLCK* myosin light chain kinase, *MNK1* MAP kinase interacting kinase 1, *NET1* neuroepithelial cell transforming gene 1 (RhoA-specific guanine nucleotide exchange factor), *P41-ARC* actin-related protein 2/3 complex 41 kDa subunit, *p47 phox* neutrophil NADPH oxidase activator 1, *p67 phox* neutrophil NADPH oxidase factor 2, *PGAM-B* phosphoglyceratemutase-B, *PGM* phosphoglucomutase, *PIX* PAK-interacting exchange factor, *Plk1* Polo-like kinase 1, *Rho GDI* Rho GDP dissociation inhibitor, *R-MLC* regulatory myosin light chain, *SHARP* SMART/HDAC1 associated repressor protein, SNAI1 snail 1 zinc finger protein, *STAT5a* signal transducer and activator of transcription 5A, *Syk* spleen tyrosine kinase, *TCoB* tubulin cofactor B;

* beta-PIX phosphorylation sites for transcript B

Table 2

Cancers with altered expression of Pak family members

Cancer type	Pak isoform	Type of alterations	References
Brain	Pak1	Increased phospho-Pak1 in cytoplasm.	[152]
Esophagus	Pak4	Protein overexpression.	[153]
Breast	Pak1, Pak4	Protein overexpression and increased nuclear localization; Gene amplification (11q13–>q14 amplicon).	[45, 83, 85, 92, 153]
Liver	Pak1	Protein and gene overexpression.	[154]
Kidney	Pak1	Protein overexpression and increased activity.	[155]
Pancreas	Pak4	Gene amplification (19q13 amplicon), protein overexpression.	[156]
Colon	Pak1, Pak4	Protein overexpression. Pak4 gene amplification (19q13 amplicon) and 2 somatic mutations.	[86, 153, 157]
Bladder	Pak1	Gene amplification (11q13–>q14 amplicon).	[158]
Ovarian	Pak1	Protein overexpression and gene amplification (11q13–>q14 amplicon).	[84, 159, 160]
Prostate	Pak6	Protein overexpression.	[161]
T-cell lymphoma	Pak1	Gene amplification.	[162]