Regulation and cellular roles of coronin proteins

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1. Introduction

1.1 Coronin proteins - structure and functions

The dynamic regulation of the cytoskeleton of a cell forms the basis of several physiological processes like cell migration, vesicle trafficking, and tissue morphogenesis (Lee and Dominguez, 2010). The coronin family of proteins is one of the major regulators of cytoskeleton. Coronins belong to the WD repeat family of proteins and contain several copies of WD repeats that form a seven bladed beta-propeller structure. Coronin proteins are highly conserved proteins and are found in all major taxa of the eukaryotic kingdom except plants. They are broadly classified into two groups: short and long coronins, depending on the beta-propeller domain(Figure 1). Short coronins contain a single beta-propeller domain followed by a unique domain of varying length. In addition, they contain a short coiled-coil domain at their C-terminus that is required for homo-oligomerization. The long coronins containa tandem beta-propeller domain and lackthe coiled-coil domain. They also contain a highly acidic domain in their extreme C-terminus that has similarity to the WASP/SCAR family members (Uetrecht and Bear, 2006).

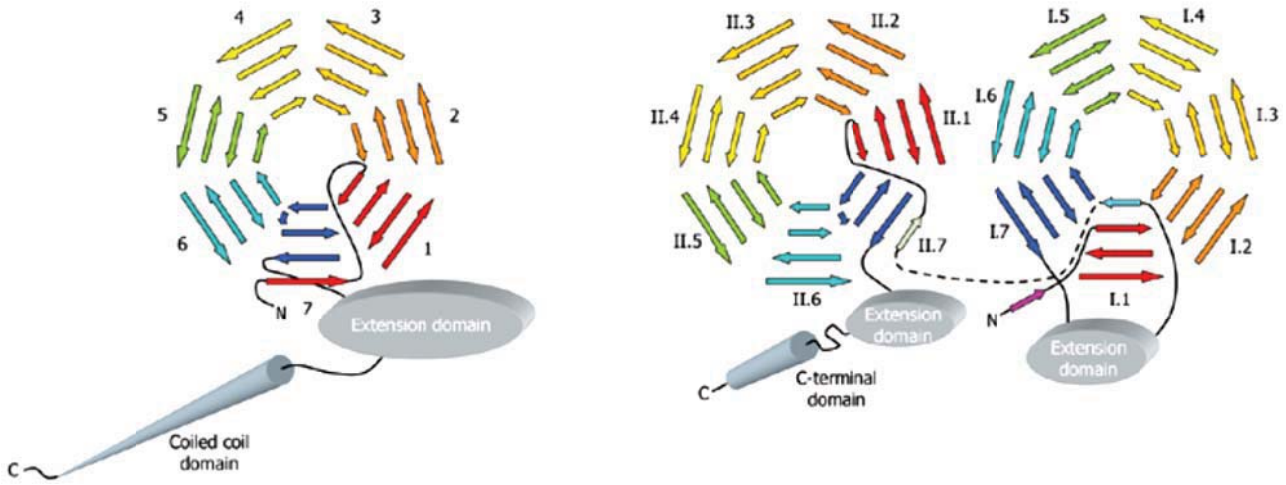

Figure1: Structural topology of the two major classes of coronins. The blades of the beta-propeller domains are colored and numbered. The coiled coil domain is shown in grey (taken from McArdle and Hofmann, 2008).

Coronins function in modulating actin dynamics by binding to filamentous actin and the Arp2/3 complex andall short coronin homologues investigated to date have been shown to bind F-actin. Coronins localize to the anterior regions of migrating cells

where they regulate the actin dynamics in the cortex (Cai et al., 2007). The single coronin in yeast binds to F-actin and microtubules in vitro and regulates dynamic actin structures such as actin patchesin vivo (Goode et al., 1999). In Drosophila, mutations in coronin disrupt the actin cytoskeleton of the embryonic imaginal disks indicating that it is essential for morphogenesis (Bharathi et al., 2004). Mammalian Coronin 7 localizes to the Golgi network and plays a role in Golgi trafficking (Rybakin et al., 2004; 2006)

The *Dictyosteliumdiscoideum* genome encodes two coronin homologues, a short coronin encoded by the *corA* gene with a conserved coiled coil domain and a longer homologue with a tandem beta-propeller domain, CRN7 encoded by the *corB* gene. *D.discoideum*coroninis thefounding member of the coronins and was identified as a co-purifying protein from acto-myosin complexes and has been found enriched in crown like projections in the dorsal surface of the cells (de Hostos et al., 1991). deHostos et al.(1993) later created a coronin deficient cell line which showed several interesting phenotypes implicating the protein in physiological processes like cytokinesis, migration, cell polarity, and morphogenesis.CRN7, a*Dictyostelium* homologue of *Caenorhabditiselegans*POD-1 has been implicated in actin driven processes and *Legionella pneumophila* internalization (Shina et al., 2010). Deletion of both coronin homologues in *D.discoideum*highlightedthe factthat even though they are involved in the same cellular processes, they act antagonistically to each other (Shina et al., 2011).

1.2 Rho GTPases

Rho-like proteins are small, monomeric GTPases of the Ras superfamily. They act as a molecular switch in the cell by cycling between an active (GTP-bound) and an inactive (GDP-bound) conformation. This activation cycle is regulated by two classes of enzymes, GEF and GAP proteins. GEFs or **G**uanosine**E**xchange **F**actors activate the Rho GTPases by exchanging GDP for GTP, and GAP or **G**TPase **A**ctivating **P**roteins increase the intrinsic GTP hydrolysis of Rho GTPases thereby effectively terminating the signal by converting them into an inactive form (GDP-bound). A third class of regulatory proteins known as GDI (**G**DP **D**issociation **I**nhibitors) regulates Rho GTPases by binding to the GDP bound (inactive) form of GTPases and prevent their spontaneous activation (Figure 2). They also bind to the isoprenyl moiety of Rho

GTPases through an immunoglobulin-like fold and regulate their cycle between the cytosol and membrane.

Once activated, the Rho GTPases can bind to several proteins known as effector molecules and transduce the signal to downstream events. Rho GTPases participate in several cellular processes like cell polarity, gene transcription, vesicular trafficking, morphogenesis, and cytokinesis (Etienn-Manneville and Hall, 2002). One of the significant aspects of Rho GTPases is their ability to link membrane receptors to the regulation of the actin cytoskeleton in the cell. Twenty mammalian Rho GTPases have been described so far. Of them,several members of the Rho, Rac, and Cdc42 sub-family were studied in detail. Mammalian RhoA regulates myosin assembly and stress fibres formation,Rac and Cdc42 regulate the formation of actin rich protrusions like lamellipodia and filopodia, respectively (Hall, 1998).

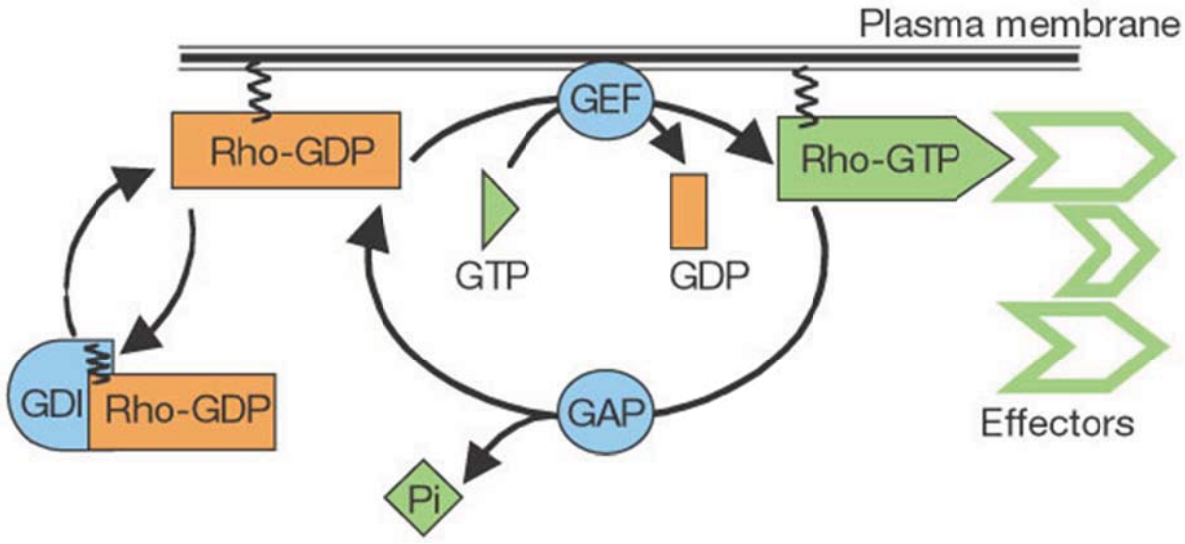

Figure 2: Rho GTPase cycle. Rho GTPases cycle between inactive (GDP-bound) and active forms (GTP-bound). GEFs catalyze the exchange reaction and GAPs inactivates them by increasing GTP hydrolysis rate. The prenylation motif of GTPases is shown in crossed line through which Rho GTPases binds to membrane(taken from Etienne-Manevilleand Hall, 2002.

The *D. discoideum* genome encodes nearly 18 Rac related GTPases, however, a true homologue of Rho or Cdc42 is absent. Some of the Rac GTPases have been studied in detail (Rivero et al., 2001, Vlahouand Rivero, 2006). The Rac1 family

consists of three members (Rac1A, 1B, 1C) and is considered as orthologues of mammalian Rac1. Rac1A, 1B, and 1C regulatethe actin cytoskeleton and cell motility (Faix et al., 1998). In addition, Rac1A and RacE are required for cytokinesis (Larochelle et al., 1997). RacB is required for chemotaxis and morphogenesis (Park et al., 2004), while RacC is implicated in phagocytosis and is regulated by phosphatidylinositol 3-kinaseactivation (Han et al., 2006). RacG is required for the regulation of cell shape, motility, and phagocytosis (Somesh et al., 2006a). RacH has been implicated in vesicular trafficking and intracellular immunity to *Mycobacterium* (Somesh et al., 2006b). Furthermore, the *D. discoideum* genome encodes numerous exchange factors (RacGEFs) and GAPs (RacGAPs) and the function of handful of them are known (Park et al., 2004, Faix et al., 1996). In addition, there are two GDI homologues present and deletion of either one or both of them results in a defective cytokinesis and contractile system (Rivero et al., 2002).

1.3 CRIB effector proteins

Rho GTPases bind to several downstream target molecules known as effector proteins. These proteins specifically interact with the GTP-bound form of Rho GTPases and this is achieved by recognizing conformational changes in the 'effector region' (switch I) of Rho GTPases (Bishop and Hall, 2000). Most of the Rac and Cdc42 effector proteins, if not all, contain a conserved CRIB motif (**C**dc42 and **R**ac – **i**nteractive **b**inding). The CRIB motif is the minimal effector binding region for Cdc42 and Rac and is 15 amino acids long with the consensus ISXPXXXXFXHXXHVG. This small motif forms part of a larger binding region (also known as PBD-**p**21-**b**inding **d**omain or GBD-**G**TPase **b**inding **d**omain) that has been shown to be required for GTPase interaction. The CRIB motif was first reported by Burbelo et al. in 1993. Subsequently, Hall and co-workers identified a host of candidate effectors proteins on the basis of homology searches to the CRIB region (Burbelo et al., 1995). The repertoire of effector proteins expanded phenomenally in the last decade or so and includes protein families having diverse sets of structure and function. Some exemplary families are kinases (ser/thr protein kinase and tyrosine kinase), actin-binding proteins, and scaffold proteins (Bishop and Hall, 2000).

The GBD of WASP (Wiscott Aldrich syndrome protein) and ACK (Cdc42 and Rac-interactive kinase) were the first reported crystallographic structures bound to GTPase(Abdul-Manan et al., and 1999, Mott et al., 1999). Subsequent mutational

studies of the CRIB motif provided a significant insight into the binding interface between GBD and GTPases. The interaction is mediated by both polar and hydrophobic contacts involving highly conserved residues in the CRIB motif. The conserved isoleucine, serine, and proline in the N terminus of the CRIB motif form hydrophobic contacts with the α5 helix of the GTPase. The conserved Asp38 in the switch I region of Cdc42 and Rac1 interacts with one of the conserved histidines (HXXH) in the CRIB motif. Mutation of either of these histidines greatly affects the interaction of CRIB effector with GTPases. The adjacent residues of the CRIB motif show less conservation among CRIB effector proteins. However, these residues make extensive contacts with the switch I and switch II regions of the GTPases and appear to be important in response to the nucleotide switch (Morreale et al., 2000).

The CRIB motif containing effector proteins bind preferentially to GTP-bound Rho GTPases and connect the activation of GTPases to a broad range of downstream responses. Once bound to the active GTPases, the activity of the CRIB effector proteins might be regulated in several ways such as activation, sub-cellular localization and others. The most common mechanism of effector activation by Rho GTPases appears to be the disruption of intramolecular auto-inhibitory interactions, which exposes functional domains within the effector protein. For example, the two related CRIB domain containing effector proteins WASP and N-WASP are regulated by intramolecular inhibition. These proteins share similar domain architecture and are involved in the relay of signals from cell membrane receptors to the actin cytoskeleton. The WASP protein contains an N-terminal GBD (GTPase binding domain) required for Cdc42 interaction, and a C-terminal VCA domain (Veprolin homology, Cofilin homology, acidic region segment) involved in Arp2/3 complex mediated actin polymerization. In an auto-inhibited state, the cofilin homology domain forms an intramolecular auto-inhibitory interaction with the N-terminal GTPase binding domain and effectively masks the VCA domain. Cdc42-GTP activates WASP by binding to the GBD and subsequently relieving the auto-inhibitory intra-molecular interactions. The relieved VCA domain then recruits Arp2/3 complex and initiates actin polymerization (Rohatgi et al., 2000). A similar mechanism of activation has also been observed in other Rho GTPase target proteins like PAK (kinase activity), mDia (actin polymerization) and several others (Hoffman and Cerione, 2000). A different mode of activation has been observed in IQGAP protein, which is involved in

actin cross-linking function where Cdc42-GTP was shown to regulate the cross-linking activity of IQGAP in vitro (Fukata et al., 1997).

In recent years, proteins with less conserved CRIB motifs have also been shown to interact with Rho GTPases in a GTP dependent manner. For example, POSH2 is an E3 ligase and scaffold protein that interacts with Cdc42-GTP through a partially conserved CRIB motif (conserved ISxP sequence) (Kärkkäinen et al., 2010). Similarly, phospholipase D2 (PLD2) contains two weakly conserved CRIB motifs in its PH domain through which it interacts with Rac2 (Peng et al., 2011). In some cases, proteins with a partial CRIB sequence use its adjacent structural modules for GTPase interaction. Par6 in *Drosophila* has been shown to be required for neuroblast cell polarity and asymmetric cell division. It contains a PDZ domain downstream to a partial CRIB motif. Both domains are required for Cdc42-GTP interaction (Garrard et al., 2003;Joberty et al., 2000). PlexinB1, a functional semaphorin receptor has been reported to contain a partial CRIB sequence embedded in its cytoplasmic domain. Deletion analysis showed that the plexin-Rac binding domain is significantly larger than the CRIB motif (Haris et al., 2000).

1.4 CRIB effector proteins in *D. discoideum*

In *D. discoideum*, several Rac effector proteins have been characterized in detail. DGAP1 and GAPA are two IQGAP-related proteins that regulate cytokinesis in *D.discoideum*. Both proteins interact with Rac1a through a conserved GRD (GTPase related domain) domain. The actin binding proteins cortexillin I and II form a quaternary complex with DGAP1 or GAPA and activated Rac1a (Faix et al., 1998;Mondal et al., 2010). Filamin, an actin cross-linking protein has been shown to interact with activated Rac1a and associates with cortexillin I (Mondal et al., 2010). Formin homology proteins regulate cytoskeletal remodeling during cytokinesis, cell polarity, and development. The diaphanous or Dia-related FH proteins (DRFs) constitute a subclass of FH related proteins and a *D.discoideum*formin homologue, mDia2, has been shown to localize to filopodia tips and regulate actin polymerization. mDia2 binds to activated Rac1a and is required for filopodia formation (Schirenbeck et al., 2005). RacC interacts with WASP and stimulates F-actin polymerization via the activation of WASP. RacE is requited for 14-3-3 localization to the cortex and 14-3-3 mediated myosin II assembly (Robinson DN, 2010). However, no direct RacEinteraction partner has beenreported so far. RacB specifically interacts with

Pakaand Pakc in its activated form and regulates myosin II assembly and chemotaxis through PAKs activation (for details see next chapter).

1.5 Pakkinases – structure and functions

PAK or **p**21-**a**ctivated **K**inases are one of the major downstream effectors of Rho GTPases. They contain an N-terminal regulatory domain and a C-terminal conserved catalytic domain. The N-terminal domain harbors the CRIB motif that is responsible for Rac or Cdc42 binding. This motif forms a part of a more inclusive domain,PBD, that contributes to overall binding affinity. The PBD also overlaps with an auto-inhibitory segment in its C-terminus known as 'inhibitory switch' that controls the basal kinase activity. Additionally, a conserved binding site for the Gβγ subunit complex of heterotrimeric G proteins exists at the extreme C terminus. PAK1 exists as a homodimer in the cells in a trans-inhibited conformation. The N-terminal regulatory domain of one PAK1 molecule binds and inhibits the C-terminal catalytic domain of the other with the PDB/CRIB region forming the dimerization interface. The binding of Rac or Cdc42-GTP to the CRIB domain relieves this inhibited conformation and subsequent trans-auto phosphorylation at many sites leads to kinase activation (Figure 3). PAKs have been shown to regulate cell shape and polarity through phosphorylation of several cytoskeletal proteins like myosin (Zhao and Manser, 2012; Bokoch, 2003).

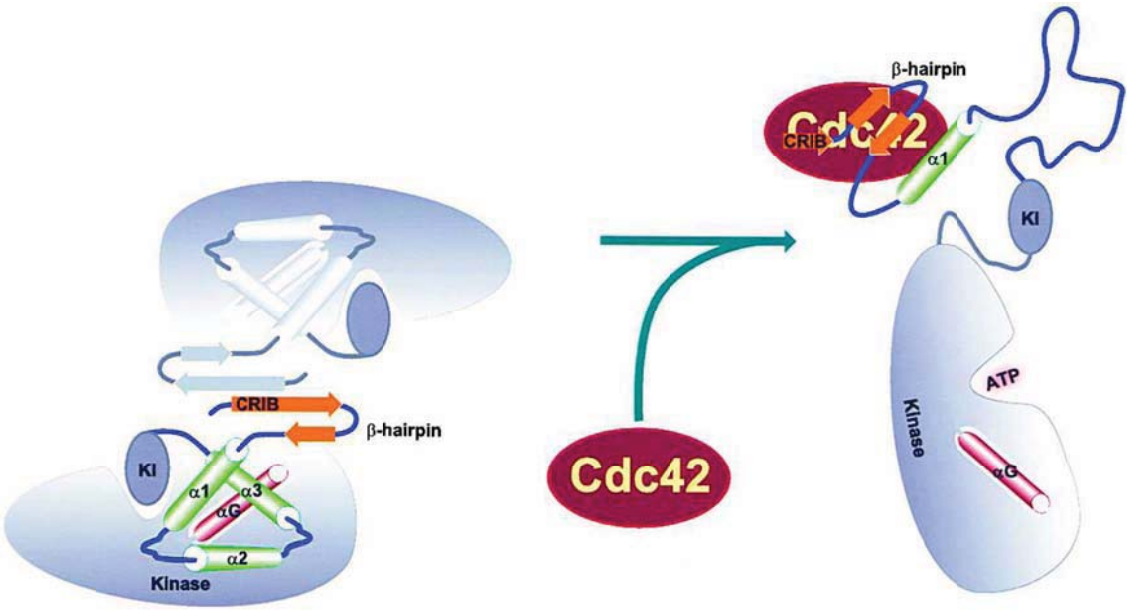

Figure3: PAK kinase activation. PAK exists as a dimer and the kinase domain is sequestered in the dimer interface. Binding of Cdc42-GTP relieves this auto-

inhibition and activates PAK. The CRIB motif and Cdc42 are highlighted in orange and red (taken from Hoffman and Cerione, 2000).

1.6 Myosin II regulation in *D.discoideum*

Myosin II is a conventional, two headed myosin that consists of two copies each of a myosin heavy chain (MHC), an essential light chain (ELC), and a regulatory light chain (RLC). A universal property of myosin II is the ability to spontaneously self-assemble into bipolar filaments. Filament assembly and dis-assembly is regulated by phosphorylation of the light and heavy chains and forms the basis for myosin II mediated regulation of cellular processes like cytokinesis and cell movement. In *D.discoideum*, myosin II heavy chain is encoded by a single gene (mhcA) and deletion of mhcAled to a plethora of defects. *mhcA*$^-$ cells showed a defective cytokinesis in suspension and were unable to develop beyond the mound stage. During chemotaxis, *mhcA*$^-$cells were unable to suppress lateral pseudopod formation and showed a cell polarity defect. Additionally, *mhcA*$^-$ cells showed a reduced cell speed due to their inability to retract the uropod (Titus et al., 1993). The myosin II in *D. discoideum* can self-assemble into bipolar filaments. The formation of these filaments is negatively regulated by phosphorylation of three conserved threonine residues in its heavy chain (Stites et al., 1998). Expression of neither a phosphomimetic mutant (3XASP) nor a non-phosphorylatable mutant (3XALA) could rescue the *mhcA*$^-$ defects demonstrating the dynamic regulation of heavy chain phosphorylation is required for myosin II functions (Bosgraaf and van Haastert, 2006, de la Roche et al., 2002).

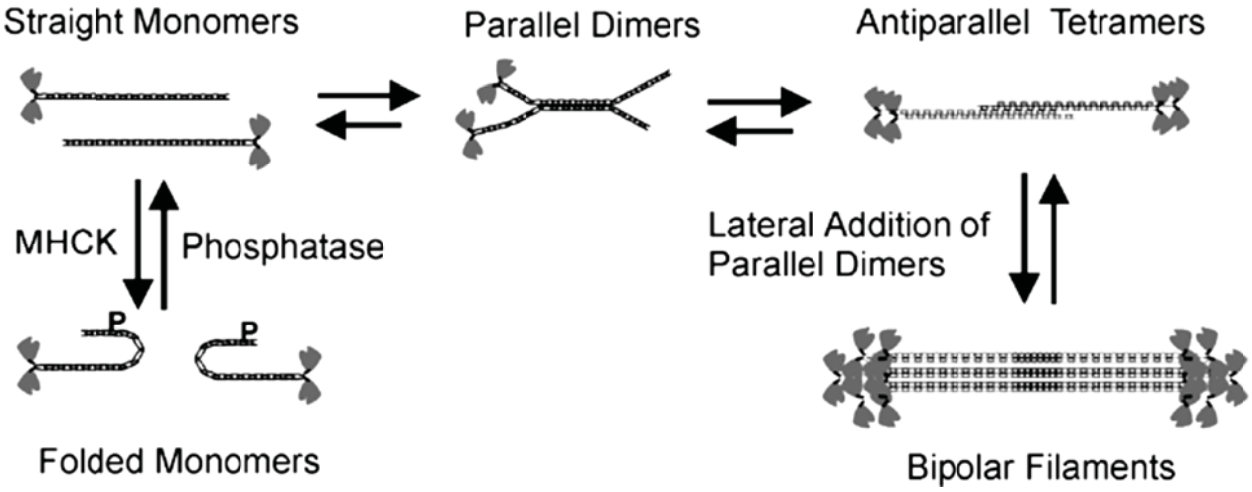

Figure 4: Myosin II regulation in *D. discoideum*(taken from de la Roche et al., 2002).

D. discoideum encodes four myosin heavy chain kinases, MHCKA through MHCKD, which are responsible phosphorylation of the three threonine residues in the MHC tail region that are critical for filament formation (Luo et al., 2001). They belong to the alpha-kinases family and apart from a conserved catalytic domain; they possess a WD repeat domain that is required for substrate targeting. Each MHCKs displays different cellular localization and was suggested to have separated functions in the cell. MHCKA is primarily localized in the anterior F-actin rich regions where it enables formation of new pseudopods by disrupting myosin filaments. MHCKC localization is dependent on myosin II and has been suggested to regulate myosin II during uropod retraction and cleavage furrow formation. MHCKB is localized in the cytosol and may serve a role in maintaining basal MHC phosphorylation levels (de La Roche et al., 2002). The molecular mechanisms underlying activation and localization of MHCKA have been studied in detail. MHCKA has been shown to be activated primarily by auto-phosphorylation and recently, it was shown that F-actin can activate MHCKA through increasing its auto-phosphorylation rate. MHCKA contains a coiled-coil domain through which it binds to F-actin and localizes to anterior regions (Steimle et al., 2001).However, it was suggested that the kinase activity one or more MHCKs was negatively regulated by upstream PAK kinases during cell migration: particularly in the posterior regions of migrating cells (Chung and Firtel, 1999).

1.7*D. discoideum* family of PAK kinases

The *D. discoideum* PAK kinase family consists of three members, PAKa, PAKb, and PAKc, with a conserved CRIB motif in the N-terminal regulatory region and a catalytic domain in its C-terminus. PAKa has been shown to localize to the posterior regions of migrating cells where myosin II is enriched (Chung and Firtel, 1999). Cells deficient in PAKa have been shown to exhibit altered F-actin cytoskeleton, defective cytokinesis and cell polarity. PAKa tagged with a membrane targeting signal was used to show that PAKa activates actin polymerization at the cortex. Most strikingly, PAKais required for myosin II assembly and it regulates myosin assembly by inhibiting one or more myosin heavy chain kinases (MHCKs), however it did not phosphorylate myosin directly. A dominant negative PAKa containing the CRIB and the catalytic domain has been shown to inhibit phagocytosis by Müller-Taubenberger et al. (2002). In

addition,this group showed that PAKa can interact with Rac1a specifically in its GTP-bound form, and that a small region in the N-terminus is required for PAK localization to the cortex. PAKb was shown as a heavy chain kinase for unconventional myosin I and is involved in the formation of phagosomes (de la Roche et al., 2005). PAKcis a PH domain containing member and localizes to the plasma membrane upon chemo-attractant stimulation. PAKc kinase activity increased upon cAMP stimulation and was activated specifically by RacB. A PAKc carrying a mutation in the CRIB motif exhibited higher basal kinase activity highlighting the role of Rac GTPases in PAK regulation (Lee et al., 2004). However, PAKccould also regulate myosin assembly. However,PAKa has been suggested to be the major player in regulation of myosin dynamics. Accordingly, Chung and Firtel(1999) suggested that PAKa localizes to the rear of the moving cells and enhances myosin assembly by inhibiting the activity of heavy chain kinases. PAKain turn is activated by phosphorylation by Akt and Rho GTPases.

1.8. Aim of this study

The aim of this work is to study the interaction specificity of coronins and Rho GTPases in the model system *D. discoideum* and

1. to analyze and characterize the putative CRIB domain of *D. discoideum* coronin proteins,
2. to understand the significance of Coronin-Rac interaction in the physiological processes of the cell,
3. to elucidate the molecular signaling pathway in which coronin and Rho GTPases are involved.

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2. Results

2.1.1 Sequence and structure of the coronin CRIB motif

Coronin proteins belong to the family of WD repeat domain containing proteins and regulate the actin cytoskeleton of the cell. Recently, it was reported that mammalian coronin proteins contain a CRIB like motif between blade 2 and blade 3 of their beta-propeller domain (Xavier et al., 2008). The CRIB domain is a small stretch of a conserved amino acids sequence that enables the proteins containing it to recognize and bind Rho GTPases (Burbelo et al., 1993). We have identified a similar motif in the *D. discoideum* coronin sequence using sequence alignment. We further compared the CRIB domain of *D. discoideum* coronin proteins with known CRIB domain sequences and found a high degree of conservation (Figure 5).

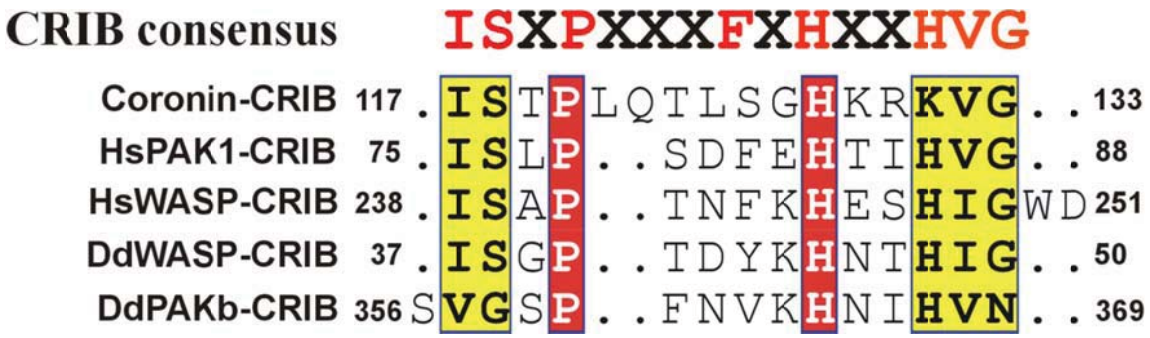


Figure 5: Sequence alignment of the CRIB domain in coronin with similar regions of selected CRIB-containing proteins. The consensus CRIB motif is shown on the top. Highly conserved sequences are shown in red boxes and similar amino acids are boxed yellow.

D.discoideum short coronin contains a highly conserved CRIB motif between amino acids 117 and 133. The N-terminal half of the CRIB motif contains hydrophobic amino acids and these amino acids are known to form extensive contacts with the α5 helix of Rho GTPases. The two conserved histidines in the C-terminus of the CRIB motif interacts with the switch I region of Rho GTPase and determines nucleotide specificity (Abdul-Manan et al., 1999). In *D.discoideum* coronin, there is a substitution in one of the conserved histidine residues and the phenylalanine (F) is completely absent (Figure 5).

D. discoideum encodes also a homologue of the long coronins, CRN7, with a tandem beta-propeller domain (Shina et al 2010). We retrieved the CRIB sequence present in each of its propeller domains and aligned them with known CRIB domains for comparison. We have also included the CRIB sequence from human coronin CRN2 and mammalian POD homologue CRN7 (Figure 7).

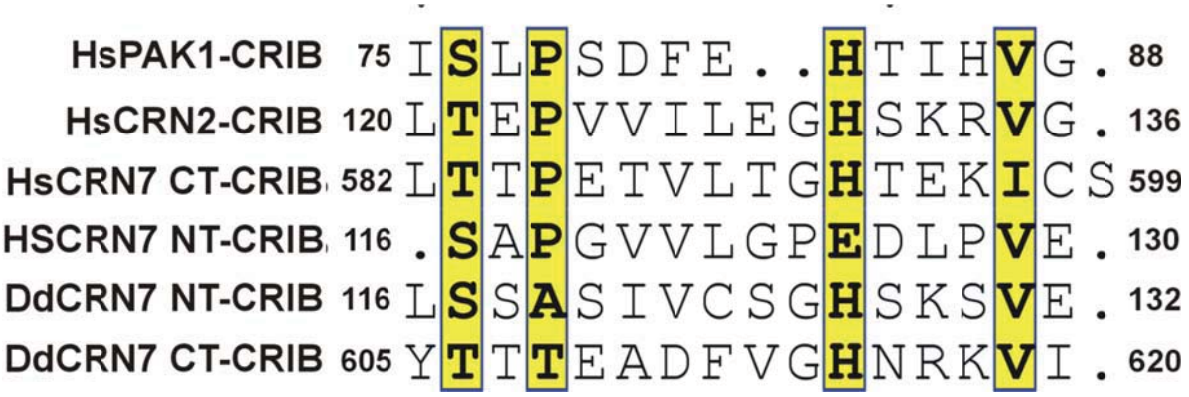


Figure 6: Sequence alignment of the CRIB domain of mammalian coronin and CRN7 homologues with similar amino acids boxed yellow.

The comparison of the CRIB domain sequence of CRN7 and mammalian CRN2 showed a somewhat lesser conservation of the critical amino acids. While all proteins share a conserved N-terminal half of the CRIB domain, the conservation in the C-terminal half is very weak.

2.1.2 Structural analysis of the coronin CRIB domain

Sequence alignment of the *D. discoideum* coronin proteins with the secondary structural elements of the recently crystallized coronin1A protein showed that the CRIB motif is in a surface accessible loop. To gain further insight into the structure of the coronin CRIB domain, we modeled the structure of *D. discoideum* coronin using the coronin1A (2AQ5) structure as a template (Appleton et al., 2006).

To investigate how well the modeled structure matches the X-ray data, the coronin model and known crystal structures (2AQ5) were superimposed on their backbone atoms and analyzed. The overall arrangements of the secondary structures in the model are in good agreement with the corresponding elements in the X-ray structure (Figure 7.1). To assess the model accuracy, the RMSD (**R**oot **M**ean **S**quare **D**eviation) values between superimposed model and crystallographic structures were

calculated. The RMSD value for the backbone atoms was found to 0.29 Ao suggesting an optimal modeling quality. To determine the stereo-chemical quality of the homology model, we used PROSA-Web, an online tool for protein structural analysis and found that the Z-score calculated by the analysis tool agrees well with good modeling quality. Furthermore, we used the Swiss-PDB viewer to view the Ramachandran plot and observed that a higher percentage of amino acids in the model lies in the favored regions of the plot (data not shown).

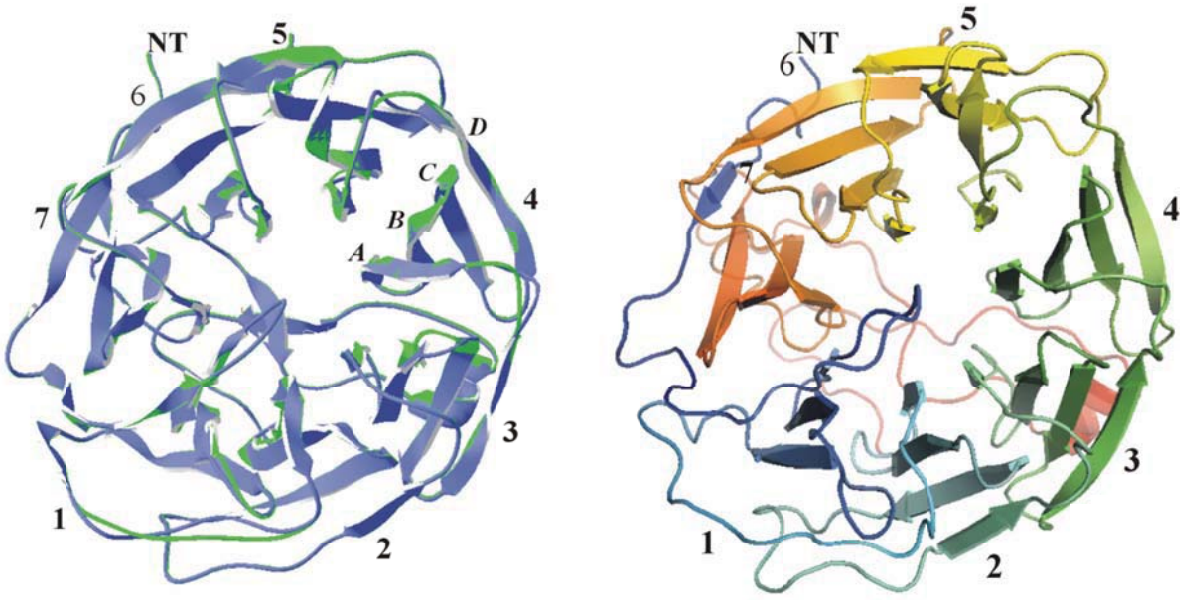


Figure 7.1: Structural modeling of the *D. discoideum* coronin protein. The *D. discoideum* coronin structure was modeled using coronin1A (2AQ5) as a template. A superimposed image of the modeled structure in green and the known structure in blue is shown at the left. At the right the final modeled structure is shown.

We have used the modeled coronin structure to determine the structure and position of the CRIB domain. We found that the N-terminal half of the CRIB domain is surface exposed while the C-terminal sequences are embedded in the beta-sheets and may be involved in hydrogen bonding with neighboring beta sheets and therefore be unavailable for interaction (Figure 7.2).

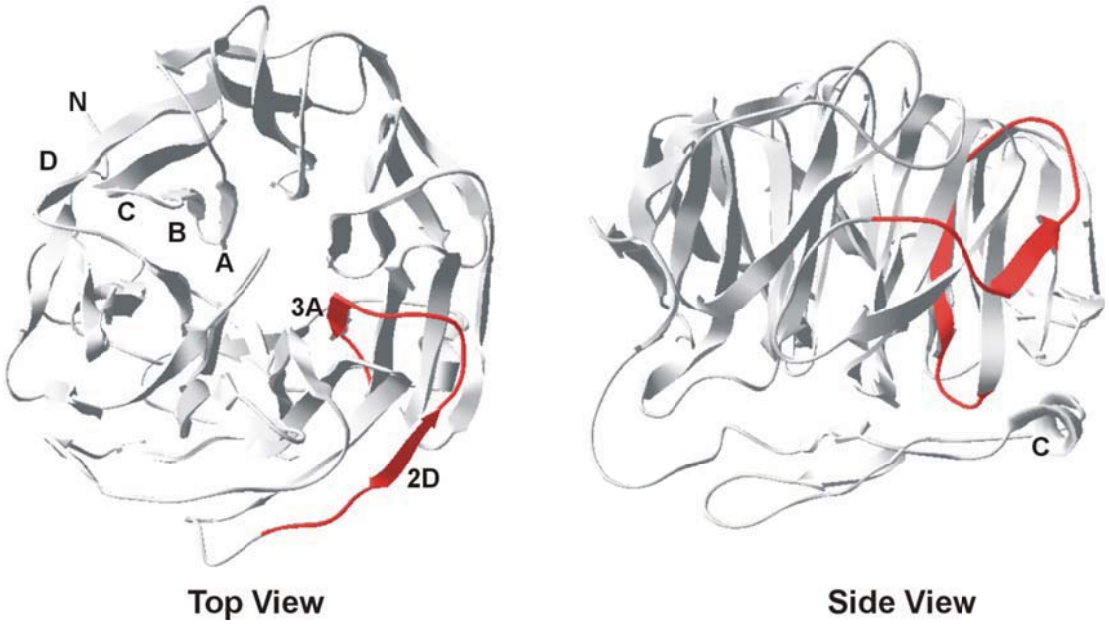
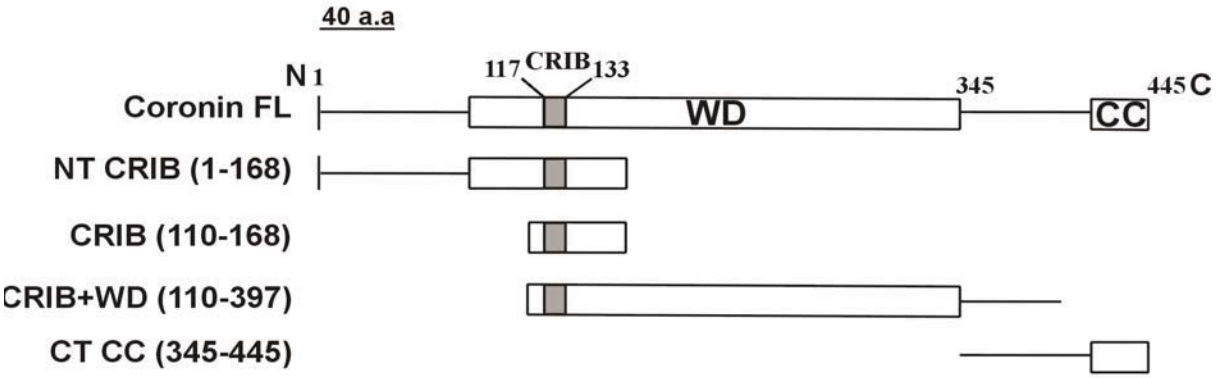


Figure 7.2: Location of the *D. discoideum* coronin CRIB domain in the modeled structure. The *D. discoideum* coronin structure was modeled using coronin1A (2AQ5) as a template. Both the top view and the side view of the structure is shown with the CRIB domain highlighted in red.

2.2.1 Expression and purification of *Dictyostelium* coronin GST-fusion constructs.

To address the biochemical properties of Coronin CRIB domain, we generated four different GST-fusion constructs. The constructs were: the CRIB domain of coronin representing the last strand of blade 2 and part of blade 3 (GST-CRIB, amino acids 110-168), a construct containing the CRIB domain together with the entire N-terminal part of coronin (GST-CRIB-NT, amino acids 1-168), a fusion construct of the core WD repeat domain (blades 2-6, amino acids 110-397) and the C terminal domain without the CRIB motif (amino acids 345-445) (Figure 8A). The DNA sequences encoding the respective regions were cloned into the bacterial expression vector pGEX 4T-2. The DNA was transfected into *Escherichia coli* strain XL1-blue. Cells were allowed to grow until the OD600 reached 0.6-0.8 before inducing them with 0.5 mM IPTG (isopropyl β-D-thio-galactoside). The induced cells were lysed and the polypeptides were purified using Glutathione-Sepharose beads (GE Healthcare) (Figure 8B).

A



B

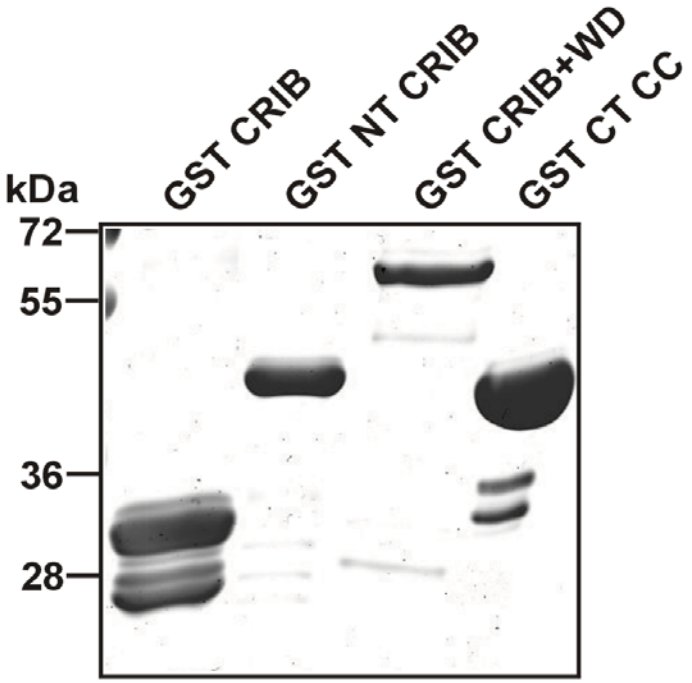


Figure 8: A. Schematic diagram showing the different GST fusion polypeptides of coronin. The position of the amino acids is indicated. The modular structure of coronin with the location of the CRIB domain is shown above for comparison. B. The different GST fusion peptides were expressed in *E.coli* XL1-blue and purified as GST fusion products using Glutathione Sepharose beads. The polypeptides were separated on a 12 % polyacrylamide gel and stained with Coomassie Blue. On the left the position of molecular weight markers is indicated.

2.2.2 Interaction of the Coronin CRIB motif with Rac GTPases

To test if the CRIB domain of coronin can interact with Rho-like GTPases of *D. discoideum,* we carried out an *in vitro* GST pull-down assay. The *D. discoideum* genome codes nearly 18 Rho-like GTPases and almost all of them belong to the Rac GTPases sub-family of Rho-like small GTPases. We expressed several of the *Dictyostelium* Rac family GTPases as GFP-fusion proteins in AX2 wild type cells: Rac1a, a homologue of mammalian Rac1 proteins, and RacE which might be the *Dictyostelium* equivalent of the mammalian Rho GTPase. Cells expressing the respective GFP-fusion proteins were lysed and the lysates were loaded onto Glutathione-Sepharose columns containing different coronin GST-fusion peptides. The columns were washed several times after two hours of incubation and the bound proteins were probed with GFP monoclonal antibody K3-184-2.

In our *in vitro* pull down experiments, the coronin GST-fusion peptides harboring the CRIB domain could precipitate GFP-Rac1a from the cell lysate while the GST control did not. This data suggests that the minimal Rac1a GTPase binding region in coronin is in fact a CRIB motif (Figure 9).

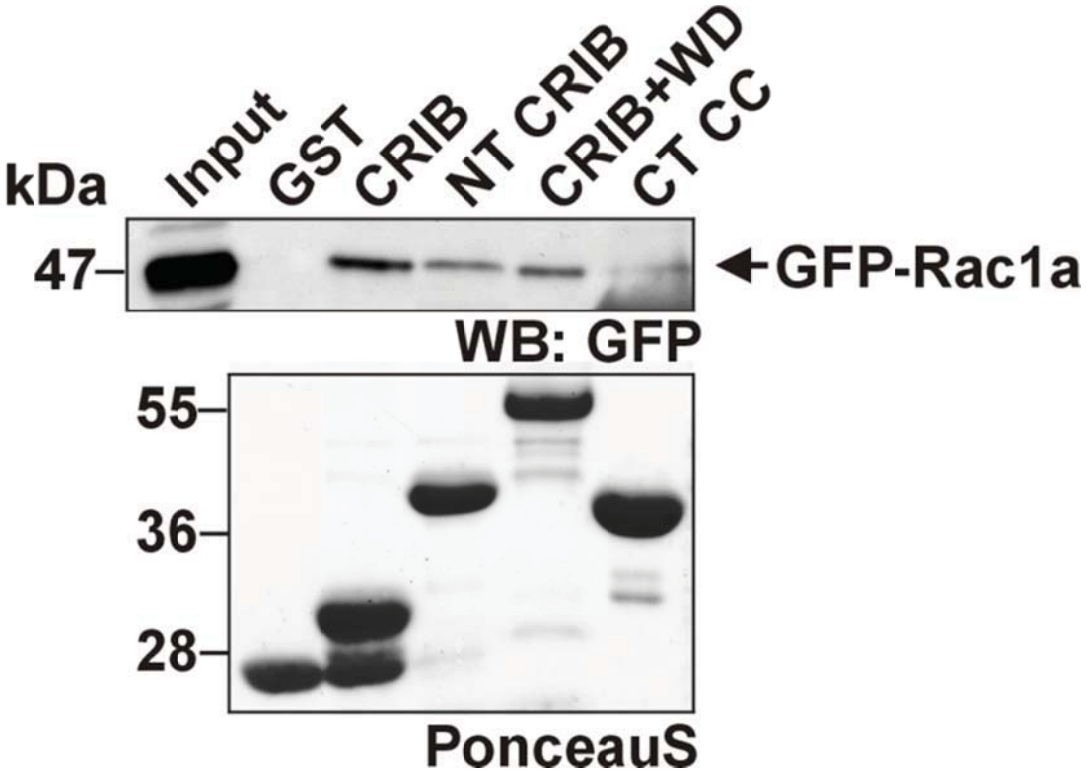


Figure 9: Glutathione-Sepharose beads coated with GST, GST CRIB, GST NT CRIB, GST CRIB+WD, and GST CT CC were incubated with AX2 cell lysates expressing GFP-Rac1a. The pull-down eluate was immunoblotted with GFP specific mAb K3-184-2.The GST fusion proteins were detected by PonceauS staining.

We further tested if this small region could also interact with other Rac GTPases in *D. discoideum* such as RacE or RacC (see below). Cell lysates of AX2 cells expressing GFP-RacE were incubated with equal amounts of GST and GST-CRIB recombinant proteins bound to Glutathione-Sepharose beads. As shown in Figure 10, the CRIB domain of coronin did interact with RacE. Some binding was observed with the GST control.

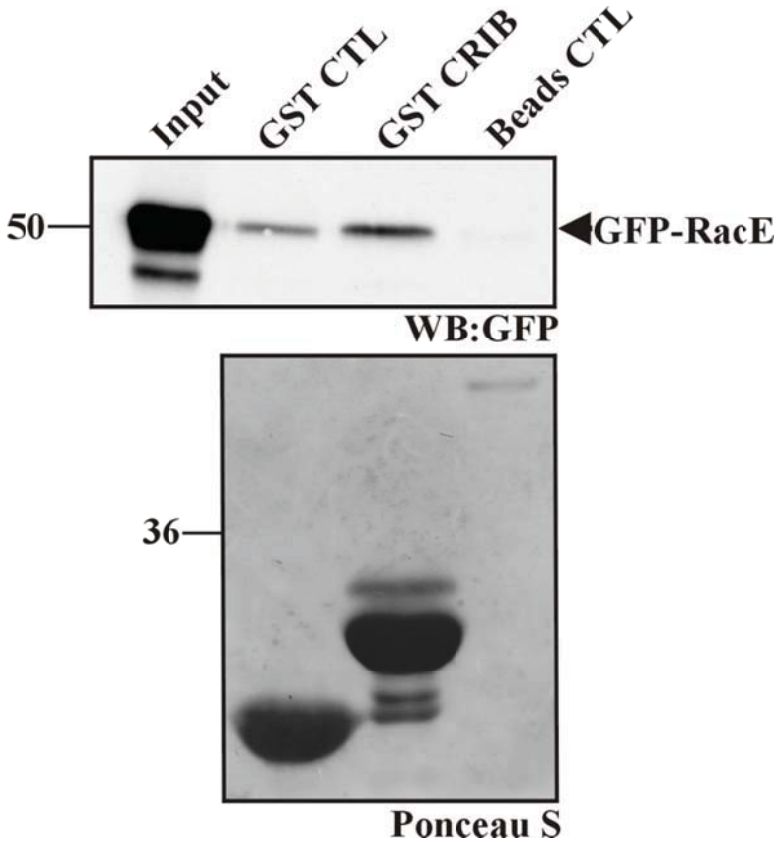
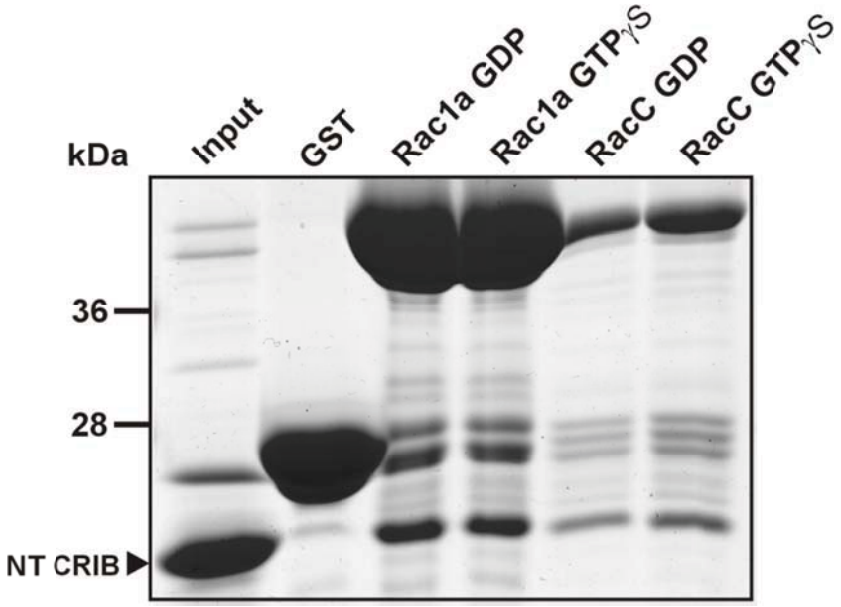


Figure 10: Glutathione-Sepharose beads coated either with GSTor GST-CRIB or uncoated beads were incubated with AX2 cell lysates expressing GFP-RacE. The bound protein was immunoblotted with GFP antibody mAbK3-184-2. The GST fusion proteins were detected by PonceauS staining. The molecular weights are indicated at the left.

2.2.3 Direct interaction of the Coronin CRIB motif with Rac GTPases

To determine whether the interaction of coronin with Rac GTPases is direct and whether coronin interacts with the Rac GTPases in their inactive (GDP bound) or active (GTP bound) states, we released the CRIB from GST-NT CRIB by thrombin cleavage and purified the CRIB fragment with an apparent molecular mass of 18.000 corresponding to aa 1-168. This purified polypeptide was further incubated with equal amounts of GST or GST-Rac GTPases preloaded with either GDP or GTPγS. After repeated washing, the bound proteins were analyzed by Coomassie Blue staining. These *in vitro* binding experiments showed that the coronin-CRIB motif interacted with *Dictyostelium* Rac GTPases Rac1a and RacC (Figure 11A). Additionally, we quantified the amount of recombinant peptide bound to either GDP or GTPγS loaded forms of Racs. Upon plotting the graph with the input set at 100%, we observed that the CRIB domain of coronin slightly preferred the active form of the Rac proteins (GTP loaded). While nearly 44.7% of the input CRIB fragment bound to Rac1a loaded with GTPγS, only 37% was bound to GDP-Rac1a. Similarly, RacC also showed increased binding with the CRIB fragment (30%) when loaded with GTPγS as compared to the GDP-RacC (27%) (Figure 11B).

A



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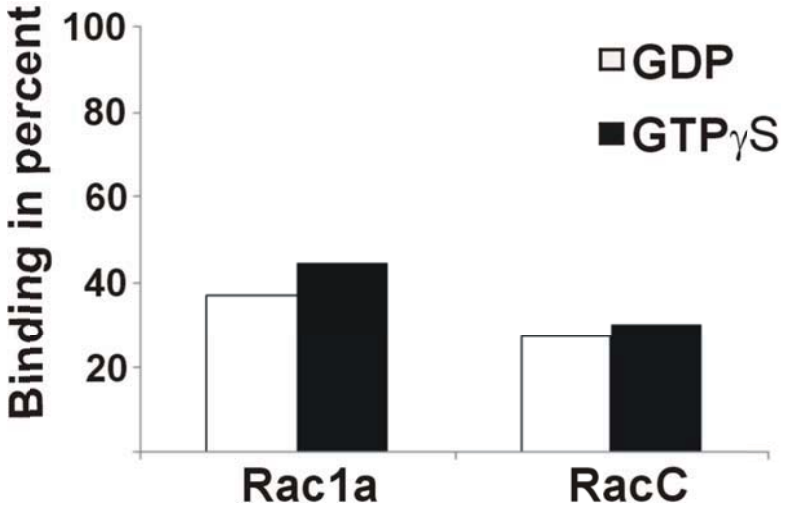


Figure 11: Direct interaction of the CRIB domain with Rac GTPases. A. Thrombin cleaved NT CRIB peptide was loaded onto columns containing GST-Rac1a and GST-RacC that were preloaded either with GDP or GTPγS. After one hour of incubation, the bound proteins were separated on 15% polyacrylamide gels and stained with Coomassie Blue. Molecular weight markers are given on the left in kDa. B. The bound CRIB peptide was quantified using imageJ and plotted in a graph with the input set at 100%. The data represent the average of two independent experiments.

These data suggest that the isolated CRIB domain of coronin has some preference for the activated form of Rac GTPases and agrees well with known CRIB-Rac interactions (Burbelo et al., 2001).

2.2.4 Subcellular localization and dynamics of the coronin CRIB domain

To further understand the *in vivo* functions of the CRIB domain of coronin, we cloned the DNA fragment coding for the CRIB domain into the GFP expression vector pBsrN2 under the control of actin15 promoter. This plasmid (GFP-cor-CRIB) was transfected into AX2 cells and the localization of the coronin CRIB domain was studied using immunofluorescence and live cell imaging. We followed the localization of GFP-cor-CRIB during random movement of *Dictyostelium* cells and observed that GFP-cor-CRIB was distributed throughout the cytosol and was recruited to the

extending pseudopod structure as shown in the time-lapse video microscopy images (Figure 12).

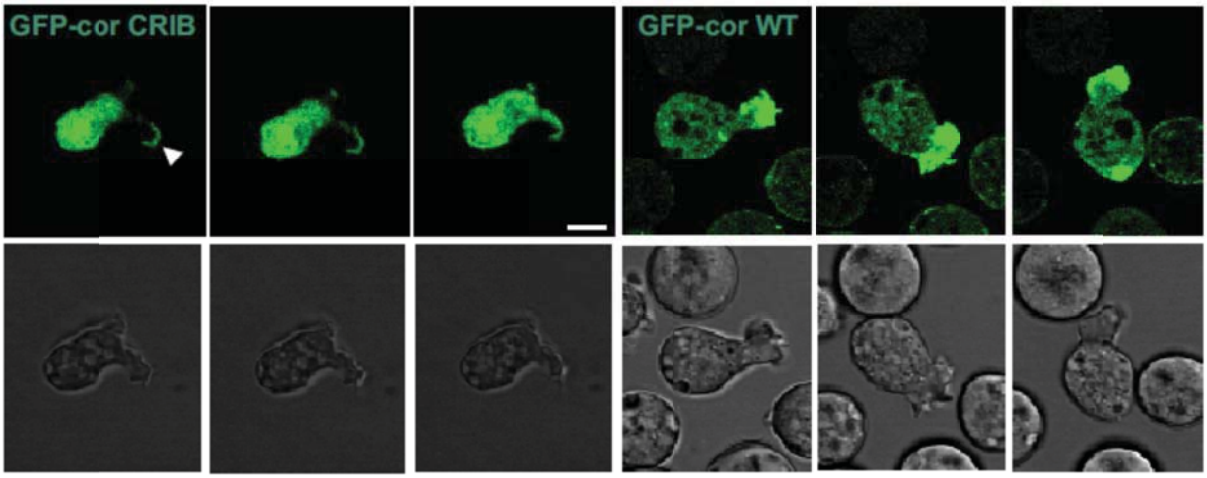


Figure 12: Localization of the coronin CRIB domain. Live cell imaging of GFP-cor-CRIB in AX2 cells. Cells were imaged with the confocal laser scanning microscope for several minutes in starvation buffer. Representative images at selected time points are shown. The recruitment of GFP-cor-CRIB to the protrusions is shown with arrowhead. Right panels, localization of GFP-tagged wild type coronin in AX2 cells. The lower panels show the phase contrast images. Scale bar, 5 µm.

Additionally, we have found that the translocation of GFP-cor-CRIB was spatially restricted and transient. GFP-cor-CRIB was recruited specifically to the rim of the extending pseudopod within a few seconds after the cell started to extend the protrusion as shown by the arrowhead in Figure 12. This translocation continued until the entire cortical region of the pseudopod was labeled with GFP-Cor-CRIB. On the other hand, the full length GFP-coronin, which was recorded for comparison, was enriched in the entire pseudopod along with cytosolic distribution as reported earlier (deHostos et al., 1991;1993; Gerisch et al.,1995) (Figure 12).

2.3 Interaction of *Dictyostelium* coronins with Rac GTPases

Considering the presence of a CRIB domain in the two *Dictyostelium* coronin proteins, we tested the interaction of *D. discoideum* Rac GTPases with coronins *in vitro*. We purified GST fused *D. discoideum* Rac GTPases expressed in *E.coli* using Glutathione-Sepharose beads and loaded them with GDP or GTPγS to mimic inactive (RacGDP) and active (RacGTP) forms of Rho GTPases. These *in vitro*

loaded GTPases were then incubated with AX2 cell lysates and after repeated washing of beads, the proteins bound were separated by SDS-PAGE and transferred to a membrane which was probed for bound coronin proteins using appropriate antibodies.

In order to test the specificity of the assay, we first conducted an *in vitro* binding assay for a known interaction partner, DGAP1. DGAP1 is a member of the IQGAP family proteins and a known effector of *D. discoideum* Rac GTPases. DGAP1 regulates several cellular processes in *D. discoideum* like cell motility, cytokinesis, and actin polymerization. It has been shown to interact preferentially with GTP loaded Rac GTPases, especially of the *D. discoideum* Rac1 family (Faix et al., 1998). GST-Rac1a bound to Glutathione-Sepharose beads was preloaded with GDP or GTPγS and incubated with AX2 cell lysates. The bound proteins were transferred to nitrocellulose membrane and probed for DGAP1 with specific antibodies. As shown in Figure 13, DGAP1 preferentially bound to Rac1a loaded with GTPγS.

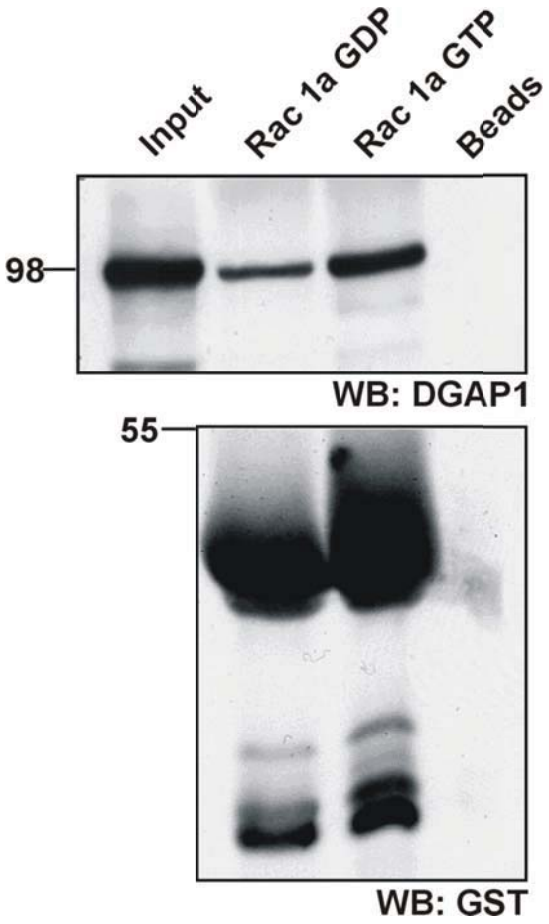


Figure 13: Rho GTPase interaction assay. GST-Rac1a bound to Glutathione-Sepharose beads was loaded with GDP or GTPγS and incubated with AX2 cell lysates. After repeated washing of beads, the bound proteins were separated on SDS-PA gels, transferred to nitrocellulose membrane and probed for DGAP1 using mAb 216-394-1. The GST fusion proteins were detected by polyclonal GST antibodies. The molecular weights are indicated on the left in kDa.

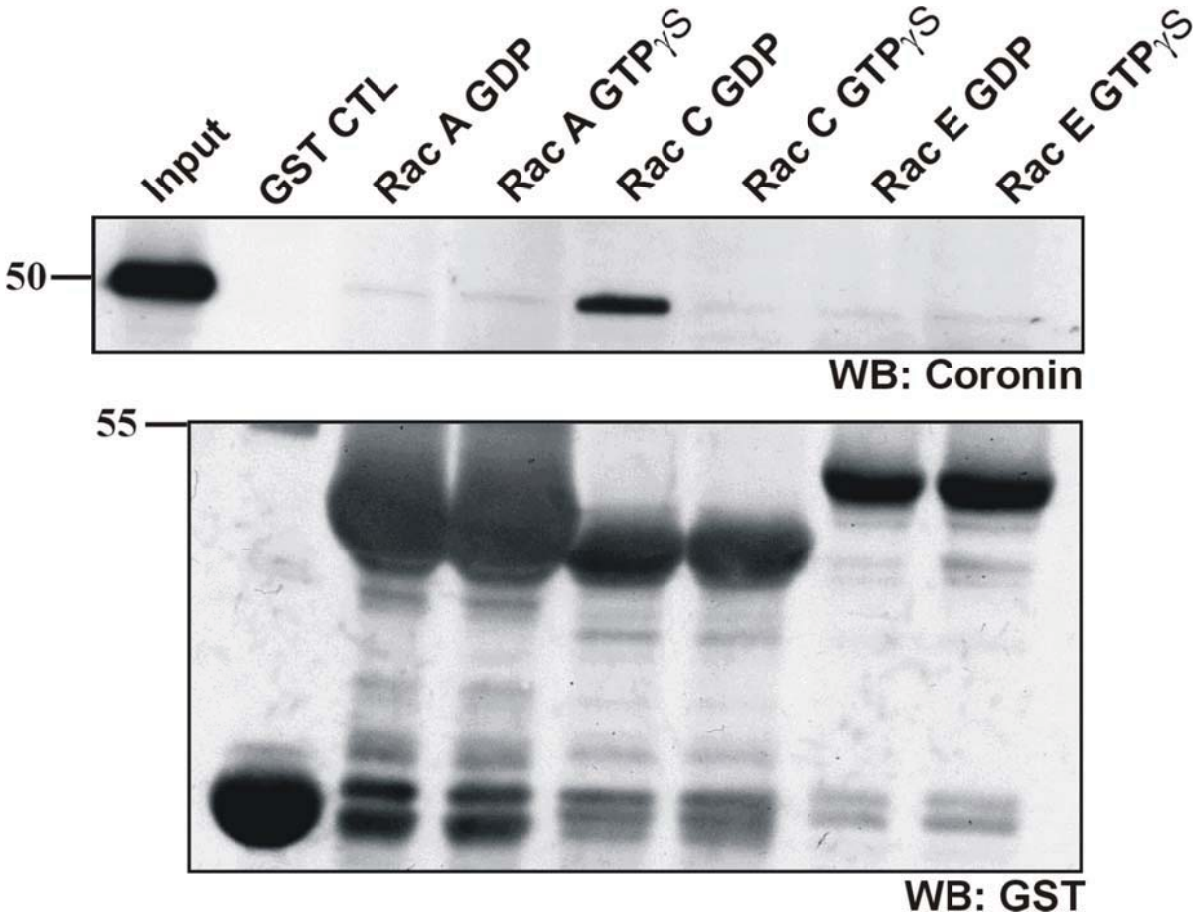


Figure 14: Coronin-Rac interaction. GST, GST-RacA, GST-RacC and GST-RacE bound to Glutathione-Sepharose beads were loaded with GDP or GTPγS and incubated with AX2 cell lysates. After repeated washing of beads, the bound proteins were resolved by SDS-PAGE and probed for coronin with mAb 176-3-6 (de Hostos et al., 1991). GST fusions were revealed by probing with polyclonal GST antibodies. Molecular weight markers in kDa are indicated at the left.

We extended this pull-down experiment to investigate the interaction specificity of *Dictyostelium* short coronin with Rac GTPases. Equal amounts of GST and a series of GST-Racs (RacA, RacC, and RacE) bound to Glutathione-Sepharose beads were

incubated with AX2 cell lysate. The bound proteins were immunoblotted with coronin antibody. Coronin bound preferentially to RacC GTPase loaded with GDP (RacC-GDP) (Figure 14).

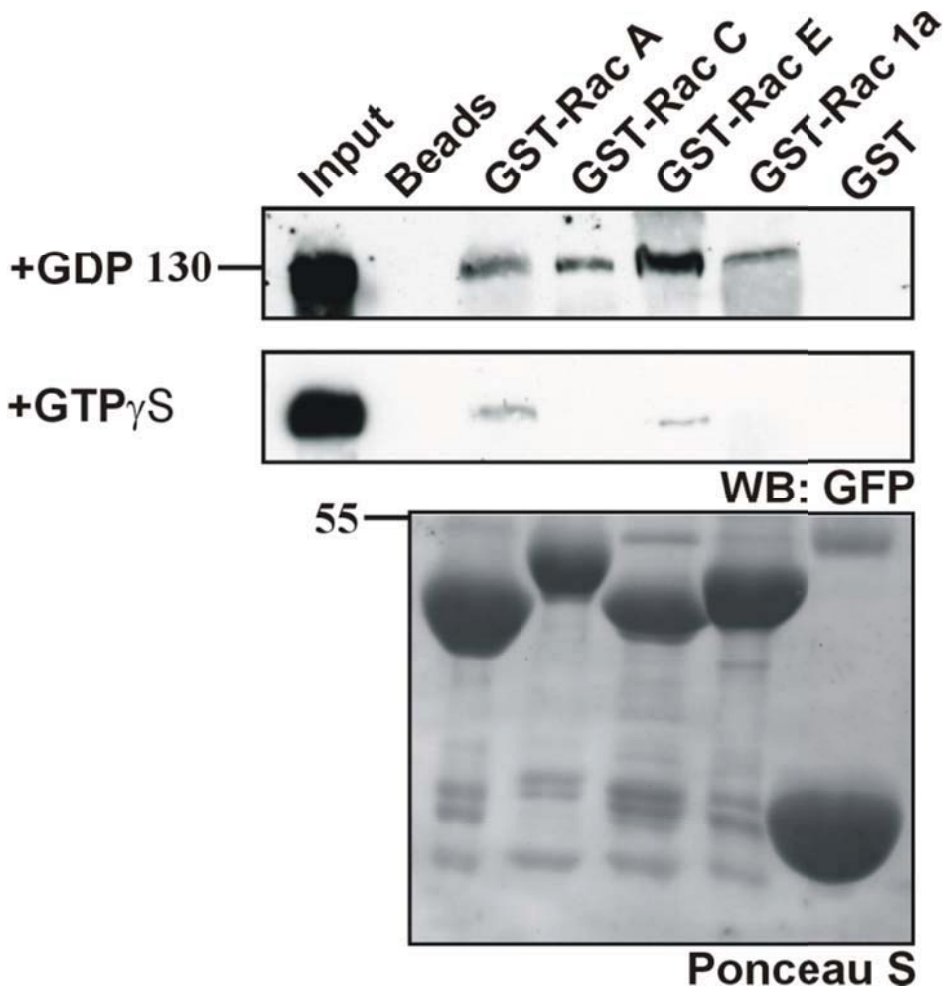


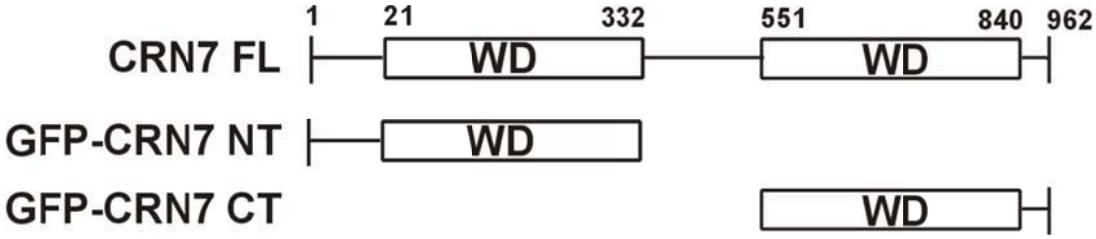
Figure 15: CRN7-Rac interaction. GST, GST-RacA, GST-RacC, GST-RacE and GST-Rac1a bound to Glutathione-Sepharose beads were loaded with GDP or GTPγS and incubated with AX2 cell lysates expressing GFP-CRN7. After repeated washing of beads, the bound proteins were transferred to a nitrocellulose membrane and probed for CRN7 using anti-GFP antibody. The proteins used in the pull down were detected by Ponceau S staining of the membrane. Molecular weight markers in kDa are indicated at the left.

The *D. discoideum* genome encodes a long coronin isoform (CRN7) with tandem β-propeller domain (Shina et al., 2010). We sought out to ascertain whether CRN7 could interact with Rac GTPases as well. GFP-CRN7 showed binding to all analysed Rac proteins (A, C, E and 1a) and, like coronin, it precipitated preferentially with Rac

GTPases loaded with GDP. For RacA and RacE some binding to the GTP loaded forms was retained (Figure 15).

In order to map the binding region of Rac in CRN7, we expressed the two WD repeat domains of CRN7 as individual fragments fused to GFP (GFP-CRN7-NT and GFP-CRN7-CT). GFP-CRN7-NT and GFP-CRN7-CT bound to certain members of the Rac GTPases with variable affinities for the GDP or GTP loaded forms. The C-terminal repeat preferred the GTP loaded forms of RacC and RacE, GFP-CRN7-NT showed an increased interaction with RacC-GTP and no binding to RacC-GDP (Figure 16).

A



B

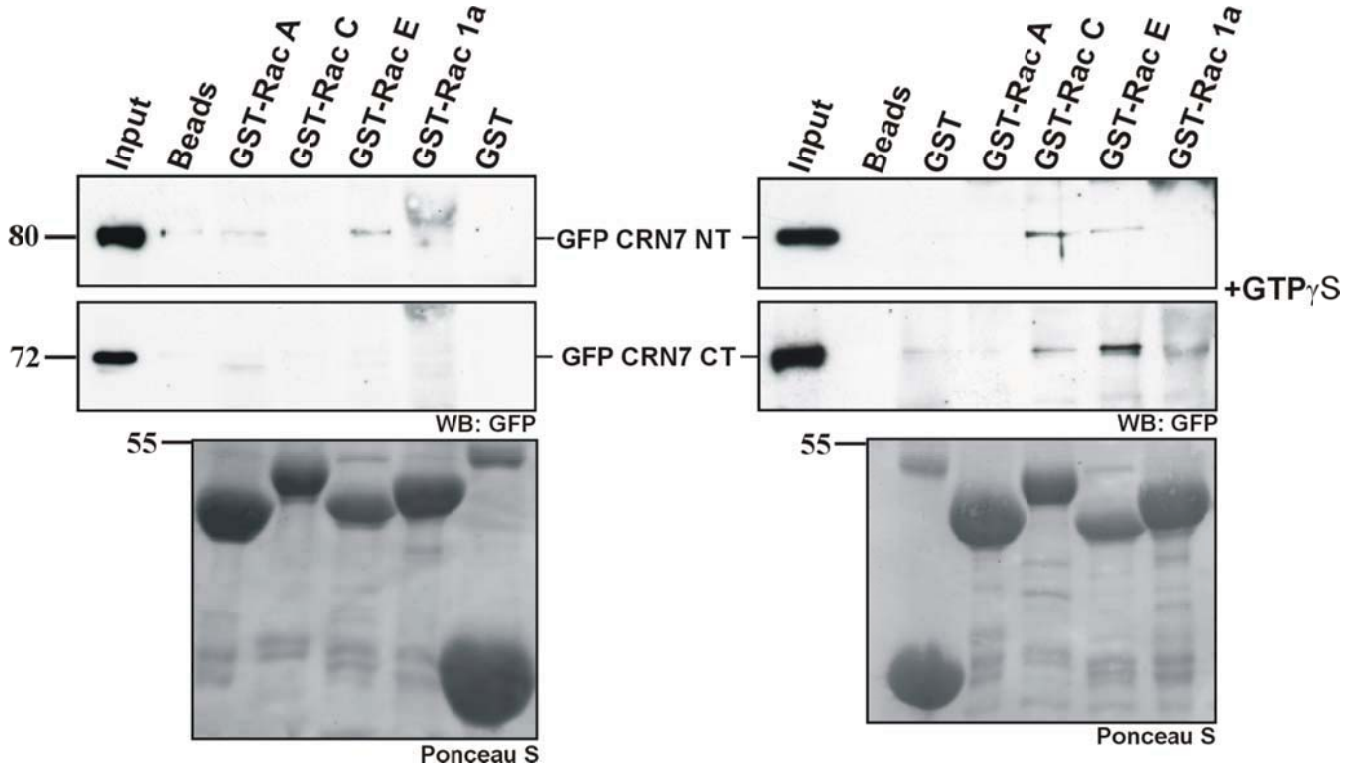


Figure 16: CRN7-Rac interactions. A. GFP fusion deletion constructs of CRN7 is shown. The figure is not drawn to scale. Modified from Shina et al.,(2010). B. CRNGST-Rac A, GST-Rac C, GST-Rac E and GST-Rac 1a bound to Glutathione-Sepharose beads were loaded with GDP or GTPγS and incubated with AX2 cell lysates expressing either GFP-CRN7-NT or GFP-CRN7-CT. After repeated washing of the beads, the bound proteins were immunoblotted with anti-GFP antibody to detect GFP-tagged proteins. The GST fusions are shown in the lower panels. Molecular weight markers in kDa are indicated at the left.

From all the above experiments we conclude that *D. discoideum* coronin proteins (coronin and CRN7) preferentially interact with Rac GTPases in their GDP bound form. However, when the individual propellers of CRN7 are expressed, they prefer active Racs (GTP-loaded).

2.4.1 Coronin regulates myosin II function

Myosin II (conventional) in *D. discoideum* plays a central role in several cellular processes like cytokinesis, chemotaxis, and development. Myosin II can self-assemble into bipolar filaments. Phosphorylation of myosin II in its heavy chain regulates the dynamics of filament assembly (Bosgraaf and van Haastert., 2006) and impaired regulation of myosin II assembly and disassembly dynamics leads to severe cell polarity and developmental defects (Mondal et al., 2008).

We performed immunofluorescence analysis in order to elucidate the function of coronins in myosin II regulation. Myosin II typically localizes to the posterior cortex of chemotaxing cells where it helps to retract the cell body and suppress lateral pseudopod formation. It was observed that the amount of myosin II associated with the cytoskeleton increases and nearly doubles during the aggregation stage. When we stained coronin mutant cells for myosin II, we observed a similar distribution of myosin II in AX2 and corB mutant cells where we found an increased cortical staining only in aggregation competent cells. In contrast, *corA*$^{-}$ and *corA*$^{-}$*B*$^{-}$ mutants showed an increased staining of myosin II in the cortex in vegetative cells comparable to the myosin II staining of aggregating cells (Figure 17).

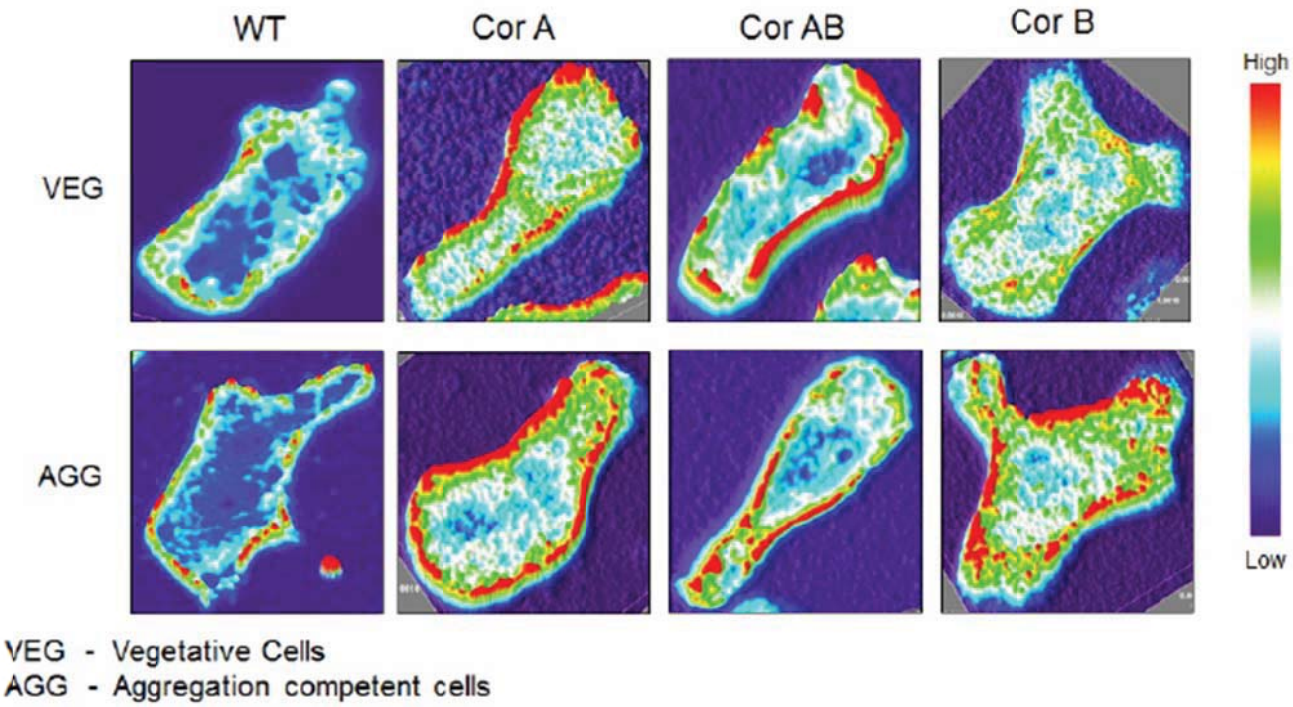


Figure 17: *Dictyostelium* coronins affect myosin assembly. Growing and aggregation competent AX2, *corA*$^-$, *corB*$^-$, and *corA*$^-$*B*$^-$ mutant cells were fixed and stained for myosin II with mAb56-396-5 (Pagh and Gerisch,1986). Images were taken with a confocal microscope. The confocal stacks were processed with imageJ using pseudo-3D projection plug-in. The z-axis represents the intensity of myosin II over the scanned area.

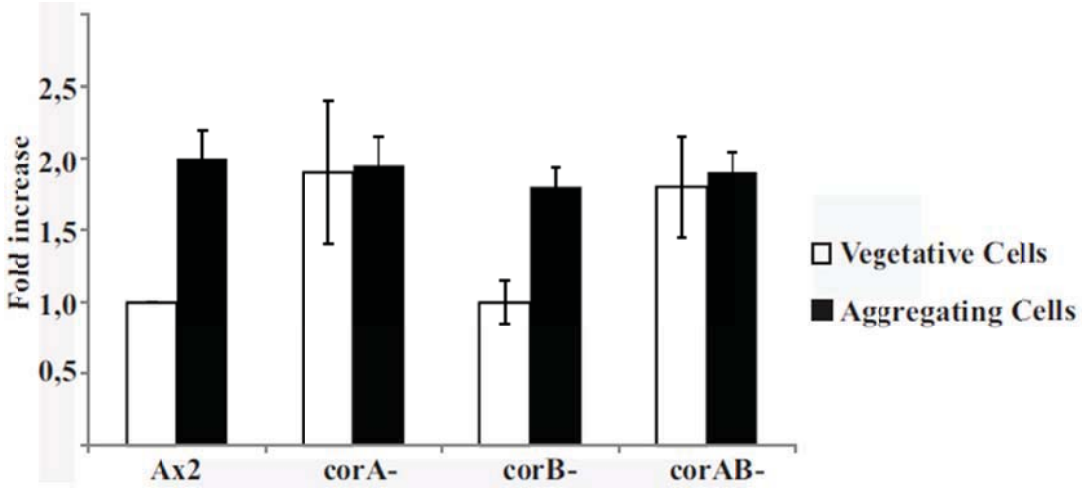


Figure 18: Coronins regulate myosin assembly. Myosin II levels in cytoskeletal ghosts from vegetative and aggregation competent cells. The bar represents

the mean of six independent experiments for vegetative and two for aggregating cells.

We further measured the amounts of myosin II recovered from detergent-insoluble cytoskeleton extracts of AX2 and coronin mutant cells. In the un-phosphorylated state, myosin II assembles into bipolar filaments and associates with the cytoskeleton. In AX2 cells, there is a twofold increase in the amount of myosin II recovered from cytoskeletal preparations of aggregation competent cells compared to growing cells reflecting the increased association of myosin II with the posterior cortical regions of chemotaxing cells. In contrast, *corA*$^-$ and *corA*$^-$*B*$^-$ mutant cells showed an elevated level of myosin II in growing cells which was comparable to that in aggregating cells. On the other hand, cytoskeletal myosin II recovered from *corB*$^-$ was comparable to AX2 cells (Figure 18).

2.4.2 *D. discoideum* coronin interacts with Rac GTPases that regulate myosin II function

Rac GTPases regulate several cellular processes through CRIB containing effectors proteins. PAK kinases are such effector proteins that are regulated by Rac GTPases. PAK kinases are activated by active Rac GTPases (GTP-bound), and in turn regulate downstream processes like myosin assembly (conventional myosin II) and myosin motor activity (unconventional myosin I) in the cell.

The *D. discoideum* genome encodes three canonical PAK kinases, PAKa, PAKb and PAKc. Two of these kinases, PAKa and PAKc, have been implicated in myosin II regulation (Chung and Firtel., 1999; Lee et al., 2004; Müller-Taubenberger et al.,2002). Additionally, these two PAK kinases are known targets for activated Rac GTPases, especially Rac1b and RacB. So we sought out to investigate whether coronin could interact with these two Rac GTPases. In pull-down assays we found that coronin bound preferentially to the GDP bound form of Rac1b and RacB (Figure 19).

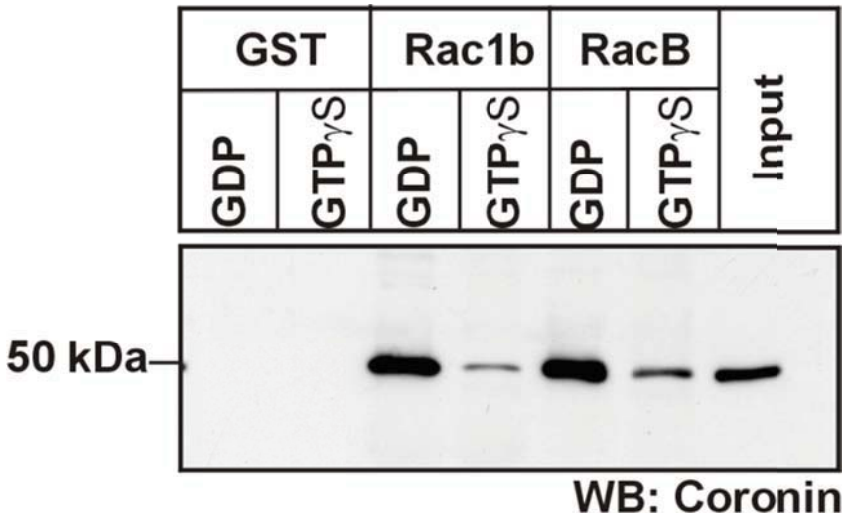


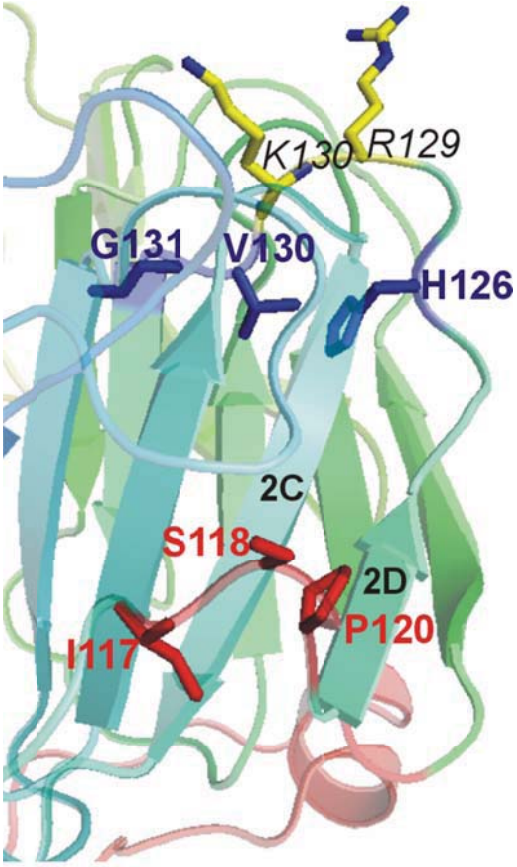
Figure 19: Coronin interacts with Rac1b and RacB. GST, GST-RacB, and GST-Rac1b bound to Glutathione-Sepharose beads were loaded with GDP or GTPγS and incubated with cell lysates from AX2. After repeated washing of beads, the bound proteins were immunoblotted with anti-coronin antibody. The molecular weight of coronin is indicated on the left.

2.5 Expression and characterization of coronin carrying a mutated CRIB motif

2.5.1 Coronin CRIB mutant constructs and expression

Effector proteins containing a CRIB domain bind preferentially to the GTP-bound form of Rac GTPases. Mutation or deletion of the CRIB domain in such proteins leads to the loss of Rac GTPases binding and subsequent impaired protein function (Lee et al., 2004). To test the importance of this motif in coronin, we created two different coronin proteins in which the conserved CRIB motif amino acids were mutated to alanine. In GFP-MUT1 the conserved residues in the N terminal part of the CRIB (ISxP) were mutated and in GFP-MUT2, the C-terminal conserved residues were mutated (H127A, V131A, G132A) (Figure 20A, B). These mutant proteins were expressed in AX2 and corA mutant cells to study the significance of coronin-Rac interaction in cellular processes.

A



B Side View

Coronin CRIB motif	**ISTPLQTLSGHKRKVG**
CRIB mutant 1	**AA..A.....**
CRIB mutant 2	**.....A.....AA**

Figure 20: A, Structure of the *D discoideum* coronin CRIB domain. Side view of the coronin CRIB motif. The mutated residues are highlighted in a ball and stick model (red and blue). Only side chains are shown for clarity. The positively charged residues adjacent to the CRIB domain (R129, K130,) are also shown. (B) Coronin CRIB mutant constructs used in our study. Conserved residues in the N terminal and C terminal part of the CRIB domain were mutated to alanine.

In order to test if the mutation of the conserved residues in the coronin CRIB motif affects the Rac binding ability, we carried out a binding assay with cells expressing different mutant proteins. To this end, equivalent amounts of lysates from GFP cor WT/AX2, GFP-MUT1/*corA*$^-$, and GFP-MUT2/*corA*$^-$ cells were loaded onto Glutathione-Sepharose columns containing Rac1b preloaded with GDP or GTPγS. The bound proteins were analyzed by western blotting with anti-GFP antibody. In these assays, the wild type GFP coronin and the GFP MUT2 protein bound preferentially to GDP-Rac1b while GFP MUT2 had completely lost its binding ability (Figure 21).

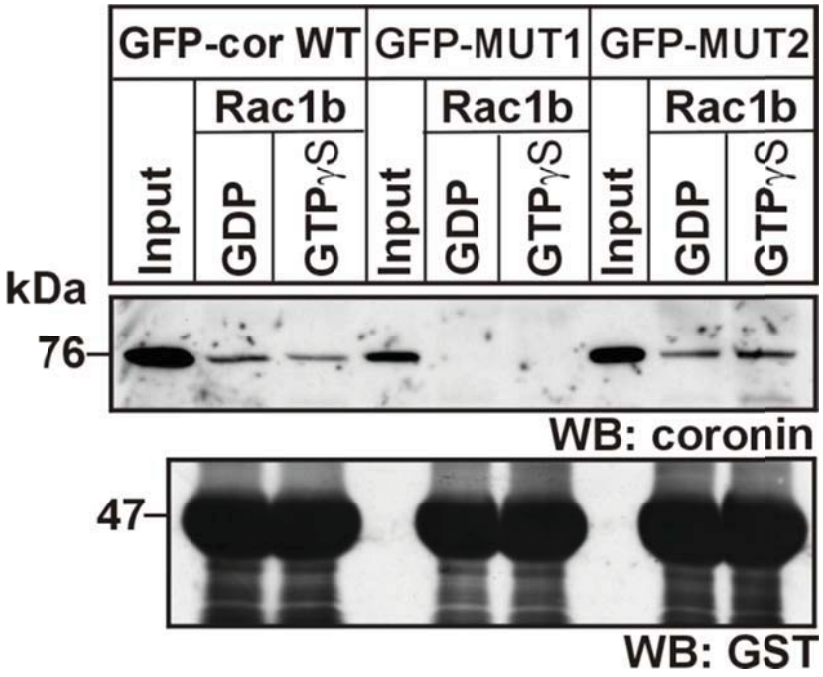


Figure 21: Interaction of CRIB mutants with Rac GTPases. GST, and GST-Rac1b bound to Glutathione Sepharose beads were loaded with GDP or GTPγS and incubated with cell lysates from GFP-cor WT/AX2, GFP MUT1/*corA*$^-$, and GFP MUT2/ *corA*$^-$cells. After repeated washing of beads, the bound proteins were immunoblotted with anti-coronin antibody mAb 176-3-6 (WB: coronin). The membrane was striped and reprobed with polyclonal antibodies against GST (WB: GST). The molecular weights are given at the left.

2.5.2 Cellular localization of coronin CRIB mutant

We then analyzed the localization of GFP-CRIB mutant proteins in fixed cells. *corA* mutant cells expressing GFP-MUT1 and GFP-MUT2 were fixed and co-stained for actin with anti-actin antibody before imaging. In *D. discoideum* coronin localizes to cortical regions and crown-like projections in growing cells (de Hostos et al., 1991). Both of the CRIB mutants showed a similar distribution and co-localized with actin in the cell cortex.

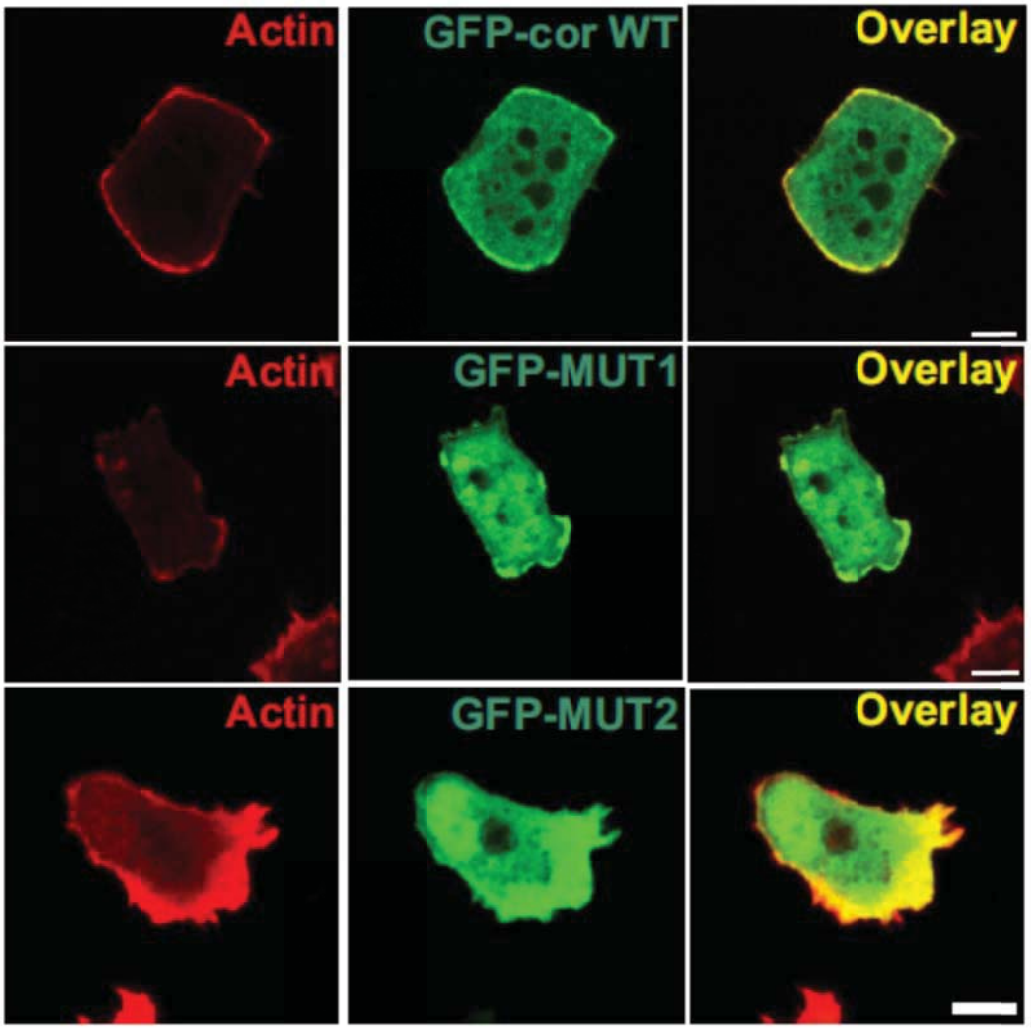


Figure 22: Cellular localization of coronin CRIB mutants. *corA*$^-$ mutant cells expressing GFP-MUT1 and GFP-MUT2 were fixed and stained for actin. The overlay images show co-localization of coronin and actin in the cortex. Scale Bar, 5 µm

2.5.3 The coronin CRIB mutant retains the biochemical properties of wild-type coronin

All short coronin homologues contain a C terminal coiled coil motif that forms the basis for their oligomerization (Spoerl et al., 2002). Additionally, this coiled coil motif has been shown to interact with the Arp2/3 complex and regulate its function. Furthermore, the coiled coil motif contains an F-actin binding site and can bundle filaments in vitro (Rosentreter et al., 2007).

To test if the mutation of the CRIB motif affects the oligomerization of coronin, we performed immunoprecipitation assays. Equivalent amounts of cells expressing GFP-cor WT and GFP-MUT2 in the AX2 wild type strain were lysed and immunoprecipitated with anti-GFP antibody bound to sepharose beads (Chromotek). We included AX2 cell lysates as a control in this assay. The lysates and precipitates were analyzed by western blots for the presence of GFP-tagged proteins and coronin. In these experiments, both GFP-cor WT and GFP-MUT2 could precipitate endogenous coronin from cell lysates indicating self-association *in vivo* (Figure 23).

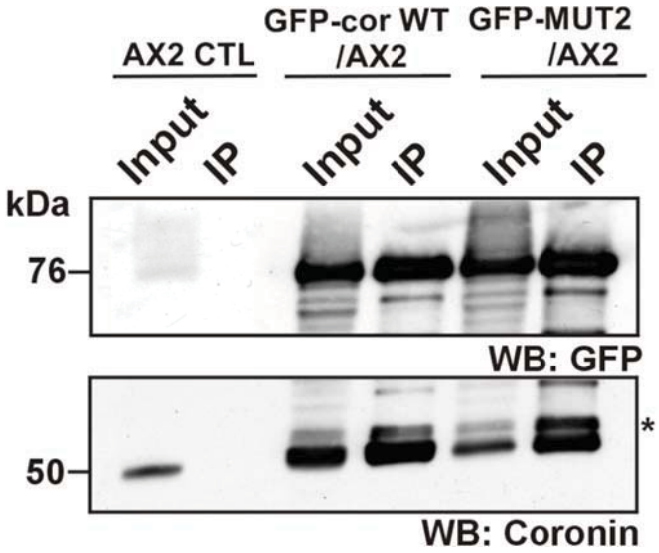


Figure 23: A coronin CRIB mutant self-associates. Equal amounts of lysates from AX2, GFP-corA WT, and GFP-MUT2 were immunoprecipitated with anti-GFP antibody. Lysate and precipitates were analyzed by western blots for the presence of GFP-tagged proteins with anti-GFP antibody for successful immunoprecipitation. The membrane was stripped and probed with anti-coronin antibody to detect self-association. The asterisk (*) indicates a degradation product of coronin.

2.5.4 Expression of Coronin CRIB mutant rescues multi-nuclearity but fails to recue the myosin II phenotype of coronin knock-out cells

We used cells expressing GFP-MUT1 and GFP-MUT2 in the *corA*$^-$ background in order to investigate the consequences of the CRIB motif mutation for coronin functions. In the following three chapters, we describe our results with regard to cytokinesis, myosin function, and phagocytosis which reveal whether the mutated protein is capable of rescuing the mutant phenotype.

Cytokinesis is a critical step in cell division and is required for cell growth and survival. Cells lacking coronin show a severe cytokinesis defect (de Hostos et al., 1993). Nearly 50% of the *corA*$^-$ cells contain three or more nuclei. In contrast, only 5% and 10% of the *corA*$^-$ cells expressing GFP-MUT1 and GFP-MUT2, respectively, showed more than 3 nuclei which was comparable to AX2 (9%) (Figure 24). Expression of GFP-corA-MUT therefore completely rescued the cytokinesis defect in corA deficient cells.

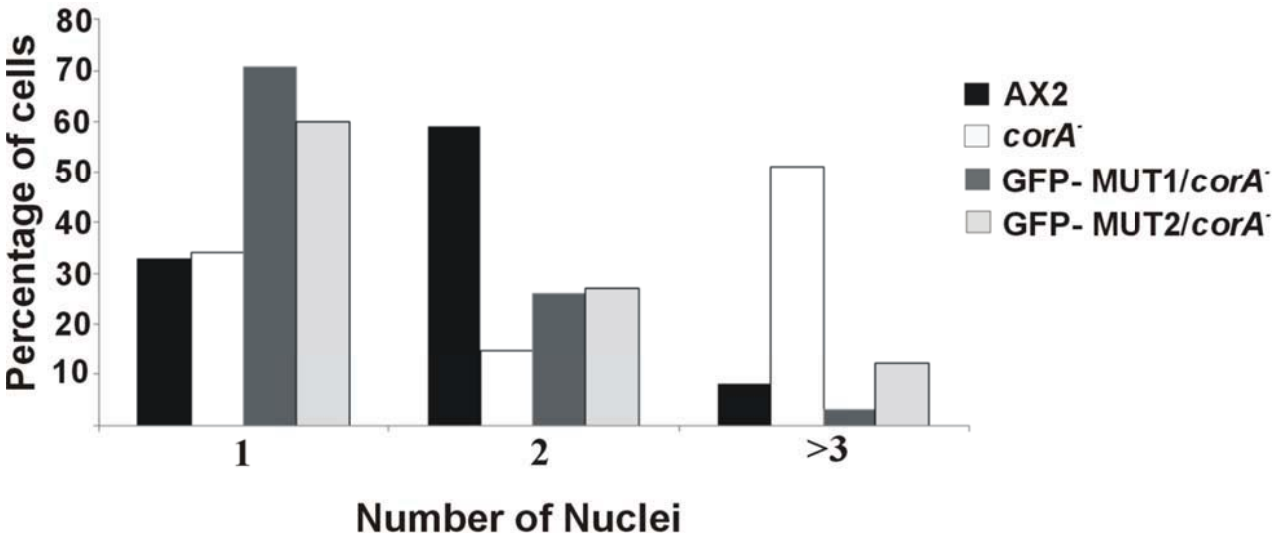


Figure 24: Quantification of nuclei. Cells were fixed with methanol and nuclei were stained with the DNA-binding dye DAPI. Nuclei in more than 500 cells were quantified for each strain.

Our immunofluorescence analysis revealed a severe myosin defect in *corA*$^-$ cells (Figure 17). To test if the expression of GFP-MUT1 and GFP-MUT2 could rescue this defect, we fixed *corA*$^-$ cells and *corA*$^-$cells expressing different mutants (rescue) and stained for myosin II. CorA mutant cells showed an increase in myosin II staining in

the cortex. This elevated cortical staining was reduced in cells that expressed GFP-corA-MUT2 while expressing of GFP-MUT1 could not rescue the myosin phenotype (Figure 25).

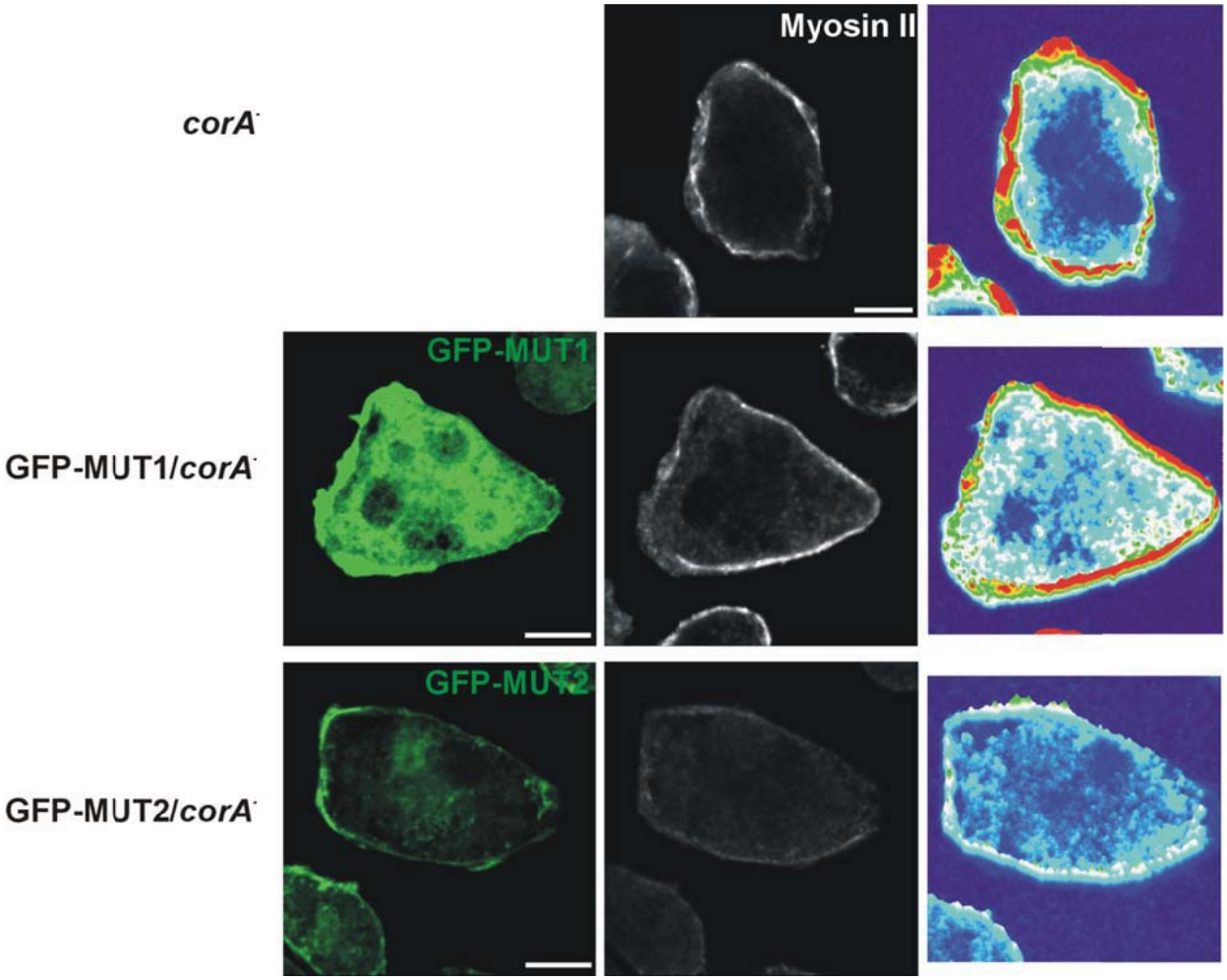


Figure 25: Coronin CRIB mutant 1 fails to rescue the myosin defect of *corA*⁻ cells. GFP-MUT1 / *corA*⁻, GFP-MUT2 / *corA*⁻ and *corA*⁻ cells were fixed and stained for myosin II. Scale bar, 5 µm.

We further prepared cytoskeletal preparations from AX2, *corA*⁻, GFP-MUT1/*corA*⁻, and GFP-MUT2/*corA*⁻ cells and quantified the amount of myosin recovered. *corA*⁻ cells showed a two-fold increase in myosin levels in growing cells compared to AX2. Expression of GFP-MUT2 in *corA* mutant cells reduced the elevated myosin II level in

such a way that it was comparable to the one of AX2 cells whereas GFP-MUT1 that is defective in Rac binding did not reduce the myosin levels (Figure 26).

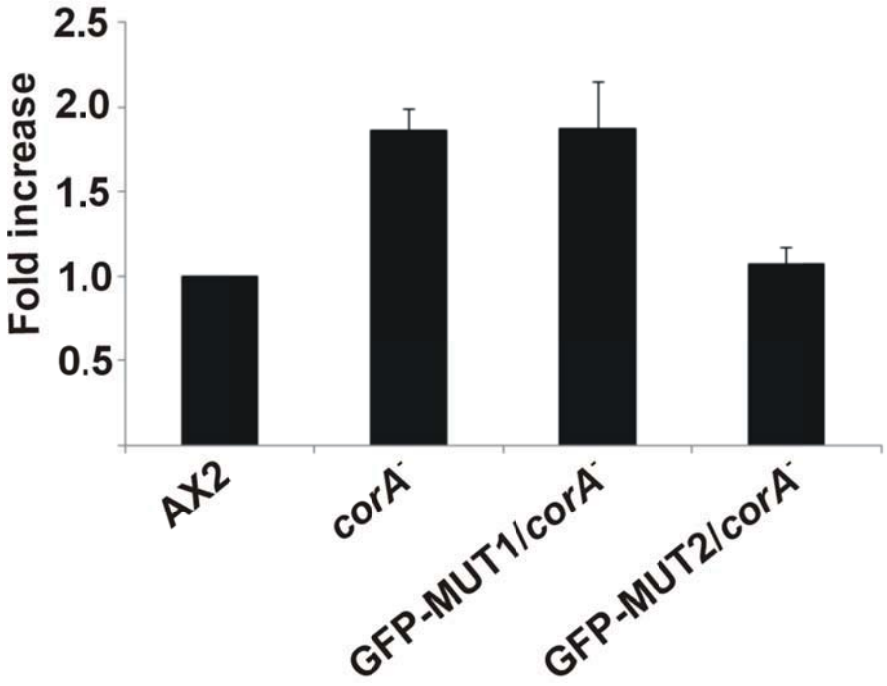


Figure 26: Expression of Coronin CRIB mutant protein fails to rescue myosin function in *corA*$^-$ cells. Myosin II levels in cytoskeletal preparations from vegetative cells. The bar represents mean of three independent experiments.

D. discoideum cells lacking coronin showed a severe defect in phagocytosis (Maniak et al., 1995). So, we tested if this defect could be rescued by the expression of GFP-coronin CRIB mutants in *corA*$^-$ cells. To this end, we quantified the uptake of yeast cells in AX2, *corA*$^-$, and *corA*$^-$ expressing GFP-MUT1 and GFP-MUT2 using TRITC-labeled yeasts. The percentage of cells containing yeast particle was 30% after 15 min incubation in AX2 cells (Figure 27). In contrast, the percentage of phagocytosing cells in the *corA*$^-$ mutant was reduced to 8%. GFP-MUT1/*corA*$^-$ (22%) cells and GFP-MUT2/*corA*$^-$cells (17%) had a similar number of phagocytosing cells compared to AX2.

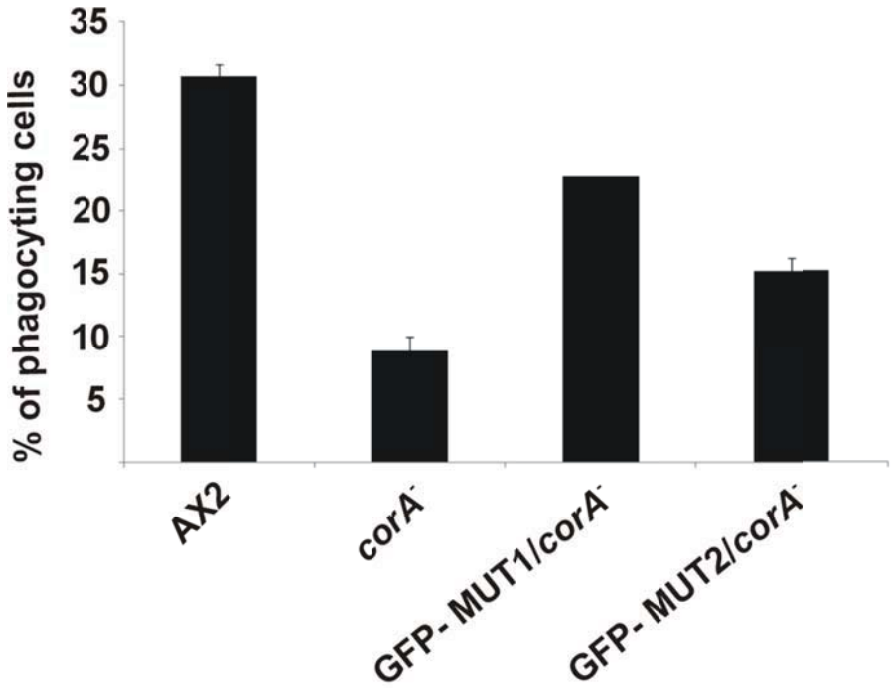


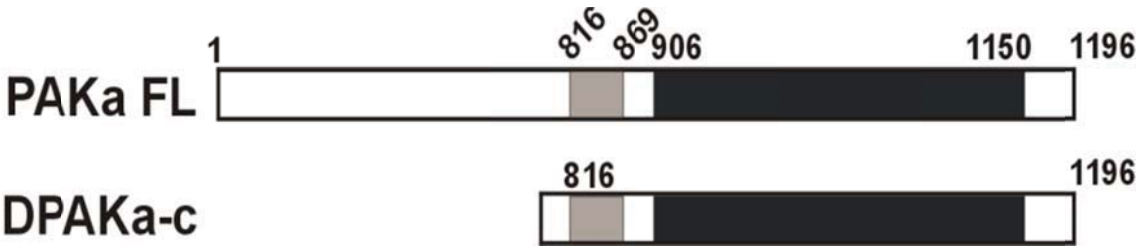
Figure 27: Phagocytosis of coronin mutants. The ratio between cells containing engulfed yeast and total number of cells counted for each strain was converted to percentage and plotted in a graph.

2.6 Expression and characterization of a dominant negative PAK in *corA*$^-$ cells

PAK kinases in *D. discoideum* regulate several cellular processes in particular myosin II assembly. Deletion of PAKa leads to abolished myosin II assembly in the cortex and a severe cytokinesis defect similar to the one observed in myosin II deficient cells (*mhcA*$^-$) (Chung and Firtel., 1999). It has been suggested that PAKa regulates the myosin II assembly through heavy chain phosphorylation. Furthermore, expression of the kinase domain of PAKa in AX2 cells showed a dominant negative effect with reduced phagocytosis (Müller-Taubenberger et al., 2002).

We have observed an elevated myosin II level in the cortex of *corA*$^-$ cells and we know that loss of coronin leads to a severe cytokinesis defect. We speculated that the myosin phenotype observed was due to increase kinase activity in the *corA*$^-$ mutant cells. Therefore we expressed a dominant negative PAKa construct (GFP-PAKa-c) in *corA*$^-$ cells and analyzed its effect with respect to cytokinesis and myosin II regulation.

A.



B.

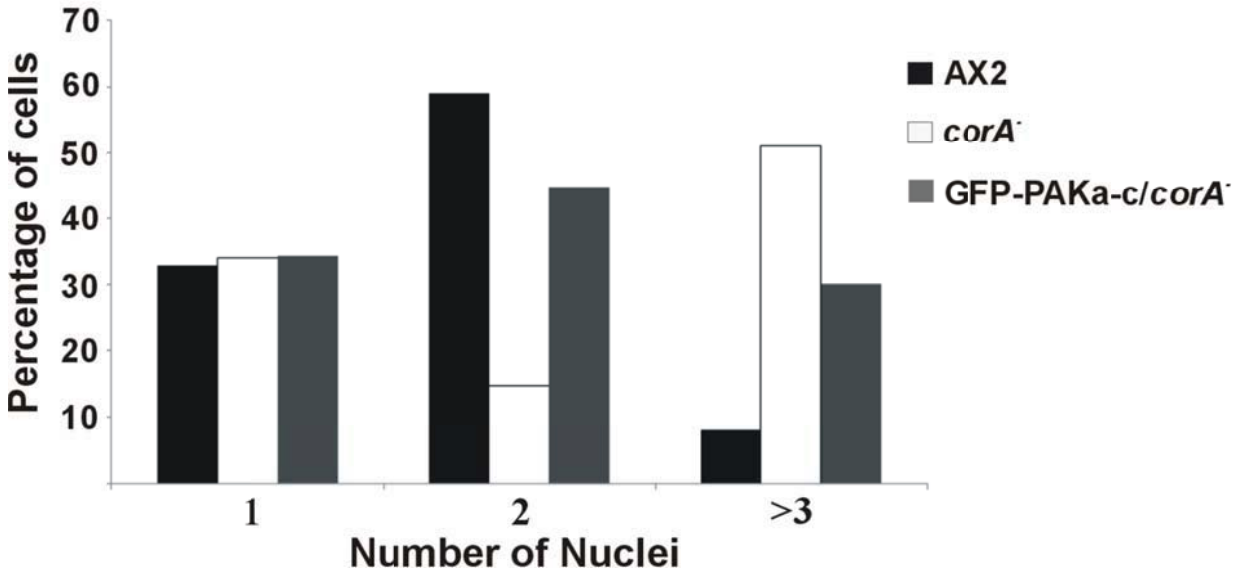


Figure 28: A. Schematic representation of PAKa dominant construct use in the study. The CRIB and catalytic domains are shown in grey and black boxes respectively. Figure is not drawn to scale. Modified from Müller-Taubenberger et al. (2003). B. Quantification of nuclei. Cells were fixed with methanol and nuclei were stained with DAPI. Nuclei in more than 500 cells were quantified for each strain.

Expression of GFP-PAKa-c in *corA*$^-$ cells partially rescued the cytokinesis defect. Whereas ~50% *corA*$^-$ cells contain 3 or more nuclei, the number was reduced in GFP-PAKa-c expressing cells to ~30% (Figure 28).

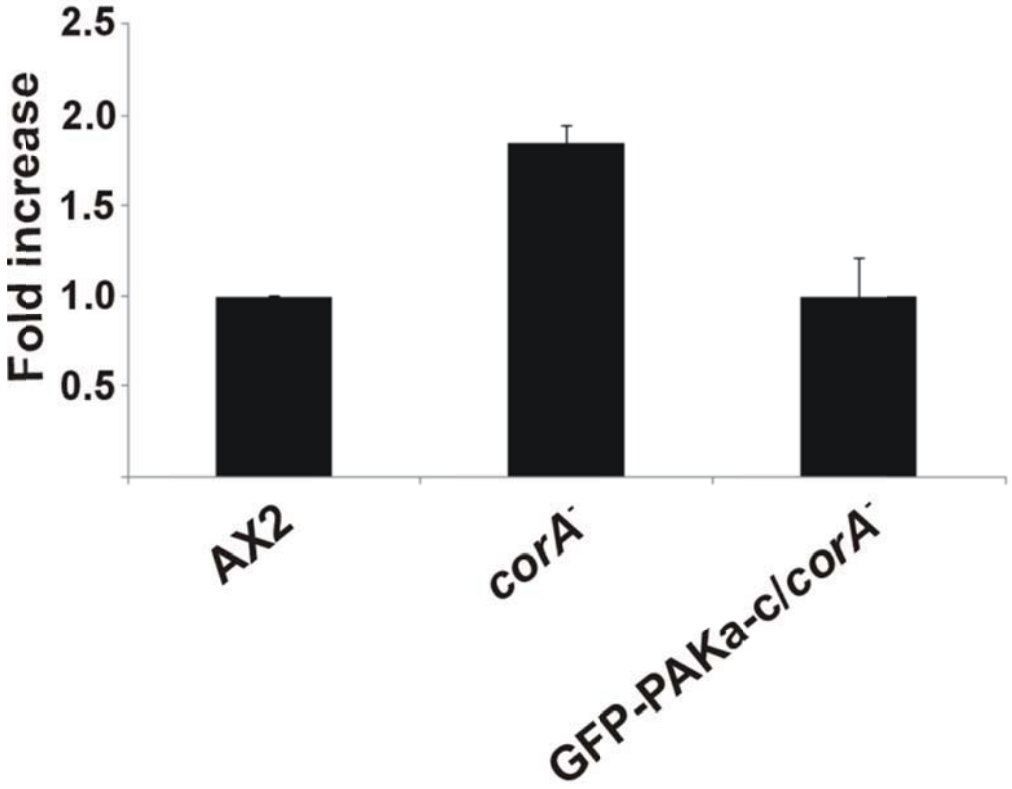


Figure 29: Expression of GFP-PAKa-c rescues myosin function. Myosin II levels in cytoskeletal preparations from vegetative cells. The bar represents mean of three independent experiments.

Next, we assessed the myosin II levels in cytoskeletal preparations. Expression of GFP-PAKa-c in *corA*$^-$ cells reduced the myosin II level to the one comparable to that of AX2 cells. These data suggests that the expression of a dominant negative PAKa in *corA*$^-$ cells interferes with the endogenous PAKa function and reduced its activity thereby alleviating myosin levels to that of the one observed in wild type cells.

2.6.1 *corA*$^-$cells show elevated Rac-GTP levels

We have shown that *D.discoideum* coronin binds preferentially to the GDP-bound form of Rac GTPases and the CRIB motif is necessary for its binding. We propose that coronin through its binding to the GDP form of Rac GTPases can regulate the availability of Rac GTPase for activation and influence the levels of activated Rac in the cells. To test this hypothesis, we carried out a pull-down assay using the CRIB domain of rat PAK (p21-activated kinase) (Filić et al., 2012) to quantify the amount of activated Rac-GTP in AX2 and *corA*$^-$cells.

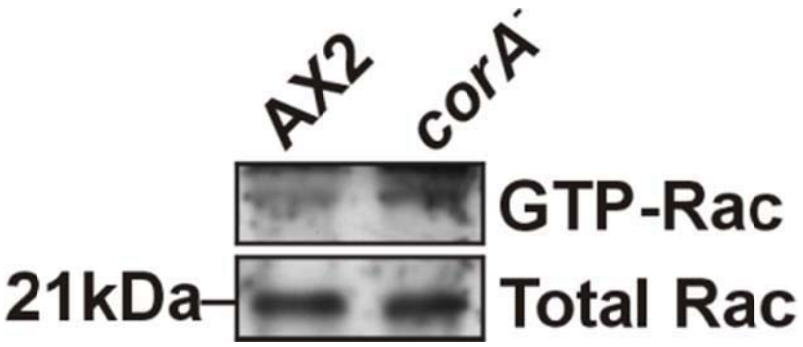


Figure 30: *corA*- cells show increased levels of GTP-Rac. Equivalent amount of AX2 and *corA*- cells were lysed and incubated with GST-PBD (p21 binding domain). After repeated washing bound proteins were immunoblotted with anti-Rac1 polyclonal antibodies. The lower panel shows the total amount of Rac in the cells.

In these assays, we found that the amount of activated Rac (GTP-Rac) was elevated in unstimulated *corA*- cells as compared to AX2 cells which suggested that coronin modulates the Rac activity in the cell.

2.6.2 *D. discoideum* coronin interacts with PAKa

PAKa in *D. discoideum* has been characterized in detail. It is required for myosin assembly in the cell and it was suggested that PAKa modulates myosin assembly by negatively regulating myosin heavy chain kinases (MHCKs) (de la Roche et al., 2002). Furthermore, in mammalian cells, coronin1A was shown to form a F-actin dependent complex with PAKa and this complex was further shown to be essential for Rac activation *in vivo* (Castro-Castro et al., 2011). So, we tested whether *D. discoideum* can bind to PAKa using pull-down assays. To this end equivalent amount of Glutathione-Sepharose beads coupled to GST, GST NT CRIB, GST CRIB+WD, and GST CT CC were incubated with AX2 cell lysates expressing GFP-PAKa. The bound proteins were analyzed by western blotting with anti-GFP monoclonal antibodies (Figure 31).

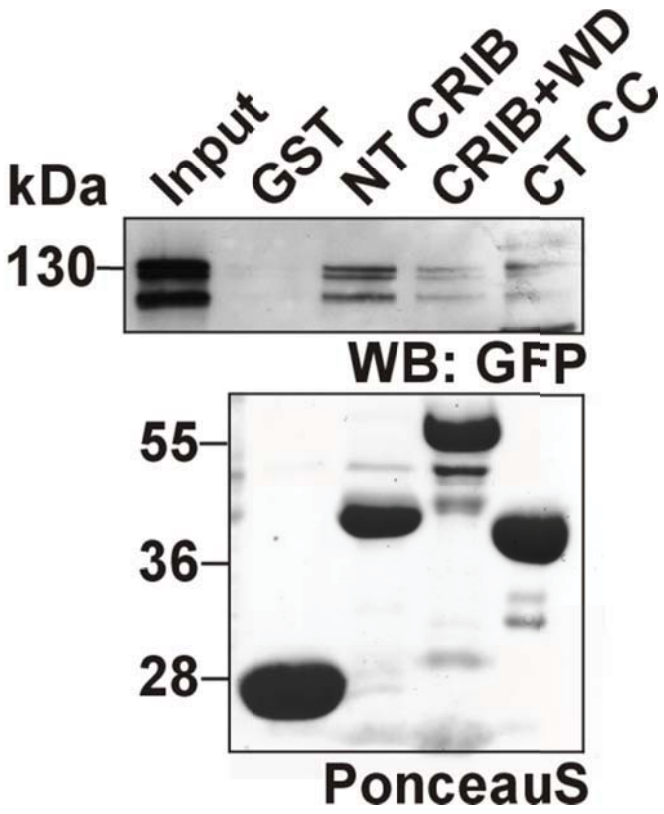


Figure 31: Coronin interacts with PAKa. Equivalent amount of GST, GST NT CRIB, GST CRIB+WD, and GST CT CC bound to Glutathione Sepharose beads were incubated with cells expressing GFP-Paka. Bound proteins were immunoblotted with anti-GFP mAb K3-184-2 (WB: GFP). In the lower panel the PonceauS stained GST fusion proteins are shown. The molecular weight markers are indicated in the left kDa.

In our pull-down experiments, we found that GST NT CRIB efficiently precipitated GFP-PAKa from the cells suggesting while weak interactions with GST CRIB+WD and GST CT CC were seen (Figure 31). It could be possible that there are multiple PAKa binding sites in the coronin molecules and that the N-terminus of the protein is the major site for interaction.

We further carried out immunofluorescence analysis to study the co-localization of PAKa and coronin. GFP-PAKa expressing cells were fixed and stained for coronin with anti-coronin monoclonal antibodies. PAKa localized in the membrane cortex as reported earlier. When these cells were imaged for coronin, we found a partial co-localization of PAKa and coronin in the cortex which would allow the formation of complexes *in vivo* (Figure 32).

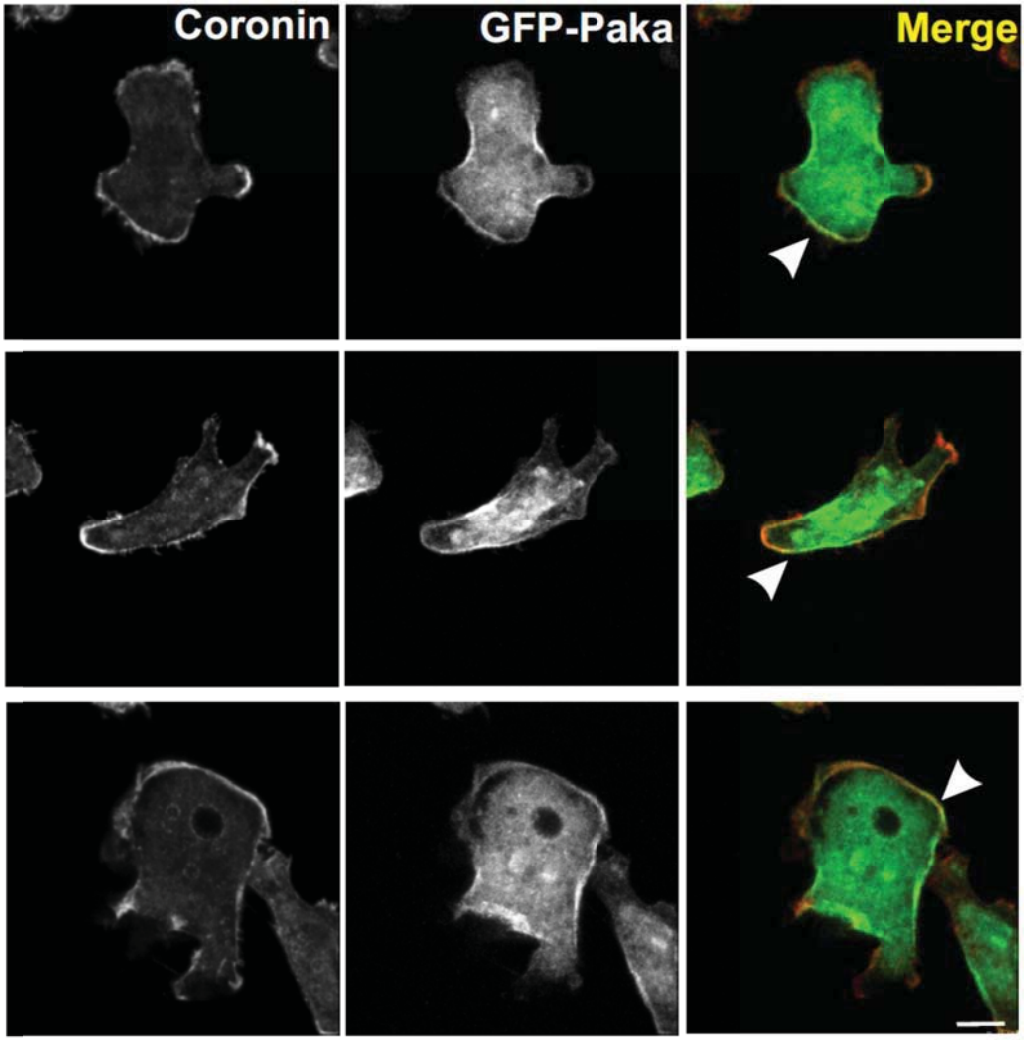


Figure 32: Coronin co-localizes with PAKa in the cortex. Cells expressing GFP-PAKa were fixed and stained for coronin with anti-coronin mAb 176-3-6. The regions of co-localization are shown with arrowhead. Scale Bar, 5 µm.

3. Discussion

A link between Rac GTPases and coronins has been suggested by earlier work. Spoerl et al. (2002) reported that in Swiss 3T3 cells the localization of human Coronin 1C (Coronin3, CRN2) was strongly influenced by expression of constitutively active or inactive Rac1. This was thought to result from changes in the actin cytoskeleton caused by the different states of Rac proteins. Furthermore, a truncated Coronin 1C containing only the core region which is composed of the WD repeats and lacking nearly all regions implied in F-actin co-localization, failed to localize to membranes and affected the shape of the cells. The cells exhibited an impaired spreading and adhesion to solid supports, whereas cell-cell adhesion was unaffected leading to a rounded or spindle-like cell shape and suppress neurite formation in neuronal cell lines (Hasse et al., 2005). Similarly, truncated *Xenopus* coronin led to impaired Rac-mediated spreading and lamellipodia formation. On this basis Mishima and Nishida (1999) had suggested that the coronin core might directly interact with a Rac GTPase and might block signal transmission to downstream effectors.

We extended the work by Xavier et al. (2008) in which a CRIB motif was identified in coronins and confirmed the presence of CRIB motifs in the *D. discoideum* coronin homologues. We further found that the CRIB motif is present in coronin species from different taxa with varying degree of conservation. When we compared the CRIB motif sequence of known effector proteins with *D. discoideum* coronins, we found a high conservation of critical amino acids as the *D. discoideum* short coronin homologue shows amino acid conservation in six positions out of eight in the typical CRIB consensus ISXPXXXFXHXXHVG (Figure 33).

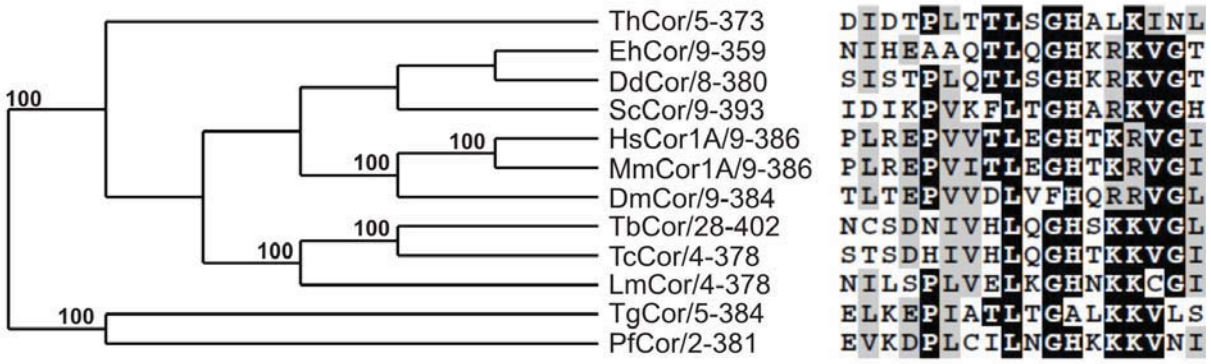


Figure 33: Conservation of CRIB domain in short coronins across taxa. Protein sequences of coronin from *H. sapiens* (P31146), *M. musculus* (O89053), *D. melanogaster* (Q7JVY0), *S. cerevisiae* (Q06440), *E. histolytica* (C4M137), *D discoideum* (P27133), *T. thermophila* (I7MIA8), *T. cruzi* (Q4D4X6), *T. brucei* (Q57W63), *T. gondii* (Q5Y1E7), *L. major* (Q4QB38), and *P. falciparum* (O44021) were retrieved and the core WD repeats (2-6) were aligned using ClustalX program (Larkin et al., 2007). A bootstrap Neighbor Joining (NJ) tree was created and the resulting cladogram is shown with supporting bootstrap values in percent. The CRIB sequence alignment of respective coronins is shown on the right side of the tree.

By cloning CRIB sequences from various effector proteins, Burbelo et al. (1995) showed that Rho GTPase binding is unaffected in sequences with one or two mutations in the eight consensus amino acids. The long coronin homologue CRN7 contains a CRIB related sequence in each of its WD repeat domains with somewhat lesser conservation.

The CRIB domain is commonly found in Cdc42/Rac effector proteins and enables them to bind to Rho GTPases in a GTP dependent manner. The CRIB motif was found to be the minimal essential domain for Rho GTPase binding, however, further analysis with mutational studies in p21 activated kinase (PAK) suggested that a larger binding region is required for Rho GTPase binding and was termed as PBD (p21 activated kinase binding domain). This domain has been routinely used as a tool to quantify cellular GTP-Rac levels because of the high affinity of this domain for Rho GTPases (Bokoch et al., 2003). We have identified the presence of a CRIB motif in a surface accessible loop between blade 2 and blade 3 of *D. discoideum* coronin proteins. In our pull-down assays with different coronin deletion peptides, all the GST fused peptides harboring the CRIB motif could precipitate GFP-Rac1a from AX2 cells. Rac1a from *D. discoideum* regulates cytokinesis and the actin cytoskeleton. The *D. discoideum* genome encodes nearly 18 Rac family GTPases (Vlahou and Rivero, 2006). So, we used this minimal GST fused CRIB peptide in a pull-down assay to identify its binding to other Rac members and found that it could precipitate GFP-RacE from AX2 cells. To further understand the *in vivo*f unctions of the coronin CRIB domain, we expressed GFP-cor CRIB in AX2 cells and followed its localization

using live cell imaging. In these experiments, we have found that GFP-cor CRIB transiently localize to the membrane of protruding pseudopods, while a full length protein GFP-cor WT was enriched in the cortex.

CRIB effector proteins binds to Rho GTPases in a nucleotide dependent manner. Rho GTPases act as a molecular switch by converting the inactive (GDP-bound) to the active (GTP-bound) conformations. Rho GTPase effectors proteins bind preferentially to the GTP-bound form of GTPases through their CRIB motif (Bishop and Hall, 2000). We have conducted pull-down assays in order to test the nucleotide specificity of coronin binding to Rho GTPases where we used a series of Rac GTPases fused to GST and loaded them with nucleotide analogues (GDP or GTPγS) to mimic the *in vivo* situation. In these experiments we found that *D. discoideum* coronin proteins preferentially interacted with GDP-bound (inactive) forms of Rac GTPases. Coronin interacted with GDP-RacC with higher affinity and CRN7 showed an increased binding to GDP-RacE. RacC has been implicated in phagocytosis and regulates actin polymerization through activation of WASP. Both of these processes were found to be impaired in *corA*$^{-}$ cells suggesting that coronin-RacC interaction might play a role in regulating them. As CRN7 contains two CRIB domains, we next asked how the individual CRIB domain interacted with Rho GTPases when expressed separately. We have used AX2 cells expressing GFP fused proteins encoding the individual beta-propeller domains of the CRN7 protein in our pull-down assays. Surprisingly, the individual beta-propeller domains showed increased binding to GTP-loaded Rac GTPases. While GFP-CRN7 NT interacted with RacC-GTP, GFP-CRN7 CT showed higher preference for GTP-RacE. This suggests an additional regulation of the interaction at the level of the full length protein.

We further tested if the interaction between coronin and Rac GTPases is direct. In our direct binding assays, we found that a thrombin cleaved fragment (NT CRIB) binds to both forms of Rac GTPases (GDP and GTP) without any preference. This data is in contrast to the full-length protein which binds preferentially to GDP-Racs. In general the conserved residues in the CRIB motif make extensive contacts with switch I and switch II regions of Rac GTPases. These residues and an adjacent

alpha-helix appear to mediate sensitivity to the nucleotide switch (Abdul-Manan et al., 1999). The CRIB motif binds preferentially the GTP loaded GTPase and exhibits reduced binding activity when the GDP form is used. In *D.discoideum* coronins the CRIB motif, although being well conserved, is not followed by an alpha-helix as in the CRIB motifs of WASP, ACK or PAK65 (Abdul-Manan et al., 1999), instead, it is embedded into beta sheets.

We studied myosin functions in the coronin mutant cells in order to understand the significance of coronin-Rac interaction. Myosin II dynamics in *Dictyostelium* is regulated by phosphorylation in its tail region by heavy chain kinases. The molecular mechanisms involved in myosin II regulation have been well studied (Bosgraaf and Haastert, 2006). Rac GTPases have been shown as significant players in myosin II regulation and now we found that coronin interact with Racs; we studied the myosin assembly dynamics in coronin deficient cells. It was shown that, during cell migration (chemotaxis) myosin accumulation in the cortex increases by twofold compared to an unstimulated cells (Mondal et al., 2008). When we stained for myosin in coronin mutant cells, we found an increased accumulation already in the growth phase cells of *corA*$^-$ and *corA*$^-$*B*$^-$ cells, while in the WT and *corB*$^-$ mutants, it increased only during aggregation. This was further confirmed by quantification of myosin in the detergent-insoluble fractions. Two Rac GTPases have been implicated in regulating myosin functions, Rac1b and RacB. These two GTPases exert their function by regulating a downstream Pak kinase activity. So, we next asked whether coronin can interact with these two GTPases in our pull-down assays. Coronin interacted with both of these GTPases in their GDP-bound form implicating that coronin might regulate myosin functions through interaction with these two GTPases.

We next assessed the significance of the CRIB motif in coronin protein functions by generating point mutations in the conserved residues. Two different expression constructs with CRIB mutations were generated. GFP-MUT1 contains a mutated CRIB motif in its N-terminal half (ISxP exchanged for AAxA) and in GFP-MUT2 the conserved amino acids in the consensus HXXXVG were mutated to alanine. In immunofluorescence studies, both of the mutants were found enriched in the cortex of cells and co-localized with actin. In addition, they were equally present in detergent

soluble and insoluble cytoskeletal fractions (data not shown) suggesting that the actin binding ability of the proteins was unaffected by CRIB mutations. As coronins can form homo-oligomers by means of a conserved coiled coil domain, we expressed these GFP-CRIB mutants in AX2 cells in order to analyze this function. In our immunoprecipitation experiments, we found that the GFP-MUT2 could form oligomers like wild-type protein.

Furthermore, we tested the binding ability of GFP-CRIB mutated coronin proteins with Rac GTPases. In pull-down assays, GFP-cor WT protein interacted with the GDP-bound form of Rac1b as observed with the endogenous protein. While GFP-MUT1 lost the ability to bind Rac1b, GFP-MUT2 behaved like wild type protein and retained its binding ability. In our structural analysis of the coronin CRIB domain, the N-terminal half of the CRIB motif was surface exposed and appeared to be more relevant for the Rac interaction as its mutation led to a loss of binding activity. When coronin deficient cells rescued with these mutants were analyzed for the myosin phenotype, we observed that the GFP-MUT2 which retained Rac binding ability could prevent the overassembly of myosin in the cortex of vegetative *corA*$^-$ cells comparable to AX2 cells. These results clearly highlight that the coronin-Rac interaction is essential for myosin functions.

Since we identified a myosin phenotype in *corA*$^-$ cells we focused here on PAKa as an effector of Rac. In mammalian cells a downstream effector of PAK is the myosin II light chain kinase whose activity is down regulated after phosphorylation by PAK1. This results in reduced myosin II activity (Sanders et al., 1999). In *Dictyostelium*, myosin assembly is controlled by phosphorylation of the myosin II heavy chain. MHCKs phosphorylate the protein in the tail region which leads to disassembly of the myosin filaments and release of myosin from the cell cortex. The activity of the MHCKs is subject to regulation by various mechanisms. In particular, Chung and Firtel (1999) suggested that PAKa affects myosin assembly in response to cAMP signaling whereby PAKa does not phosphorylate myosin II directly but regulates it in a negative fashion through regulation of MHCK. In PAKa null cells the level of cytoskeletal myosin was reduced to ~65% of wild-type level and constitutively active PAKa led to enhanced cytoskeletal myosin already in growth-phase cells. PAKs are activated by GTP-bound Rac proteins and for PAKa binding to several Racs including

Rac1a and Rac1b has been shown. For this interaction the CRIB domain was responsible (Chung and Firtel, 1999; Müller-Taubenberger et al., 2002; Park et al., 2004).

In our work we found up-regulated cortical myosin levels already in *corA*$^-$ growth phase cells which exhibited a strong cortical staining for myosin that did not increase further during development. Enhanced myosin content in the cortex suggested that MHCK is not active and cannot phosphorylate myosin heavy chain and release it from the cortex. MHCK inactivation might be achieved by an overactive PAKa. PAKa like myosin resides in the cortex during aggregation. It is a GTPase effector and its activity is regulated by active Rac GTPase. We propose that in the *corA*$^-$ the balance between GDP-bound and GTP-bound Rac is altered due to the loss of coronin. In fact, the Rac-GTP levels were increased as demonstrated by pull downs with the PDB of rat PAK1 which binds only activated Rac (Filić et al., 2012). This leads to an overactive PAKa in the cytoskeleton which inhibits MHCK resulting in overassembly of myosin in the cortex (Figure 17). Intrigued by the link between PAK and myosin we tested this hypothesis and expressed in *corA*$^-$ cells a dominant version of PAKa, PAKa-c, consisting of the CRIB domain and the kinase domain. In contrast to full length PAKa this protein is located in the cytosol (Müller-Taubenberger et al., 2002). This resulted in normal myosin levels in the mutant during growth phase. As PAKa is not known to form (homo-) oligomers it is unlikely that the PAKa protein in the cortex is influenced by PAKa-c in its activity. PAKa-c rescued the phenotype because it sequestered active Rac to a large extent in the cytoplasm. This might restore the wild type phenotype of PAKa activation in the cortex and proper regulation of the myosin heavy chain kinase.

Cytokinesis is a crucial step in the mitotic process. Coronin deficient cells showed severe cytokinesis defects with the majority of cells being multinucleated, a phenotype similar to MHCA$^-$ cells (de Hostos et al., 1993). The multinuclearity defect in MHCA$^-$ cells cannot be rescued by the expression of a non-phosphorylatable form of myosin II (3XALA). Phosphorylation of myosin II in its tail region is essential for myosin disassembly and it was suggested that myosin II needs to disassembles first from the cortex before its assembly in the cleavage furrow to play its role in furrow

constriction (de La Roche., 2002). Since we observed a higher accumulation of myosin in the cortex of *corA*$^-$ cells, we speculated that the cytokinesis phenotype in *corA*$^-$ cells could be due to deregulation in myosin dynamics which in turn is a result of higher PAKa kinase activity. As the expression of dominant negative PAKa, PAKa-c rescued the myosin functions in *corA*$^-$ cells, we counted the number of nuclei in these cells and found that there was a partial rescue in cytokinesis. While 50% of the *corA*$^-$ cells had more than 3 nuclei, this is reduced to 30% in PAKa-c expressing cells suggesting that the cytokinesis phenotype of *corA*$^-$ cells is in part due to the deregulation in myosin assembly dynamics.

A recent analysis has shown that in mammalian cells Pak1 forms a complex with coronin1A in an F-actin dependent manner. In this report, coronin1A was suggested to act as a scaffolding protein and form a complex with Pak1 and RhoGDI thereby enhancing the release of Rac from the GDI for its activation (Castro-Castro et al., 2011). To analyse this for *Dictyostelium* PAK and coronin we first tested if there is a binding site for PAKa in coronin by pull-down assays. We found that the GST-NT CRIB could precipitate efficiently GFP-PAKa from AX2 cells suggesting the presence of a PAK binding site in the coronin N-terminus. RhoGDI was absent from the complex. In immunofluorescence analysis, we found a partial co-localization of PAKa and coronin in the cortex. It was shown previously that PAKa localizes to the rear of migrating cells like myosin while coronin is enriched in the leading fronts suggesting that only a fraction of PAKa may exist in a complex with coronin *in vivo* (Chung and Firtel, 1999).

Coronin proteins, a WD repeat containing family, are well known for their role in regulating actin cytoskeleton dynamics (Clemen et al., 2008). Here, we report that *D. discoideum* coronins interact with Rac GTPases in their GDP-bound form. Since Rac GTPase activation stimulates PAK kinases, and activated PAKs regulate the myosin cytoskeleton, we propose that coronin through its binding to GDP-Rac regulates Rac activation and thereby myosin dynamics. In support of this hypothesis, we show that, (1) the CRIB domain of coronin is essential for Rac interaction, (2) myosin II assembly is upregulated in *corA*$^-$ cells and expression of a dominant negative PAK is

sufficient to rescue the myosin phenotype in *corA*$^-$ cells, (3) a coronin CRIB mutant defective in Rac binding fails to rescue the myosin phenotype, and (4) *corA*$^-$ cells display elevated Rac-GTP levels. In a recent report, Caveolin 1, a principle component of caveolae membrane, has been shown to use a similar mechanism to regulate Rac activities in the cell (Grande-Garcia at al., 2007). As Rac GTPases are implicated in regulating various physiological processes, we believe that the coronin-Rac interaction may have more than one role in *D. discoideum*. Taken together, we propose a model in which coronin proteins in *D. discoideum* play a role in regulating Rac GTPase activities in the cell. Whereas coronin regulates the activation of Rac GTPases in a global manner, CRN7 may spatio-temporally inhibit Rac GTPase activities.

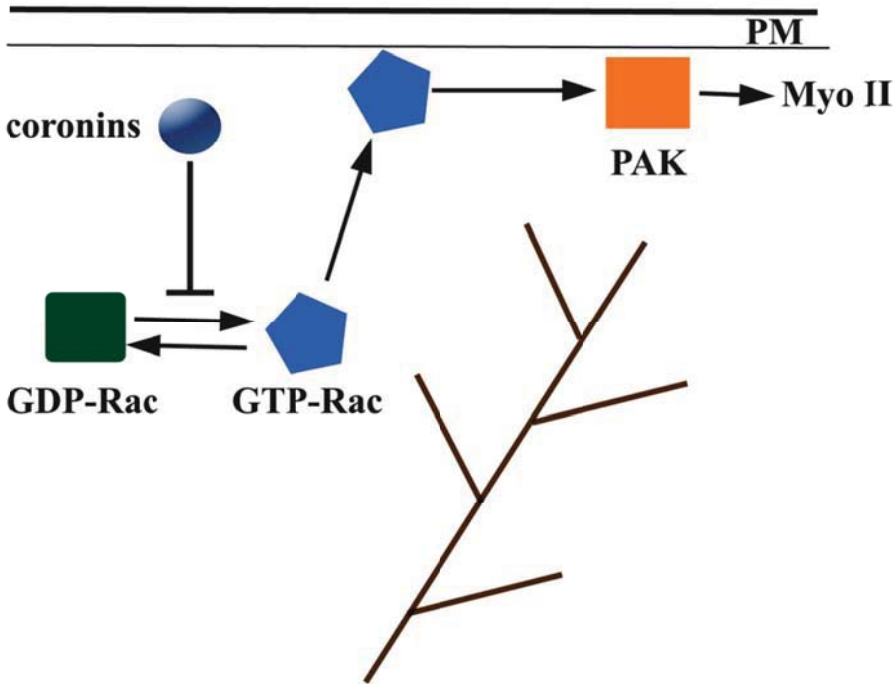


Figure 34. Model highlighting the roles of coronin-Rac interaction in the cell. Rac GTPases cycle between inactive GDP-bound (in green) and active GTP-bound conformations (in blue). Activated Rac GTPases bind to the plasma membrane and activate PAKs (in orange) at the cell cortex. The activated PAKs modulate the myosin cytoskeleton. Coronins shown as blue ball bind to GDP-bound Rac GTPases and prevent their activation. Coronins can also regulate the actin cytoskeleton (shown as criss-crossed lines) either directly or through PAK activation (not shown).

4. Materials and Methods

4.1 Materials

4.1.1 Oligonucleotide Primers

Forward Primers

cor-5‘ For	**5‘-GGATCC**ATGTCTAAAGTAGTCCGTAGTAGTAAATAT-3‘
cor-330 For	**5‘-GGATCC**GAAGGTGGTTTAACCGACTCAATCTCAACC-3‘
cor-499 For	**5‘-GGATCC**ACTGTTGAAGGTCACTCTGATATGATCACT-3‘
cor-1029For	**5‘-GGATCC**TTCCGTGTACCAAGAAAATCTGATATCTTC-3‘

Reverse Primers

330 Rev	**5‘-CCCGGG**TGGGATACCCCAAATACAAATGTTACAATC-3‘
504 Rev	**5‘-CCCGGG**AACAGTGGTTAAATTCTTACCTTGTTCAAC-3‘
1029 Rev	**5‘-CCCGGG**TGAGATTGGTTCAACGGTGAATGGAGTAAC-3
1188 Rev	**5‘-CCCGGG**AGCTGAAGCTTTTTTGACAAAACCACCAGCTAAAC-3‘
3‘ Rev	**5‘-CCCGGG**TTGGTGAGTTCTTTGATTTTGGCATCCTTTTTAAC-3‘

Primers for Site-Directed Mutagenesis

MUT1 For

5’-
CCCAGAAGGTGGTTTAACCGACTCAGCCGCCACCGCACTCCAAACTTTATCTGG
TCACAAGAGAAAGG-3’

MUT1 Rev

5’-
CCTTTCTCTTGTGACCAGATAAAGTTTGGAGTGCGGTGGCGGCTGAGTCGGTTA
AACCACCTTCTGGG-3‘

4.1.2 Primary Antibodies

anti- coronin mAb 176-3-6	-	de Hostos et al., 1991
anti-actin mAb act1-7	-	Simpson, and Spudich, 1984
anti-myosin mAb 56-396-5	-	Pagh and Gerisch,1986
anti-GFP mAb K3-184-2	-	Noegel et al., 2004
anti-Rac1a pAb	-	Filić et al., 2012

4.1.3 D. discoideum strains used in the study

corA KO	-	de Hostos et al., 1991; 1993
corB KO	-	Shina et al., 2009
corAB KO	-	Shina et al., 2010
AX2 expressing GFP-Rac1a	-	Faix et al., 1998
AX2 expressing GFP-RacE	-	This study
GFP-crn7 NT	-	This study
GFP-crn7 CT	-	This study
GFP-cor CRIB	-	This study
GFP-cor WT	-	This study
GFP-MUT1	-	This study
GFP-MUT2	-	This study
GFP-MUT1 in corA$^-$	-	This study
GFP-MUT2 in corA$^-$	-	This study
GFP-PAKa in AX2	-	Müller-Taubenberger et al., 2002
GFP-PAKa-c in corA KO	-	This study

4.2 Methods

4.2.1 Growth of *Dictyostelium* strains

D. discoideum strain AX2 was used as wild type strain. Cells were cultured either in petri dishes or in suspension culture (160 rpm) at 22^{0}C with appropriate antibiotics. All strains were grown and maintained as described (Claviez et al., 1982).

4.2.2 Cloning, expression and purification of GST and GFP fusion proteins

A vector coding for full length coronin with GFP fused to the C-terminus under the control of actin15 promoter was described previously (Gerisch et al., 1995). This plasmid was referred to as GFP-cor WT. The GFP-coronin MUT1 and MUT2 constructs were created by site directed mutagenesis using appropriate primers and the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the sequence verified. Cloning of GFP-PAKa and generation of a dominant negative PAKa (GFP-PAKa-c) was described earlier (Müller-Taubenberger et al., 2002). The plasmids were transformed into AX2 and *corA* knock-out cells by electroporation. Transformants expressing the respective GFP-tagged proteins were selected by using G418 (Geneticin) at 2µg/ml. The expression levels were determined by western blots. For the expression of different coronin deletion constructs as GST fusion proteins, appropriate coding sequences were PCR amplified and cloned into the expression vector pGEX 4T-2 (GE Healthcare) using BamHI and XmaI sites. GST fusion proteins were expressed in *E. coli* strain XL1 blue and purified from the soluble fraction using Glutathione Sepharose affinity columns (GE Healthcare). A 200 bp fragment encoding the CRIB domain of coronin was cloned into the expression vector pBsrN2 (Blau-Wasser et al., 2009) and introduced into AX2 cells. The transformants were selected with blasticidin at 1.5 µg/ml (MP Biomedicals, Eschwege, Germany). Rac proteins were expressed as GST- and GFP-tagged fusion proteins.

4.2.3 Loading of Rac GTPases with GDP or GTPγS

For direct interaction assays, GST, GST-Rac1a, and RacC bound to Glutathione Sepharose columns were loaded with GDP or GTPγS in nucleotide exchange buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM EDTA, and 1 mM DTT) for 1 hour at 4^{o}C. Equivalent amounts of thrombin cleaved fragment (NT CRIB) was added to the preloaded columns and incubated for 1 hour at 4^{o}C. After repeated washing, the bound proteins were separated by SDS-PAGE (15% acrylamide) and visualized with Coomassie Blue. For interaction of full length coronin with Rac GTPases, AX2 cells ($5x10^{7}$) were lysed by sonication in lysis buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT, and 1% NP40 with EDTA-free protease inhibitor cocktail (Roche)) and equivalent amounts of cell lysates were added to columns containing GST, GST-Rac1b, and GST-RacB preloaded with either GDP or GTPγS. After 2 hours of incubation at 4^{o}C, beads were washed with wash buffer

(lysis buffer without protease inhibitors) and pull-down eluates were analyzed in western blots with anti-coronin mAb 176-3-6 (de Hostos et al., 1991). Interaction of DGAP1 with Rac1a was analyzed as described (Faix et al., 1998). Probing was with mAb 216-394-1(Faix and Dittrich, 1996).

4.2.4 Immunoprecipitation and pull down experiments

For coronin self-association studies, equivalent amounts of AX2 cells ($5x10^7$) expressing GFP-cor WT and GFP-MUT2 were lysed by pipetting several times in 500 µl lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.5% NP40 with protease inhibitor cocktail) and incubated on ice for 20 minutes. 500 µl of dilution buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA) was then added to the clarified lysate and incubated with 20 µl of GFP-TRAP beads (ChromoTek, Martinsried, Germany) for 2 hours at 4^{o}C. The beads were washed and the immunoprecipitates were analysed by western blots with anti-GFP mAb K3-184-2 (Noegel et al., 2004) and anti-coronin mAb 176-3-6 (de Hostos et al.,1993). Interaction of Rac1a GTPase with coronin fragments was investigated by pull-down assays. AX2 cells expressing GFP-Rac1a ($5x10^7$) were lysed (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 1 mM DTT, and 5% glycerol) supplemented with protease inhibitors (Sigma) and incubated with equivalent amounts of GST, GST-CRIB, and GST-NT CRIB, GST-CRIB+WD, and GST-CT CC fusion proteins bound to Glutathione Sepharose beads for 2 hours at 4^{o}C. The pull-down eluates were immunoblotted with GFP specific mAb K3-184-2.

For determining the levels of activated Rac in *D. discoideum* cells, a pull down with the GTPase-binding domain (GBD) from rat PAK1 kinase (GST-PAK-GBD) which specifically interacts with the GTP-bound form of Rac1 was carried out followed by western blot analysis with polyclonal antibodies against Rac (Filić et al., 2012).

4.2.5 Mutant analysis

Immunofluorescence was performed as described (Mondal et al., 2008). Cells were fixed by ice cold methanol (5 min, -20^{o}C). Actin was recognized by mAb act-1 (Simpson et al., 1984). For myosin II staining, vegetative and aggregation-competent cells were fixed using ice cold methanol and stained for myosin II using mAb 56-396-2 (Pagh and Gerisch, 1986). Fixed cells were imaged using confocal laser scanning microscopy (TCS SP5 Leica). For surface plot rendering (Figure 6A), scanning

parameters for AX2 were used to image *corA*$^-$ cells and the image stacks were processed with imageJ plug-in for pseudo-3D representation, in which the z-axis represents intensity. Phagocytosis assay for *corA*$^-$ and rescue cells were performed as described (Shina et al., 2011).

4.2.6 Miscellaneous methods

Isolation and analysis of cytoskeletal proteins were done as described earlier (Mondal et al., 2008). To analyse CRIB sequences of coronin, CRIB motifs of HsPAK1 (Q13153), HsWASP (P42758), DdWASP (Q7KWP7), DdPAKb (Q869N2), HsCRN2 (A7MAP1), HsCRN7 (P57737), DdCRN7 (Q55E54) were retrieved and aligned using clustalW2 online program (Thompson et al., 1994). The aligned sequence was processed using ESPript 2.2 (Gouet et al., 1999) for representation. To study the sequence conservation of CRIB domain in short coronins across taxa., proteins sequences of coronins from *H sapiens* (P31146), *M musculus* (O89053), *D melanogaster* (Q7JVY0), *S cerevisiae* (Q06440), *E histolytica* (C4M137), *D discoideum* (P27133), *T thermophila* (I7MIA8), *T cruzi* (Q4D4X6), *T brucei brucei* (Q57W63), *T gondii* (Q5Y1E7), *L. major* (Q4QB38), and *P falciparum* (O44021) were retrieved and the core WD repeats (2-6) were aligned using ClustalX program (Larkin et al., 2007). A bootstrap Neighbor Joining (NJ) tree was created and the resulting cladogram is shown with supporting bootstrap values in percent.

5. Summary

Coronin proteins constitute a subfamily of WD repeat domain containing proteins represented by two homologues, coronin and CRN7, in the amoeba *D. discoideum*. Deletion of either one or both coronins led to severe defects in several physiological processes, such as cytokinesis, migration, and morphogenesis. It was suggested that coronin proteins in *D. discoideum* have both unique and redundant functions in the cell. However, the molecular mechanisms involved are largely unknown. We study here the CRIB (Cdc42- and Rac-interactive binding) motif of the coronin proteins in *D. discoideum*. It is located in a surface accessible area of the beta-propeller and provides a binding site for Rac proteins. Binding to GDP-loaded Rac is more efficient than to GTP-Rac. Through mutational studies, we identified residues important for Rac binding. Furthermore, we show here that *corA*$^-$ cells are defective in myosin II assembly and show increased cortical myosin already in the growth-phase whereas in wild-type cells the level increases only in the aggregation phase. Myosin II assembly is regulated by phosphorylation of its tail region through myosin heavy chain kinases (MHCKs). Their activity depends on the activation state of the serine/threonine kinase PAKa, a CRIB motif containing protein. We find that the myosin defect of *corA*$^-$ mutants can be rescued by expression of the coronin CRIB domain and by a dominant negative PAKa (PAKa-c) which is not targeted to the cell cortex like the wild-type protein and cannot exert its inhibitory function on MHCK. Importantly, a CRIB mutant deficient in Rac binding fails to rescue the myosin phenotype in *corA*$^-$ mutants highlighting the importance of coronin-Rac in regulation of myosin functions. In addition, we show that coronin interacts with PAKa though its N-terminus and that the binding site does not overlap with the CRIB domain. We propose that coronin through its affinity for GDP-Rac regulates the availability of GTP-Rac for activation of PAKa which then affects myosin assembly.

Zusammenfassung

In dieser Arbeit wird die Bedeutung des CRIB (Cdc42- and Rac-interactive binding) Motivs im WD Repeat enthaltenden Protein Coronin untersucht. Diese Motiv ist an der Oberfläche des Proteins lokalisiert und somit zugänglich für Interaktionen mit RhoGTPasen. Coronin bevorzugt Rac-GDP vor Rac-GTPγS, und Mutationen im CRIB Motiv führen zu einer Aufhebung der Rac Bindung. Basierend auf der Präferenz von Coronin für RacGDP könnte Coronin als Regulator der Rac Aktivierung fungieren. Diese Hypothese sollte mit Hilfe einer Coronin defizienten *Dictyostelium discoideum* Mutante (*corA*$^-$) überprüft werden. In dieser Mutante sind die Mengen an aktiviertem Rac erhöht, ferner weist sie einen Myosin Defekt auf. *corA*$^-$ Zellen besitzen eine erhöhte Menge an kortikalem Myosin bereits in der Wachstumsphase, wohingegen in Wildtyp-Zellen dies erst in Zellen in der Aggregationsphase auftritt. Kortikales Myosin ist wichtig für die Polarisierung der Zellen in der Aggregationsphase und für eine effziente Chemotaxis. In *D. discoideum* ist die Bildung von kortikalen Myosinfilamenten durch Phosphorylierung des C-Terminus durch Myosin-schwere-Ketten-Kinase (MHCK) reguliert. Die Aktivität der MHCK ist abhängig vom Aktivierungsstatus der PAKa (p21-activated kinase). PAKa besitzt ein CRIB Motiv und wird durch RacGTP reguliert. Interessanterweise kann der Myosindefekt der *corA*$^-$ Mutante nicht durch ein Coronin aufgehoben werden, das eine mutierte CRIB Domäne besitzt. Dieses Mutantenprotein ist auch nicht in der Lage, mit Rac Proteinen zu interagieren. Eine dominant negative PAKa (PAKa-c), die nicht mehr im Zellkortex lokalisiert ist sondern im Zytosol, führt dagegen zu einer Reduktion von kortikalem Myosin in der Wachstumsphase in *corA*$^-$ Zellen. Unsere Ergebnisse führen zu einem Modell, bei dem Coronin die Menge an Rac kontrolliert, die für eine Aktivierung zur Verfügung stehen. Dadurch kann es Prozesse beeinflussen, die von RacGTP abbhängig sind wie die Aktivierung der PAKa.

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7. Abbreviations

CRIB	-	cdc42/Rac interactive binding
PAK	-	p21- activated kinases
GDP	-	Guanosine di-phosphate
GTP	-	Guanosine tri-phosphate
GTPγS	-	Guanosine 5′-[γ-thio] triphosphate tetralithium salt
GEF	-	Guanosine Exchange Factors
GAP	-	GTPase activating proteins
GDI	-	Guanosine dissociation inhibitors
GBD	-	GTPases binding domain
PDB	-	p21 activated kinase binding domain
ACK	-	Cdc42 and Rac-interactive kinase
VCA	-	Verprolin homology, Cofilin homology, acidic region segment
GRD	-	GTPase related domain
mhcA-	-	myosin heavy chain A knock-out cells
MHCK	-	Myosin Heavy Chain Kinases
Akt	-	Proteins kinase B (PKB)
IPTG	-	isopropyl β-D-thio-galactoside
MUT1 or 2	-	coronin protein mutated in CRIB domain

8. Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen und Abbildungen, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht ist, sowie, dass ich eine Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Frau Prof. Dr. Angelika A. Noegel betreut worden.

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