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The PAKs come of age
Celebrating 18 years of discovery

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Protein kinases are versatile signaling molecules that are involved in the regulation most physiological responses. The p21-activated kinases (PAKs) can be activated directly by the small GTPases Rac and Cdc42 and are among the best characterized downstream effectors of these Rho proteins. The structure, substrate specificity and functional role of PAKS are evolutionarily conserved from protozoa to mammals. Vertebrate PAKs are particularly important for cytoskeletal remodeling and focal adhesion assembly, thereby contributing to dynamic processes such as cell migration and synaptic plasticity. This issue of *Cellular Logistics* focuses on the PAK family of kinases, with ten reviews written by researchers currently working in the field. Here in this introductory overview we highlight some of the most interesting recent discoveries regarding PAK biochemistry and biology. The reviews in this issue cover a range of topics including the atomic structures of PAK1 and PAK4, their role in animals as assessed by knockout studies, and how PAKs are likely to contribute to cancer and neurodegenerative diseases. The promise remains that PAK inhibitors will emerge that validate current pre-clinical studies suggesting that blocking PAK activity will positively contribute to human health.

Focus on PAK Kinases

PAKs were first discovered in 1994 in a screen for proteins that interact with the small G-proteins Rac1 and Cdc42.[1] It turned out that PAKs are a prototype target in that their Cdc42/Rac interaction-binding (CRIB) domain is found not only across the family but also in non-kinase effectors such as Wiskott-Aldrich syndrome protein (WASP).[2] PAKs are found in all eukaryotes, with budding yeast Ste20 kinase the first in its class to be described.[3] The biology of these diverse PAKs from protozoa to man are described in the article by Manser and Zhao in this issue.[4] Early genetic and biochemical studies of Ste20 showed that this PAK signals from Cdc42 to the pheromone-responsive MAP kinase pathway in budding yeast.[5] In both budding and fission yeasts the PAKs play an important negative feedback role to limit Cdc42 activation at the growing tip (or tips in the case of bipolar cells).[6]

Although PAK signaling does stimulate MAP kinase activation in mammalian cells, the mechanisms are somewhat different to those described in yeast, in that PAK primarily feeds into the canonical pathway through Raf-1, a major target of mammalian Ras not found in yeast. Raf1 can be activated by PAKs through Ser338 phosphorylation (as outlined in this issue by Ye and Field[7]). One interesting new target for mammalian PAKs is the atypical MAPK ERK3, which has an unusual activation loop. The vertebrate MAP kinases ERK1/ERK2, JNK, p38 and ERK5 all contain the conserved T-x-Y motif in their activation loop which is dually phosphorylated by members of the MAP kinase kinases family. PAK1/2/3 modifies ERK3 Ser-189 (or ERK4 on equivalent Ser-186)—the single site needed for kinase activation.[8] The role of ERK3/4 in cell proliferation is not yet resolved, but one well studied downstream target is MAP kinase-activated protein kinase 5 (MK5).

PAKs come in two flavors, which are denoted group I and II: the group I kinases in man comprise PAK1–3. The PAK1 (rat PAKα) is the best studied isoform, but is not found in all cell types or tissues.[9] The ubiquitous PAK2(γ) is under-studied as it is extremely toxic in *E. coli*, even in the context of mammalian expression vectors.[10] PAK3(β) exists as four alternate spliced forms in neurons.[11] The presence of PAK3 exons (b and c) renders the kinase constitutively active and decreases interaction with GTPases, and may promote heterodimerization with PAK1.[12] Curiously the PAK3 gene locus has been massively amplified in the zebra finch.[13] Various group I PAKs have been knocked out in mice, flies and worms. In this issue, Manser and Zhao provide details on invertebrates[4] while the phenotypes of mice knockouts are described in detail by Kelly and Chernoff.[14]

The group II PAKs include the ubiquitous PAK4 and the brain-enriched PAK5 and PAK6; there are ~10 times fewer publications featuring these kinases than for the group I PAKs. Recently a small widely expressed alternate spliced isoform of human PAK4 (termed PAK4b) was identified that contains only 68 residues of N-terminal regulatory sequence.[15] This sequence nonetheless contains a functional CRIB and auto-inhibitory domain (AID) also found in the larger PAK4a, and that is related the PAK1 AID. Thus PAK4 has finally lived up to its billing as a "p21-activated kinase." Interestingly there appears to be no auto-phosphorylation event associated with Cdc42-driven PAK4

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activation.[15] Audrey Minden's article in this issue describes in detail the role of group II kinases and their functions as revealed by studies using knockout mice.[16]

Pioneering studies on protein kinases were performed in muscle extracts. The regulation of smooth muscle contraction is activated by phosphorylation at Ser-19 of the regulatory light chain subunits of myosin II (MLC2). PAK1 has been shown to input on this pathway, attenuating the contraction of skinned smooth muscle by inhibiting the calcium regulated myosin light chain kinase (MLCK).[17] Myosin II is also important in controlling cell contractility in most non-muscle cells. The RhoA effector Rho-associated kinase (ROCK) and Cdc42 effector myotonin-related Cdc42-binding kinase (MRCK) are key MLC2 kinases in this context.[18] In *C. elegans* ROCK, PAK1 and MRCK homologs act redundantly to promote proper embryonic elongation via epidermal and muscle cells.[19] A constitutively active form of CeMLC only rescues loss of MRCK, indicating that ROCK and PAK1 have other targets in this process. PAK1 is part of the mechanosensory signaling module that responds to tension in these cells.[20] The cardiac muscle of PAK1-knockout mice are superficially normal; however, PAK1 KO hearts show reduced MLC2 phosphorylation after ischemia and reperfusion.[21] The specific defects in cardiac development and function due to loss of PAK1 function are described by Ke and coworkers in this issue.[22]

The immuno-localization of PAKs in cultured cells, being a static picture, can be rather uninteresting. In reality these kinases move between different cellular compartments, and can be found at the plasma membrane, in cell adhesions and in the nucleus.[23] The dynamic targeting of PAKs probably contribute significantly to their ability to act on substrates. Parrini's article in this issue provides some insight into the design of biosensors that can be used to visualize PAK1 localization and activation in live cells.[24] These tools are valuable probes to study PAKs in cells, and ultimately in model organisms. In cultured cells group I PAKs are targeted to cell adhesions via the PAK-interacting exchange factor PIX. In an important proteomic paper describing the composition of focal adhesions, βPIX emerged as a protein that maintains adhesions in an "immature" state.[25] PAK phosphorylates βPIX at Ser340[26] but the role of this modification is not understood. Other PAK targets within focal adhesions are not yet been established. Although paxillin is the binding partner for the PAK1/βPIX/GIT complex at adhesions complex it is not phosphorylated by PAK1 at Ser272[27] as previously suggested. Inhibiting PAK decreases focal adhesion turnover[28] and exactly how PAK/PIX regulates focal adhesion turnover and maturation will no doubt throw up some interesting stories.

The Potential of PAK Inhibitors

Partial structures for PAKs are known, and these structures can greatly aid the design of specific PAK inhibitors. Jha and Strauss, in this issue, provide insight into the features of PAKs revealed from the X-ray structures of the catalytic domains and the complex of the auto-inhibitory domain with inactive PAK1.[29] Such static molecular pictures are complemented by NMR analysis,[30] as well as all-atom in silico molecular dynamic (MD) simulations.

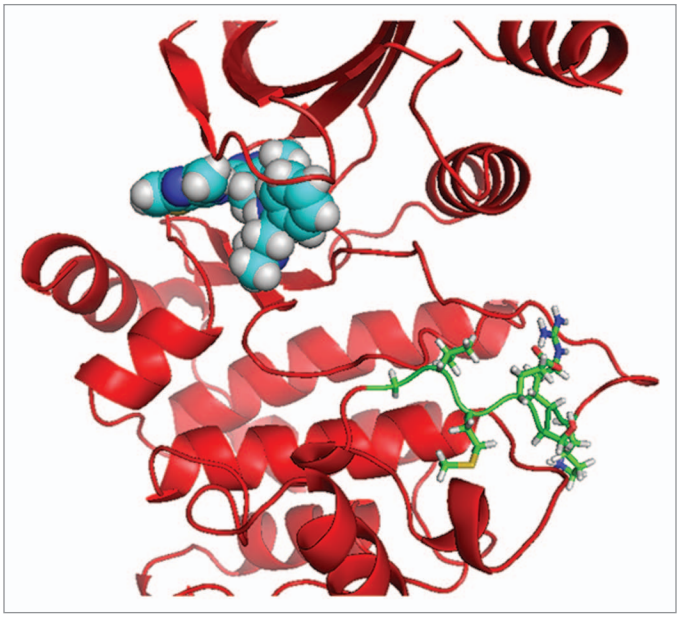

Figure 1. A representation of the complex between PAK1 and inhibitor PF-3758309. The structure of PAK1 structure was based on the conformation previously described.[10] The PF-3758309 complex was docked using HADDOCK/CNS, based on the position of ATP in the binding cavity of the PAK4: PF-3758309 complex. Parameters for the drug were generated by PRODRG. The docked structure was then minimized employing the CHARMM forcefield for the protein and the PRODRG generated parameters for the drug moiety. The position of the kinase activation loop is shown in green.

In the past four years these computationally taxing simulations have moved from the realm of super-computers to the desktop.[31] In 1992 such solution-based MD simulations were first used to explain how the coordination of water molecules was key to the mechanism of Ras GTP hydrolysis,[32] and indeed other Ras-like proteins. The utility of this technique is illustrated with lapatinib a high affinity inhibitor of EGFR and HER2, with weak affinity for ErbB4. Although the crystallographic contacts of lapatinib are essentially identical with these three kinases,[33] MD simulations correctly trace the higher affinity of EGFR vs. ErbB4 for lapatinib to water molecules interacting with EGFR Cys775.[34] Similarly the conformational behavior of the active phosphorylated PAK1 catalytic domain has been modeled by MD simulation.[10] The simulations demonstrate how the key activation loop phosphate is orientated to PAK1 Lys308, at the end of the αC helix, thus holding this helix in an active conformation; the model correctly predicts why the commonly used PAK1(T423E) phospho-mimetic is not active, as it lacks this Lys308 interaction. The MD-optimized structure of PAK1 in complex with the pan-PAK inhibitor PF-3758309 developed by Pfizer is illustrated in **Figure 1**. The kinase is in a "closed" conformation and the binding of the drug in the ATP binding pocket closely resembles interactions seen for PAK4 in complex with PF-3758309 (pdb 2X4Z).

Much of the interest in PAKs centers on their role in cancer, and the possibility that PAK inhibitors may be useful in clinic. The article by Ye and Field in this issue discusses the ways that

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Dr. Jeffrey Field is professor of Pharmacology at the University of Pennsylvania Perelman School of Medicine. He earned a BA in biology from Columbia University and a PhD from the Albert Einstein College of Medicine with Dr. Jerard Hurwitz. During postdoctoral studies with Dr. Michael Wigler at the Cold Spring Harbor Laboratories, he isolated the first known Ras effector, the yeast adenylyl cyclase. To isolate cyclase, he developed a technology known as epitope tagging, or HA-tagging, which has since been used in thousands of laboratories to isolate other proteins. In his own lab at the University of Pennsylvania, where he has worked since 1993, he established the central role of PAK kinases in Ras signaling and cell transformation. His current work centers on the role of the cytoskeleton in transformation and survival as well as mechanisms of smoking carcinogenesis.

About Dr. Ed Manser

Dr. Ed Manser obtained his PhD (Biophysics) at the National Institute for Medical research in London, working on the microtubule cytoskeleton under the supervision of Dr. Peter M. Bayley. He secured a postdoctoral position in 1986 at the Institute of Neurology, London, to undertake research in the new field of molecular neurobiology. In Singapore he developed an interest in the biochemical pathways downstream of the newly discovered Rho small GTPases. His team was supported until 2009 by the GSK-IMCB Singapore Research fund, during which time groundbreaking discoveries in the field of Rho signal transduction were made. This included the identification and isolation of effector protein kinases of the ACK, PAK, ROCK and MRCK families. Subsequently associated proteins for PAK1 such as PIX and GIT were uncovered. These function in complexes to drive cell shape changes, motility and cytokinesis in many cell types, including neurons. Ed has published a number of "citation classics" (> 400 citations) on these Rho effectors. Currently, he holds joint appointments as a Principal Investigator in the Astar-Neuroscience Research Partnership and in the Institute of Medical Biology.

PAKs are amplified, overexpressed or activated in many cancers to drive the growth of tumors.[7] PAKs, most often PAK1 and PAK4, are overexpressed in certain cancers[35] in which they promote the growth and maintenance of tumors. This review also addresses some of the targets and signaling pathways that PAKs are using to drive tumor growth, reviewing studies primarily on PAK1. However, PAK4 may be the most important isoform in human cancer as it is the only isoform that will reliably cause tumors when ectopically expressed.[36] The links between PAK4 function and cancer is reviewed by Minden, also in this issue.[16]

Since blocking PAK was anticipated to selectively affect cancer cells, a number of academic labs and pharmaceutical companies have developed small molecule PAK inhibitors. Most compounds are ATP competitive, but IPA-3 is an unusual allosteric inhibitor that prevents PAK activation by Cdc42.[37] Such compounds provide the first small molecule probes to study PAK function, although the PAK AID peptide[38] remains the gold standard in cell culture. The pan PAK inhibitor PF-3758309 developed by Pfizer[39] is in clinical trials for cancer, although it may not proceed due to poor bioavailability. The range of PAK inhibitors under development are described in this volume by Coleman and Kissil in this issue.[40] It is becoming clear that there is essential cross-talk between tumor cells, the vasculature, and immune cells during tumor progression. In this context PAK inhibitors might be able to simultaneously modulate all three cell types for a positive outcome. There have been a number of recent successes with protein kinase inhibitors to treat cancer, most notably against the prototype Ras-target BRaf;[41] the development of suitable PAK inhibitors may add to this list.

Inhibiting PAKs may also be a route to modulate host-pathogen responses. An emerging area is the role of these kinases in pathogen responses. For example PAKs have long been suspected to be required for efficient HIV infection.[42] One of the most interesting recent findings of PAK function regards the enterohemorrhagic *E. coli* O157:H7. In the infection process, this strain uses the type III effector EspG protein to interfere with membrane trafficking at the level of the Golgi apparatus. In a yeast two-hybrid screen, PAKs (isoforms 1, 2 and 3) were found as its relevant host substrates.[43] The structure of EspG with a small region of the PAK AID (the region that also binds to fragile-X proteins), at 2.8 Å resolution provides the first example of allosteric kinase activation by a bacterial effector. Of relevance to a possible broader role for PAK in the Golgi, EspG can simultaneously bind PAK2 and the small G-protein Arf1. The kinase target(s) of PAK2 in the context of the Golgi apparatus are not known, but could be revealing. Other roles for PAK in the life cycle of viruses, bacterial pathogens and malaria parasites is discussed by Semblat and Doerig in this issue.[44]

PAKs are highly expressed in the brain where they are needed for both its development and in synaptic function. Mutations in PAK3 are associated with familial cognitive disorders,[45] and PAKs in their active state can directly interact with the fragile X mental retardation protein FMR1,[46] which coordinates activity-dependent protein translation in spines.[47] Based on studies in flies and mice it is suggested that drugs that inhibit group I PAKs would

be able to reverse some of the behavioral and physical defects associated with fragile X syndrome. In addition, PAKs have been implicated in the neurodegenerative disorders of Alzheimer and Huntington diseases. These important findings are reviewed by Ma et al. in this issue.[48]

As can be judged from the timing of this special focus, it has taken us a good many years to grasp how these kinases contribute to cell function, with the underlying PAK biochemistry still open to unexpected findings. For example, recent evidence points to PAK4 being constitutively phosphorylated on the activation loop Ser474;[15] this explains why antibodies directed toward pS474 should fail to detect changes in PAK4 activity. There remains a plethora of discoveries ahead since the number of well-defined PAK (kinase) targets are limited (an updated list of published targets is in the review by Ye and Field[7]). Three important facts emerge from the seminal report describing PF-3758309.[39] First that PAK inhibition is well tolerated by cells and animals; second, PAK inhibitors can block many more tumors than would be predicted from expression studies and third, surveys of signaling pathways perturbed by PF-3758309 identified almost all known PAK pathways, as well as several new ones not typically associated with PAK. No doubt the availability of small molecule probes and knockout mice to explore PAK signaling will reveal new PAK targets, pathways and functions.

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