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Minireview

Flipping the Switch: The Structural Basis for Signaling through the CRIB Motif

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The Rho family GTPases function as tightly regulated molecular switches governing critical cellular functions. The activity of these proteins is controlled by their GTP binding and hydrolytic cycle such that binding of cellular GTP induces an active conformation capable of interacting with downstream effector molecules and initiating a cellular response. The conserved CRIB (for Cdc42- and Rac-interactive binding) motif is the hallmark of an important subset of effectors for Cdc42 and Rac (Figure 1). This conserved sequence forms part of the limit binding domain for Cdc42 and Rac (also called the PBD for p21 binding domain, and GBD for GTPase binding domain), and residues of the CRIB sequence are essential for the interaction of these effectors with GTP-bound Cdc42 or Rac (Rudolph et al., 1998; Owen et al., 2000). This region was first identified by Lim and colleagues (Manser et al., 1994) as a conserved sequence in two Cdc42 effectors, the serine/threonine kinase PAK-1 (p21-activated kinase 1) and the tyrosine kinase ACK-1 (activated Cdc42-associated kinase 1). Subsequently, Hall and coworkers identified a host of candidate Cdc42/Rac effector proteins on the basis of sequence homology to this region and coined the term CRIB motif (Burbelo et al., 1995). PAK, ACK, and a third more recently appreciated Cdc42 effector, WASP (Wiscott-Aldrich Syndrome protein), are the most well-studied CRIB motif–containing proteins and provide an important basis for our understanding of Cdc42/Rac-mediated signaling.

The CRIB motifs, or more accurately the PBD domains, of these three effectors connect the activation of Cdc42 and Rac to a broad range of downstream responses. One important outcome of PAK activation is the initiation of a kinase cascade leading to the activation of stress-responsive nuclear MAP kinases like the c-Jun kinase and p38/HOG1 and accompanying changes in gene expression (reviewed by Bagrodia and Cerione, 1999). Other important substrates for the PAKs include the serine/threonine kinase Raf, which may serve to link Cdc42 or Rac to Ras-induced oncogenesis, as well as myosin light-chain kinase and other cytoskeletal components participating in Cdc42- and/or Rac-mediated actin rearrangements. The WASP family of proteins (Bi and Zigmond, 1999) interact with the Arp2/3 complex to initiate the polymerization of actin filaments. Two closely related family members, WASP and N-WASP, contain a conserved CRIB motif that enables the binding of Cdc42 to stimulate actin polymerization (Symons et al., 1996). Mutations in WASP that lead to actin cytoskeletal defects in hematopoietic cells are responsible for the clinical characteristics of Wiscott-Aldrich syndrome, including platelet abnormalities and immunodeficiency. A third family of CRIB domain–containing proteins is represented by the nonreceptor tyrosine kinases ACK-1 (Manser et al., 1993) and ACK-2 (Yang et al., 1997), which are highly specific targets for Cdc42. While these tyrosine kinases have yet to be linked to a specific pathological condition, it is likely that they will play important roles in neuronal cell function as they are highly expressed in brain and have been implicated in integrin-coupled signaling.

There seems little doubt that understanding how the activated form of Cdc42 or Rac triggers the activation of its CRIB domain–containing target/effectors will provide important molecular insights into the signaling functions of these GTP binding proteins. A remarkable feature of the GTP binding proteins in their capacity as signaling switches is the subtlety of the structural changes that distinguish their GDP-bound "off" state from their GTP-bound "on" (active) state. These structural changes are basically confined to two limit regions denoted as switch I and switch II. With the recent emergence of the structures for the inactive and active states of CRIB domain–containing targets (reviewed below), we see that while the conformational alterations in the GTP binding proteins are relatively minor, these changes lead to dramatic structural alterations in their signaling targets. As will become evident below, this type of conformational change/amplification has important consequences for the initiation of signal propagation.

In a recent issue of *Cell*, the Mayer and Harrison groups reported the structure of the serine/threonine kinase PAK in its basal (autoinhibited) state (Lei et al., 2000), providing the basis for an intriguing and somewhat unexpected mechanism for its activation by Cdc42/Rac. This structure, as well as the corresponding structure for WASP recently described by Rosen and collaborators (Kim et al., 2000), reveals that these proteins contain a structurally conserved autoinhibitory module that maintains them in an inactive conformation. Comparisons with recent NMR structures of Cdc42/PBD complexes (see below) suggest that the binding of Cdc42 leads to a reorganization of the inhibitory domain and a concomitant activation of their effector signaling activities. While the structure of the core inhibitory module in the basal state, and the corresponding rearrangement of this domain that accompanies Cdc42 binding, are nearly identical for PAK and WASP, these two effector proteins differ in critical aspects of their activation mechanisms. In this minireview, we will discuss the conformational changes associated with Cdc42-mediated activation of PAK and WASP, as well as consider the broader implications of this new structural information for understanding how a GTP binding protein like Cdc42 utilizes these targets to effect cellular signaling responses.

Identification and Structural Analysis of the Autoinhibitory Interactions in PAK and WASP

Previous biochemical studies of WASP-related proteins pointed to an intramolecular autoinhibitory interaction between the N-terminal PBD domain and the C-terminal VCA domain (named for verprolin homology, cofilin homology, and acidic regions) (Miki et al., 1998). In order to undertake the structural analysis of this protein by NMR spectroscopy, Kim et al. carefully pared down the

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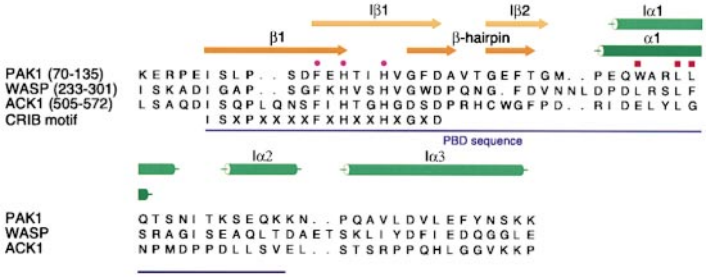

Figure 1. Sequence Alignment of PAK, WASP, and ACK

The sequences of PAK and WASP in the region of the IS domain are aligned based on their structures, while ACK was positioned by alignment with the CRIB domain alone. The secondary structural elements of the autoinhibited and activated conformations of the PAK IS domain are shown above the alignment in light and dark colors, respectively. The PDB sequence used for determining the Cdc42-bound structure is underlined in blue. Residues in PAK that bind Cdc42 upon activation and are also involved in either the dimer interface or in stabilizing the fold of the IS domain are indicated as magenta circles and red squares, respectively.

full-length WASP molecule and delineated the limit domain of the PBD that was capable of binding to the VCA region. Likewise, a short sequence within the cofilin homology region (CHR) was identified as the limit domain required for interaction with the PBD. The authors prepared a fusion protein between the PBD and the CHR domains, linking the two domains with a short flexible peptide. Thus, their NMR structure represents a minimal autoinhibitory unit for WASP and presumably reconstructs interactions critical for maintaining the basal state of the full-length protein.

Lei et al. similarly engineered a minimal autoinhibitory structure for PAK. Capitalizing on the finding that an N-terminal fragment of PAK including the CRIB domain was able to bind and inhibit the full-length kinase (Frost et al., 1996), they were able to crystallize a complex between the independently expressed kinase and inhibitory domains of PAK. The kinase domain of PAK adopts the familiar two-lobed structure seen in all known kinases (Johnson et al., 1996). A surprising result, however, is that PAK exists as a dimer in the crystal structure. This finding was unanticipated by previous work but elegantly confirmed biochemically for soluble full-length PAK using gel filtration, analytical ultracentrifugation, and dynamic light scattering. Moreover, the dimerization interface is formed by a pair of β strands, one from each monomer, which generate an antiparallel sheet, and part of this interface corresponds to the conserved CRIB motif. Within the dimer lies an inhibitory structure nearly identical to that first described for WASP. The ability of the conserved CRIB motif to function as part of a common regulatory element influencing two very divergent signaling proteins warrants a closer look.

Autoinhibition by the PBD: The Inhibitory Switch (IS) Domain

The structures of the autoinhibited forms of WASP and PAK reveal that the PBD is embedded in a larger, structurally conserved, inhibitory domain responsible for maintaining these proteins in a basal (autoinhibited) state. The inhibitory domain is referred to as the inhibitory switch (IS) due to the conformational changes induced by Cdc42 that lead to effector activation (described below). Remarkably, the structure of the IS domains in the autoinhibited forms of PAK and WASP are very similar and function as part of a compact folded module comprised of three architectural elements (Figure 2). Two of these structural elements are derived from the IS domain itself, an N-terminal β hairpin that immediately follows the conserved CRIB sequence and a central bundle of three α helices. The WASP structure contains an additional helix C terminal to this bundle, while in PAK this helix is replaced by a peptide segment that is responsible for inhibiting the kinase activity. The third critical structural component of the inhibited fold is an additional helical segment that is distinct from the IS domain. In the case of WASP, this additional helix corresponds to the limit peptide of the cofilin homology region that retains binding activity toward the PBD. The interaction of the IS domain with this cofilin-like helix is directly responsible for the inhibition of WASP activity. Mutagenesis studies have implicated a conserved basic sequence within the cofilin homology region (CHR) of WASP in Arp2/3 binding (Bi and Zigmond, 1999). Residues within the conserved basic sequence of the CHR contribute to intramolecular interactions between this helix and the IS domain in the autoinhibited conforma-

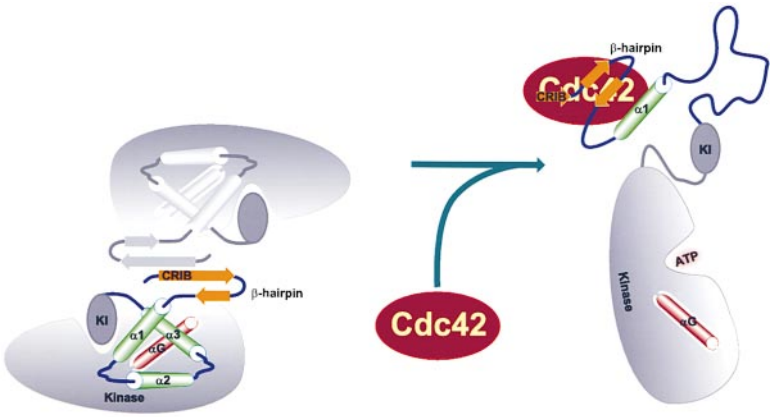

Figure 2. Schematic Illustration of the Conserved Structural Changes of the IS Domain upon Cdc42 Activation

The structure of the inhibited conformation of the PAK IS domain, including the additional helical segment (helix G), is in the colored portion on the left. The conformational changes within the IS domain associated with activation upon Cdc42 binding are shown in color on the right. These colored structural elements of the IS domain are conserved in the WASP structure while other nonconserved elements of the PAK structure are shown in gray.

tion of WASP. Presumably, the intramolecular interaction of the IS domain with the CHR helix is incompatible with binding to the Arp2/3 complex and, in the absence of Cdc42 binding, maintains WASP in an inactive state.

The autoinhibitory mechanism for PAK appears to be more complex (Figure 2). Inhibition of the kinase activity is achieved by sterically occluding the active site through dimerization, as well as through the placement of a kinase inhibitory (KI) segment in the active site. However, stabilizing the KI segment in the active site is a set of interactions between the folded IS domain of PAK and helix G from the large lobe of the kinase, completely analogous to the packing of the CHR helix against the IS domain of WASP. Residues involved in contacts between the IS domain of PAK and helix G were anticipated to be critical in the autoinhibitory mechanism from studies showing that mutations at these positions yield a constitutively active kinase. Once properly oriented, the KI segment of PAK stabilizes the inhibitory conformations of two key structural components of the active site (helix C and the activation loop) in a manner similar to that seen in other autoinhibited kinases (Johnson et al., 1996). Additionally, a lysine residue from the KI segment further blocks the active site by forming salt bridges with two catalytically important aspartate residues.

An important unresolved question is whether these inhibitory contacts in PAK occur in an intramolecular fashion or if dimerization is required for intermolecular inhibition. The dimer in the crystal structure is comprised of four independent domains, two kinase domains, and two IS domains, and it is not possible to determine from the structure which pair of kinase and inhibitory domains will be covalently connected in the full-length protein. Another important question is the role of dimerization in the biological function of CRIB domain–containing proteins. It is tempting to speculate that the CRIB domain represents a general dimerization motif; however, the monomeric nature of the WASP structure argues against this conclusion. Perhaps the autoinhibition of PAK indeed occurs via an intermolecular mechanism, such that contacts between the kinase and inhibitory domains are important in stabilizing dimer formation. It is possible that certain CRIB-containing effectors require the additional levels of control provided by dimerization, or that there are some circumstances in which the heterodimerization of two different target/effectors will provide an advantage by coordinating different effector outputs.

Activation by Cdc42/Rac

Comparisons of the structures describing the activated (GTP-bound) form of Cdc42 complexed to various CRIB domain–containing peptides from WASP, PAK, and ACK (Abdul-Manan et al., 1999; Mott et al., 1999; Gizachew et al., 2000; Morreale et al., 2000), with those for the autoinhibited forms of PAK and WASP, reveal an intriguing model for activation that invokes a restructuring of the PBD peptide and a dramatic unfolding of the remainder of the IS domain. All of these structures show nearly identical sheet-like contacts between the first PBD β strand, including the CRIB motif, with the β2 strand and C-terminal portion of the switch I region in Cdc42. The other important set of interactions observed for the Cdc42/WASP-PBD complex (Abdul-Manan et al., 1999) and for one of the Cdc42/PAK-PBD (Morreale et al., 2000) complexes are those of the β hairpin and first α helix of the PBD with the switch regions of Cdc42. These interactions are responsible for driving the structural rearrangements of the IS domain. In the basal state, the β hairpin and α1 helix form a layer of secondary structure that packs against the remainder of the IS domain. The binding of Cdc42 extracts this entire layer from the IS domain such that the GTP binding protein forms contacts with the face of the β hairpin and α1 helix that formerly contributed to the hydrophobic core of the folded IS domain. In the case of WASP, the α1 helix packs against the helical region of the switch II domain of Cdc42, thus disrupting part of the binding site for the CHR helix and contributing to the release of this inhibitory interaction. More importantly, this mode of Cdc42 binding is sterically incompatible with the folded IS domain and is likely to drive the unfolding of this domain, completely eliminating the inhibitory interactions and giving rise to effector activation.

The contacts between the IS domain and helix G of PAK are not directly inhibitory as they are for the WASP CHR helix; rather, these contacts serve to stabilize the KI segment in the kinase active site. Unfolding the IS domain would presumably destroy these stabilizing contacts and release the KI segment, thus activating the kinase. Moreover, the β1 strand of the PAK-PDB is critical for the formation of the dimerization interface and continues as an extended strand into the β hairpin of the IS domain in the autoinhibited structure. Cdc42 binding disrupts this strand, creating a β bulge centered around residue 85 of PAK. While the β hairpin observed in the autoinhibited structure is preserved, the β bulge effectively rotates the N-terminal portion of the first β strand away from the core of the IS domain such that the dimerization interface is occluded. Thus, PAK activation appears to involve both the unfolding of the IS domain and disruption of the dimer.

The structural flexibility of the IS domain is likely to play an important role in the activation mechanism. In the absence of either of its binding partners, the IS domain of WASP is unstructured (Kim et al., 2000). This structural "plasticity" allows the IS domain to flip between the inhibitory conformation representative of the basal (autoinhibited) state and a distinct activated structure that is promoted and/or stabilized by the binding of Cdc42. Such intrinsically disordered domains are an emerging theme in signal transduction, allowing signaling proteins to convert between distinct structures or between folded and unfolded states, thus resulting in the stimulation of key cellular activities (Wright and Dyson, 1999).

Additional Levels of Regulation

The structures for key effector/targets like PAK and WASP represent a critical step forward in understanding the molecular basis by which GTP binding proteins like Cdc42 initiate signaling events. It is likely that multiple levels of complexity underlie the regulation of these effectors, as the structural data hints at potential mechanisms by which these effectors may integrate information from a variety of upstream regulatory cues. Both PAK and WASP contain potential phosphorylation sites within their IS domains that probably become accessible upon activation by Cdc42 or Rac and may provide for the extended activation of PAK or WASP even after the dissociation of their activating GTP binding proteins. In addition, both PAK and WASP are comprised of a series of signaling modules capable of additional protein–protein interactions that are likely to influence their activity. PAK uses a nonconventional proline-rich sequence

to bind members of the Cool (for cloned out of library)/PIX (for PAK-interactive exchange factor) family of proteins (reviewed by Bagrodia and Cerione, 1999). This sequence lies between the IS and kinase domains and was not included in the structure of the autoinhibited form of PAK; however, it may lie close to the dimerization interface. The p50Cool-1 isoform has been shown to inhibit PAK activity, suggesting that the binding of p50Cool-1 may stabilize the position of the KI segment and potentially block the ability of Cdc42 to disrupt dimerization. In contrast, a longer splice variant, p85Cool-1/β-PIX, does not inhibit PAK activity, and a related protein, Cool-2/α-PIX, appears to directly stimulate PAK. The extended C termini of the larger Cool/PIX proteins may disrupt the PAK dimer or destabilize the position of the KI segment in the active site. The PAK activating Cool/PIX proteins also interact with other signaling proteins, most notably members of the Cat (Cool-associated tyrosine phosphosubstrate)/Git (G protein–coupled receptor kinase interactor)/PKL (paxillin–kinase linker) family, providing additional possibilities for regulating PAK activity. Still other protein–protein interactions such as the binding of Nck to the N-terminal proline-rich sequence of PAK, or the binding of G protein βγ subunits to the C-terminal helix of the kinase domain, may also function to regulate PAK by influencing its monomer–dimer equilibrium.

Similarly, WASP may be regulated through interactions between its polyproline-rich sequence and profilin or SH3 domain–containing proteins. Kim et al. make the important observation that competition between folding of the WASP IS domain and Cdc42 binding exacts a "thermodynamic penalty" for unfolding this stable domain upon activation (this is also presumably true of PAK). As a consequence Cdc42 binds rather poorly to the full-length protein ($K_D \sim 40$ μM). This affinity might be dramatically enhanced by a cooperative unfolding of the IS domain through the binding of other activators, allowing even tighter control of these signaling events.

Conclusion

When considering the various CRIB domain–containing effectors for Cdc42 and Rac, one wonders about the generality of the mechanism that has emerged from the structural studies of PAK and WASP. The proline-rich sequence of ACK following the CRIB motif is not likely to fold like the IS domain of PAK and WASP, and even within the PAK family this mechanism is not likely to be universal, as the bulk of the IS domain is missing in PAK4. As we enter the age of structural genomics, it will be important to heed the message relayed by the PAK and WASP structures. Structural conservation within the CRIB motif of WASP and PAK does not guarantee similar modes of regulation, with dimerization in one case and purely intramolecular contacts in another. Moreover, the individual folds of isolated domains may not always reveal the nuances that these domains exhibit within their macromolecular context. It is likely that the details of these nuances, as they are teased out of larger and more complex structures, will continue to provide critical insights into the basis of signal propagation by Cdc42/Rac and related molecular switches.

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