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NMR binding and crystal structure reveal that intrinsically-unstructured regulatory domain auto-inhibits PAK4 by a mechanism different from that of PAK1

Wei Wang [a], Liangzhong Lim [a], Yohendran Baskaran [b], Ed Manser [b], Jianxing Song [a,c,*]

[a] Department of Biological Sciences, Faculty of Science, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore
[b] sGSK Laboratory, Institute of Molecular and Cell Biology, SINGAPORE 138673.
[c] Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore

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ABSTRACT

Six human PAK members are classified into groups I (PAKs 1–3) and II (PAK4–6). Previously, only group I PAKs were thought to be auto-inhibited but very recently PAK4, the prototype of group II PAKs, has also been shown to be auto-inhibited by its N-terminal regulatory domain. However, the complete auto-inhibitory domain (AID) sequence remains undefined and the mechanism underlying its auto-inhibition is largely elusive. Here, the N-terminal regulatory domain of PAK4 sufficient for auto-inhibiting and binding Cdc42/Rac was characterized to be intrinsically unstructured, but nevertheless we identified the entire AID sequence by NMR. Strikingly, an AID peptide was derived by deleting the binding-unnecessary residues, which has a Kd of 320 nM to the PAK4 catalytic domain. Consequently, the PAK4 crystal structure complexed with the entire AID has been determined, which reveals that the complete kinase cleft is occupied by 20 AID residuescomposed of an N-terminal α-helix and a previously-identified pseudosubstrate motif, thus achieving auto-inhibition. Our study reveals that PAK4 is auto-inhibited by a novel mechanism which is completely different from that for PAK1, thus bearing critical implications for design of inhibitors specific for group II PAKs.

1. Introduction

By direct binding to a variety of "effector" protein kinases including the p21-activated kinases (PAKs), the Rho-family GTPases such as Rac and Cdc42 control many cellular functions, comprisingcytoskeletal organization, morphological change, cell motility, and cell-cycle progression. PAKs function as hubs to interact with multiple partners, and have been demonstrated to be extensively involved in cancer, brain function, and virus infection. In human, the PAK family is composed of six members, which share conserved catalytic and Cdc42/Rac binding (CRIB) domains. They can be further classified into groups I (PAK1, -2, and -3) and II (PAK4, -5, and -6), based on the domain organizations and regulatory properties [1–7]. Previously, only group I PAKs have been demonstrated to be auto-inhibited and activated by Cdc42 or Rac binding. However, very recently PAK4, the prototype of group II PAKs, was also shown to be auto-inhibited by the N-terminal region (20–68) [7]. Moreover, the N-terminal residues 9–68 which are highly conserved in allthree group II PAKs are sufficient for auto-inhibiting PAK4 and binding Cdc42/Rac (Fig. 1A) [7].

Structurally, PAKs share with other non-PAK kinases the core catalytic domain constituted by two subdomains called the kinase lobes, namely N- and C-lobes, in between which is a long cleft called the kinase cleft which accommodates the catalytic machinery including substrate- and ATP-binding pockets [5,8,9]. Previously the crystal structure of the auto-inhibited PAK1 was determined to be a homodimer with each kinase domain bound to an N-terminal auto-inhibitory domain (AID), and consequently the auto-inhibitory mechanism was established [5]. On the other hand, the molecular mechanism underlying theauto-inhibition remains largely elusive for group II PAKs. Only very recently, the determination of PAK4 structures reveals that six residues Arg49-Val54 of the N-terminal regulatory domain are bound to the substrate-binding pocket of the kinase cleft as a pseudosubstrate [9].

However, three key questions remain unanswered for PAK4: 1. what is the solution conformation of the N-terminal regulatory domain in the free state? 2. What is the entire AID sequence? 3. How does the entire AID complex and auto-inhibit the PAK4 catalytic

* Corresponding author at: Department of Biological Sciences, Faculty of Science, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore. Fax: +65 6779 2486.
E-mail address: bchsj@nus.edu.sg (J. Song).

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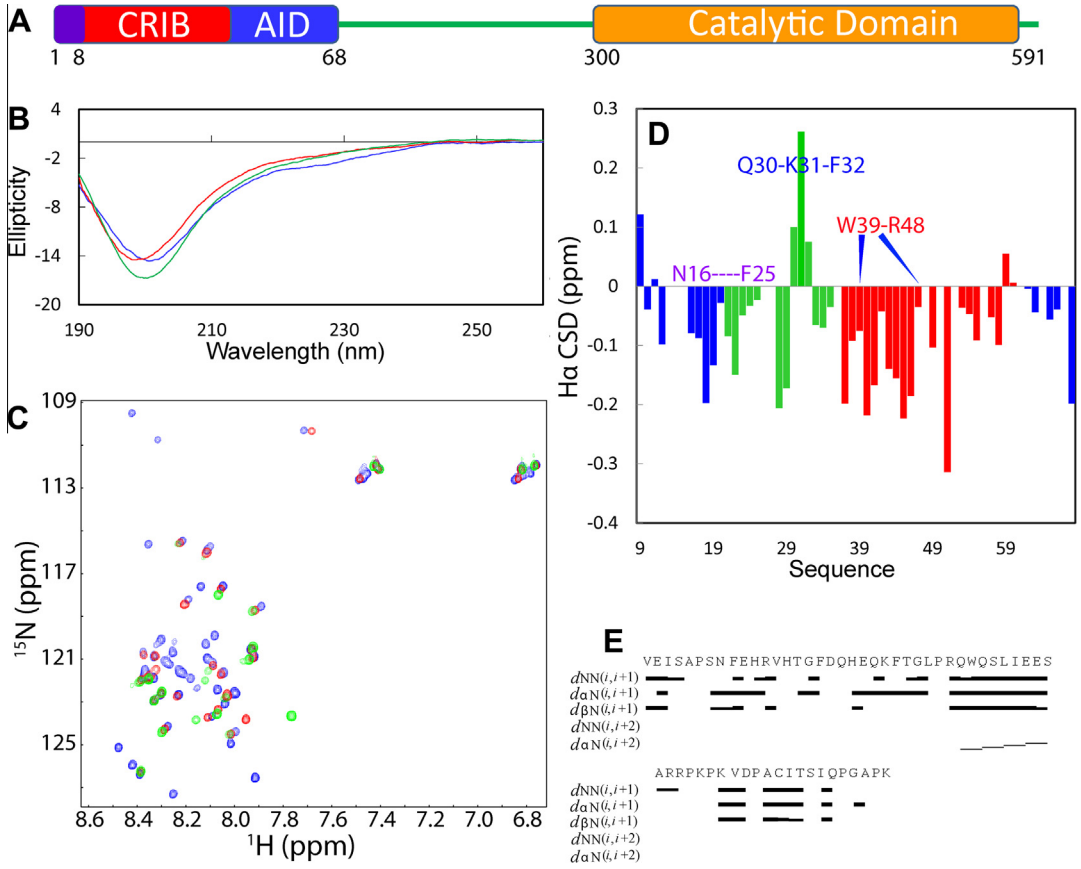

Fig. 1. The N-terminal regulatory domain of PAK4 is predominantly unstructured. (A) Domain organization of PAK4 including the nuclear localization signal over residues 1–8 (NLS, purple), Cdc42/Rac binding (CRIB, red) domain and auto-inhibitory domain (AID, blue) over residues 9–68, as well as catalytic domain (light brown) over residues 300–591. (B) Far-UV CD spectra of three peptides with a protein concentration of 25 μM, over residues 9–68 (blue), 29–61 (red) and 36–60 (green) at 25 °C in 5 mM phosphate buffer (pH 6.3). (C) Two-dimensional ^{1}H–^{15}N NMR HSQC spectra of three peptides with a concentration of 100 μM in 10 mM phosphate buffer (pH 6.3) over residues 9–68 (blue), 29–61 (red) and 36–60 (green) at 25 °C on a Bruker 800 MHz NMR spectrometer. (D) Residue specific Hα chemical shift deviations (CSD) for the full-length regulatory domain (9–68). Green bars are used for representing CRIB residues, red for AID residues, and blue for the rest. (E) NOE connectivity pattern of the full-length regulatory domain (9–68) defining secondary structures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

domain? Here we addressed these questions by a combined use of CD, ITC, NMR spectroscopy and X-ray crystallography.

2. Materials and methods

2.1. Cloning, expression and purification ofproteins

Three peptides (9–68, 29–61, 36–60) derived from the N-terminal regulatory domain of the human PAK4 with the nuclear localization signal (1–8) deleted were cloned into pGEX-4T-1 vector while the human PAK4 catalytic domain was cloned into pSY5M vector. Detailed protocols for protein expression and purification have been described in Supplementary material.

Isotope-labeled proteins for NMR studies were generated as we usually conducted [10–12]. The purity of the protein samples was verified by SDS–PAGE, and their molecular weights were confirmed using a Voyager STR matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Applied Biosystems).

2.2. Circular dichroism (CD) spectroscopy and titration calorimetry (ITC)

All circular dichrosim (CD) experiments were performed on a Jasco J-810 spectropolarimeter as we previously described [10–12].

ITC was used to characterize the binding of three peptides with the catalytic domain of PAK4 with a Microcal VP ITC machine (GE). The catalytic domain was placed in a 1.4 ml sample cell while the peptides were loaded into a 300 μL syringe. The titration data were fitted by using the built-in software ORIGIN to obtain thermodynamic binding parameters as we conducted on other proteins [12].

2.3. NMR experiments

All NMR experiments have been performed at 25 °C on an 800 MHz Bruker Avance spectrometer. For sequential assignment, a ^{15}N-labeled NMR sample of the full-length regulatory domain (9–68) at a protein concentration of 500 μM was prepared in 10 mM sodium phosphate buffer (pH 6.3) in the presence of 10 mM dithiothreitol (DTT) and 10% D_2O for NMR spin-lock. Three-dimensional NMR ^{15}N-edited HSQC-TOCSY and HSQC-NOESY spectra were acquired and the subsequent analysis led to the sequential assignment. NOE connectivities were derived from HSQC-NOESY spectrum.

For HSQC titrations, NMR samples of three ^{15}N-labeled peptides at protein concentrations of 200 μM were prepared in 10 mM sodium phosphate buffer (pH 6.3) in the presence of 5 mM DTT and the catalytic domain was dialyzed to the same buffer. The two-dimensional NMR ^{1}H–^{15}N HSQC spectra were collected for three peptides in the absence and in the presence of the unlabeled