CPNA IS AN ACTIN FILAMENT DEPOLYMERIZING PROTEIN

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ABSTRACT

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by Mingxi Han

Copines make up a multigene family of calcium-dependent, phospholipid-binding proteins. Copine proteins consists of two C2 domains at the N terminus followed by an “A domain” similar to the von Willebrand A domain found in integrins. The C2 domain is a calcium-dependent membrane-binding motif, while the A domain is thought to be a protein-binding domain. We are studying copine protein function in the model organism, *Dictyostelium discoideum*, which has six copine genes, *cpnA*- *cpnF*. Previous research on one of the copine proteins in *Dictyostelium*, CpnA, suggests that CpnA interacts with actin. However, it is not clear, whether CpnA can directly bind to actin. The goal of this project is to determine if CpnA is able to bind to actin monomers and/or filaments and if CpnA is able to regulate actin polymerization and/or depolymerization *in vitro*. First, we created DNA constructs to express a GST-tagged CpnA in *Dictyostelium* and then transformed the newly made plasmids into wild-type *Dictyostelium* cells. We used glutathione agarose chromatography to purify GST-CpnA from *Dictyostelium* cells. Using F-actin binding assays, we found that CpnA is able to cause depolymerization of the F-actin in the presence, but not absence of calcium. In addition, we did immunoprecipitations with cells expressing a GFP (Green fluorescent protein)-tagged CpnA and a GFP-tagged A domain of CpnA and an anti-GFP antibody to isolated these fusion proteins to

determine whether F-actin and/or G-actin could co-precipitate. Our preliminary data suggests that both the full-length CpnA and the A domain of CpnA are able to bind F-actin, but not G-actin. Our results indicated that CpnA can bind to F-actin in a calcium-dependent manner and acts as an F-actin depolymerizing protein.

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CHAPTER I

INTRODUCTION

Copines

In 1998, Creutz *et al.,* (1998) found two new related proteins, which he called copines, by isolating calcium-dependent membrane binding proteins from *Paramecium*. Since then, copines have been found in a variety of eukaryotic organisms, including *Dictyosteliumdiscoideum, Caenorhabditiselegans, Arabidopsis thaliana, Paramecium, Musmusculus*and *Homo sapiens* (Creutz *et al*., 1998; Damer *et al*., 2005). All of these organisms have multiple genes in the copine family with humans having eight copine genes. The wide distribution of copines and their high conservation in evolution indicates that copines may play key roles in various cellular processes; however, the exact function of copines is unknown (Tomsig *et al.,* 2003).

Copines are soluble, calcium-dependent phospholipid-binding proteins that are characterized by two C2 domains at the N-terminal region followed by an "A domain" at the C-terminal region. The C2 domain functions as a calcium-dependent membrane binding domain. The "A domain" is thought to function as a protein binding domain and is similar to the von Willebrand A (VWA) domain also found in integrins (Creutz *et al.,* 1998).

The C2 domains are found in many other proteins. Those with multiple C2 domains, like synaptotagmin and rabphilin, are involved in membrane trafficking. Those with a single C2 domain, like protein kinase C and phospholipase C, are

involved in cell signaling. Having C2 domains suggests that copines may function in either membrane trafficking or cell signaling (Tomsig *et al.,* 2002).

Tomsig *et al.,* (2003) identified protein binding partners of human copines using the yeast two-hybrid system. Tomsig *et al.,* (2003) found that the A domain of several human copines can interact with many different intracellular proteins, including those involved in cell signaling, cytoskeleton function, and transcriptional regulation. Human copines are expressed differentially in different tissues suggesting that they have specific functions in particular tissues. Copines I-III are present in all tissues, copineIV is highly expressed in the aorta and the heart, copineV is expressed in the heart and the lymphatic system, and copineVI is only expressed in the brain (Cowland *et al.,* 2003).

Copines have been studied in both *C. elegans* and *Arabidopsis*. In *C. elegans*, the *gem-4* gene, which codes for a copine protein, has an antagonistic relationship with the gon-2 gene. The*gon-2* gene codes for acation channel of the TRPM (transient receptor potential ion channels melastatin) family that is necessary for the division of gonadal precursor cells. Therefore, gem-4 is thought to have two possible roles, as either an inhibitor of GON-2, or a regulator of GON-2 trafficking to the plasma membrane. In *C. elegans*, there is no significant phenotype found with the *gem-4* mutation. GFP labeled gem-4 localizes to the plasma membrane (Church & Lambie 2003).

Plant copines have been studied using *Arabidopsis thaliana* as a model. *BONZAI1 (BON1), also calledCPN1*is required for normal growth of *Arabidopsis thaliana* (Hua *et al.,* 2001). BON1 and its associated protein BAP1 appear to play a significant role in regulating cell expansion and division at low temperatures. In addition, *CPN1/BON1* gene mutants have a defect in disease resistance when grown under harsh and high humidity conditions. However, the ability of disease resistance could be increased when the mutants lived in the permissive and low humidity conditions. The function of Cpn1 may be to regulate responses to severe conditions, like low or high humidity. In addition, Cpn1may act as a suppressor of defense responses, such as the hypersensitive cell death defense response (Jambunathan *et al.,* 2001, 2003; Hua *et al.,* 2001). The Cpn1/BON-1 protein labeled with GFP was found to be associated with the plasma membrane.

The analysis of mutant copine homolog phenotypes in *Arabidopsis thaliana* and *Caenorhabditiselegans* indicates that *copines* are very important in many different cell functions, such as development, growth, apoptosis, and pathogen defense.

Dictyosteliumdiscoideum Is a Model Organism to Study *Copines*

The protozoan *Dictyostelium discoideum* is a eukaryotic, haploid amoeba that inhabits the forest soil and feeds on diverse species of bacteria (Raper *et al.,* 1935, 1936). *Dictyostelium* is easy to culture and is amenable to genetic manipulation. The *Dictyostelium* genome contains many homologous genes to those in more derived

eukaryotes (Eichinger *et al.,* 2005; Heidel *et al.,* 2011). Therefore, *Dictyostelium* is used as a model organism to study many biochemical and genetic processes (Schaap *et al.,* 2011).

In starvation conditions, *Dictyostelium*, which normally exists as a single celled organism, will go through a specific developmental cell cycle in 24 hours to form multicellular fruiting body structures containing environmental resistant spores on top of a thin stalk. When the environment is depleted of nutrients, the amoebae will release cAMP. The cAMP can be sensed by the cells by way of the cAMP receptor cAR1 (Klein *et al.,* 1988). Cells will move toward the aggregation center and, in about 10 hours, will form a mound (Franca-Koh *et al.,* 2006). At 14-16 hour, the cells in the mound will form a finger-like structure, and from 16-18 hours, the finger may fall over to form a slug that can move towards the light and heat (Kessin *et al.,* 2001; Raper *et al.,* 1984).By 19-20 hours, the slug will undergo culmination to form a fruiting body. The fruiting body is made up of 20% of the cells that die to form a stalk that support the remaining 80% of cells that form a ball of spores. The main function of the stalk is to raise the spores to facilitate the dispersal of spores by passing organisms (Bonner 1982; Huss 1989).

Dictyostelium discoideum is used to study cytokinesis, cell motility, phagocytosis, chemotaxis, signal transduction, and cell differentiation. The NIH (National Institutes of Health) has chosen *Dictyostelium* as one of the model organisms in the Model Organism Initiative.

CpnA in *Dictyostelium*

Six copine genes in the *Dictyosteliumdiscoideum* genome have been identified by Dr. Cynthia Damer's lab: copine A-copine F (*cpnA-cpnF*). All six *Dictyostelium* copine proteins contain two C2 domains followed by a VWA domain. The expression patterns of all six copine genes has been detected during development by using real time reverse transcription-PCR (RT-PCR). All the copine genes were expressed in vegetative cells and each gene exhibited a distinct expression pattern throughout development. Many of the copines showed an up regulation of mRNA expression at developmental transitions, suggesting that copines are important regulators of *Dictyostelium* development (Damer *et al.,* 2007).

We have focused our studies on one of the copines, copineA (*cpnA*). CpnA is expressed throughout development and it can bind to membranes in a calcium-dependent manner *in vitro* (Damer *et al.,* 2005). Also, GFP-tagged CpnA can bind to membranes in a calcium-dependent manner. Expressed in wild-type *Dictyostelium* cells, GFP-CpnA was found in the cytoplasm without binding to membranes. However, GFP-CpnA was found to transiently (1-10s) bind to the plasma membrane and intracellular vacuoles in a subset of starved cells. In these cells, GFP-CpnA was observed to transiently bind to membranes multiple times over a short period of time (Damer *et al.,* 2005). In fixed cells, GFP-CpnA was found bound to the plasma membrane and intracellular organelles including contractile vacuoles, organelles of the endolysosomal pathway, and phagosomes. This result indicates

that CpnA may play a significant role in the function of these organelles (Damer *et al.,* 2005).

In order to study the function of CpnA in *Dictyostelium*, a *cpnA*-knockout strain was created using homologous recombination. The *cpnA*-cells exhibited normal growth rates, but had a slight cytokinesis defect. *cpnA*- cells also formed very large contractile vacuoles in water, which indicates that they have a defect in contractile vacuole function. However, *cpnA*- cells had endocytosis and phagocytosis rates similar to wild-type cells (Damer *et al.,* 2007). These studies showed that CpnA plays a role in cytokinesis and contractile vacuole function.

When starved, the *cpnA*- cells are able to aggregate to form mounds and slugs, but cannot form fruiting bodies (Damer *et al.,* 2007). In addition, *cpnA*- cells exhibit delayed aggregation and form large mounds. The large mounds formed by *cpnA*- cells do not break apart into several slugs, as the wild-type slugs do (Flegel *et al.,* 2011). This study suggests that *cpnA*- cells may have a possible defect in cell adhesion. The cells may adhere to each other too strongly. The result could also explain the delay in aggregation during development.

When *cpnA*- cells are mixed with a small percentage of wild-type cells, they are able to form fruiting bodies, but with short stalks. The more wild-type cells mixed with *cpnA*- cells, the longer the stalks would be. The wild-type cells were found all over the chimeric slug, and within the fruiting bodies' stalks and spore heads. All these results suggest that CpnA is involved in the regulation of aggregation and

culmination during *Dictyostelium* development. In addition, CpnA may play a role in prestalk cell differentiation (Smith *et al.,* 2010; Flegel *et al.,* 2011).

In fact, all the developmental defects observed in the *cpnA*- cells can be rescued by mixing the *cpnA*- cells with wild-type cells. The short-stalked fruiting bodes were formed when the mutants mixed with 10% wild-type cells. Moreover, as the percentage of mixed wild-type cells increased, the number of the new forming fruiting bodes increased and also the length of the stalks (Smith *et al.,* 2010). This result suggests that *cpnA*- cells have an ability to respond to the signals that have been secreted by wild-type cells. However, the *cpnA*- cells cannot secrete this signaling molecule (Smith *et al.,* 2010). In addition, the fruiting body formation defect found in *cpnA*- cells can be rescued by depleting extracellular calcium. Ethylenediaminetetraacetic acid (EDTA), a water-soluble solid, can be used to chelate extracellular calcium, and then the *cpnA*- cells can form short-stalked fruiting bodies in the absence of calcium. The results suggest that *cpnA* may have a role in releasing the molecule that is regulated by calcium, which is important in the differentiation of stalk cells (Smith *et al.,* 2010).

During the development of *Dictyostelium*, slugs will move towards heat and light to find a suitable place to form the fruiting body (Bonner 1998). This migration is called phototaxis and thermotaxis. Slugs formed from *cpnA*-cells exhibit defects in phototaxis and thermotaxis (Flegel *et al.,* 2011). No difference was observed in the distance that slugs moved between wild-type and *cpnA*-cells, indicating that the

cpnA- slugs have a defect in sensing the heat and light and not in movement (Smith *et al.,* 2010).

Protein binding partners of CpnA

To determine the exact functions CpnA may play in these various activities, we carried out experiments to identify the binding partners of CpnA. We used two different techniques: column chromatography and immunoprecipitation. Both techniques have identified actin as a possible protein-binding partner of CpnA (unpublished results).In addition, Tomsig *et al.,* (2003) identified actin as a protein that interacts with several of the human copines using the yeast two-hybrid system. We also stained both *cpnA*- cells and wild-type cells with rhodamine-phalloidin and observed that *cpnA*- cells have more actin filaments than wild type cells (Hanqian Mao, unpublished observations), suggesting that CpnA may play a specific role in actin filament disassembly.

Actin was first discovered in 1887 by extracting it from muscle (Halliburton1887). It is one of the most abundant proteins found in the cytoplasm of eukaryotes. Actin is able to form filaments (F-actin), which are one of the three main components of the cytoskeleton. Actin has two forms, monomeric molecules of a single protein (G-actin) and polymeric filaments (F-actin). Actin filaments can be polymerized and depolymerized very quickly. Actin filaments build large networks in cells and are involved in many cellular functions such as cell shape, cell adhesion, cell migration, cytokinesis, phagocytosis, and signal transduction (Huber 2013). The

actin protein has also been found to localize to the nucleus (Grummt 2006). The location of actin can be regulated by signal transduction pathways, and the actin filaments are especially stable in muscle (Eckert 2002).

To determine if CpnA binds directly to actin and/or actin filaments and if CpnA has a role in actin assembly or disassembly, we must first develop a system to isolate and purify CpnA. The GFP-CpnA could be expressed in wild-type *Dictyostelium* cells (Damer *et al.,* 2005). However, it would be hard to purify GFP-tagged CpnA. Consequently, we expressed a GST (Glutathione S Transferase)-tagged version of CpnA in *Dictyostelium* and purified it using glutathione agarose chromatography. First, we ligated the *cpnA* cDNA into a plasmid containing the GST gene to express a CpnA with an N-terminal GST tag and a C-terminal HIS-tag. The newly made plasmid was transformed into wild-type *Dictyostelium* cells for expression. The fusion protein was successfully expressed and purified by glutathione agarose chromatography. Once a pure fraction of CpnA was obtained, we performed F-actin binding assays to test the direct interaction between CpnA and actin.

We also used immunoprecipitations to test for the interaction between CpnA and actin. These experiments would show if the actin can bind specifically to the A domain of CpnA by using cells expressing GFP-tagged CpnA and GFP-tagged A domain.

Our results indicate that GST-CpnA is able to directly bind to F-actin and cause depolymerization in a calcium-dependent manner. The GFP-Ado (A domain of CpnA)

and GFP-CpnA can bind to F-actin, but are not able to interact with G-actin. Our conclusion is that CpnA can act as an F-actin depolymerizing protein by binding to F-actin.

CHAPTER II

MATERIALS AND METHODS

Creation of DNA Constructs to Express GST and HIS-tagged CpnA

The plasmid, pDXA-GST (203) was obtained from the Dicty Stock Center. This vector, pDXA-GST (203) is used to express proteins with an N-terminal GST tag and a C-terminal HIS-tag. The pDXA-GST plasmid was digested with the restriction enzyme *KpnI* at 37^{o}C for an hour. Then the *cpnA* cDNA was cut from the pTX-GFP plasmid with *KpnI*, gel purified, and ligated into the pDXA-GST plasmid using the PromegaLigaFast rapid DNA ligation system. The ratios of insert to vector were3:1 and 6:1**.** The ligations were transformed into DH5α library efficiency *E. coli* cells**.** The *E. coli* cells were thawed on ice and mixed with 2μl of the ligation reaction. The cells were incubated on ice for 30 minutes and heat-shocked for 45 seconds in a 42^{o}C water bath. Next, the cells were placed on ice for 2 minutes and then 0.9ml of room temperature S.O.C medium was added. All the tubes were put into the incubator shaking at 225 rpm at 37^{o}C for 1 hour. The cells were spread on Luria Broth (LB) agar plates with ampicillin (0.1mg/ml) and incubated at 37^{o}C for 20 hours. Colonies were picked and cultured in 2ml of LB with ampicillin (0.1mg/ml) shaking at 225 rpm at 37^{o}C overnight. The plasmids were extracted from the bacteria cells using a Qiagen mini-prep kit and were screened for the correct plasmid by a restriction digest with *EcoR*V and a double of digest with *BamH*I-HF and *Sal*I-HF. The concentration of the plasmids after the mini-prep was estimated using the Nano-drop. A Qiagen maxi-prep

was used to obtain a large amount of plasmid for transformation into *Dictyostelium* cells.

Dictyostelium discoideum Cell Culture

*Dictyosteliumdiscoideum*NC4A2, an axenic strain derived from the wild-type NC4 strain, cells were cultured on plastic petri dishes in HL-5 media (0.75% protease peptone, 0.75% thiotone peptone, 0.5% Oxid yeast extract, 1% glucose, 2.5mM Na_2HPO_4, and 8.8mM KH_2PO_4, pH 6.5) supplemented with penicillin-streptomycin at 60 Units/ml at 20^{o}C.

Transformation of Plasmids into *Dictyostelium discoideum* Cells

Dictyostelium cells were harvested in HL-5 media and counted by using a hemocytometer. The cells were pelleted at 1000rpm for 5 minutes at 4^{o}C. The supernatant was discarded. The cells were washed with 10ml of H-50 buffer (20mM HEPES, 50mM KCl, 10mM NaCl, 1mM $MgSO_4$, 5mM $NaHCO_3$, 1mM NaH_2PO_4, pH 7.0) twice. Cells were resuspended in H-50 buffer to a concentration of 5×10^7cells/ml. Then 100μl of the cells were mixed with10μg of plasmid DNA and placed into chilled 1mm electroporation cuvettes that were put into the electroporator (Eppendorf2510). The cells were pulsed twice 5 seconds apart. The voltage was set at 1400, 1450, 1500 and 1550 mV and the time constant was 1.0. After pulsing, the cells were placed on ice for 5 minutes and then plated with HL-5 media containing

penicillin-streptomycin and incubated at 20^{o}C. After 24 hours, the HL-5 media was replaced with HL-5 supplemented with G418 media (1μg/ml).

Western Blot

To screen for the expression of the GST-tagged CpnA in the newly transformed cell lines, a Western Blot was performed. Cells were harvested from plates, treated with sample buffer, boiled, and then run on a 12% polyacrylamide gel at 2x10^{6} cells/per lane. The gel was transferred to PVDF membrane and then incubated with a 1:2000 dilution of CpnA antibody (polyclonal rabbit antisera against a recombinant fragment consisting of the first C2 domain of CpnA) as the primary antibody. A 1:2000 dilution of a horseradish peroxidase-conjugated anti-rabbit antibody was added to the membrane as the secondary antibody. An ECL chemiluminescence kit along with the Gel Logic 2200 for imaging was used to detect the CpnA protein on the blot.

Glutathione Agarose Chromatography

Dictyostelium cells expressing GST-tagged CpnA were harvested from plates and counted with a hemocytometer. The cells were pelleted at 2500rpm for 5 minutes at 4^{o}C and the supernatant was discarded. Lysis buffer (5mM EGTA, 1%Triton X-100, 1mM DTT and 1X protease inhibitor dissolved in PBS) was used to resuspend the pelleted cells and the cell suspension was incubated on ice for 30 minutes. Glutathione agarose beads (Sigma, 50mg) were suspended in 5ml of DI water, placed on the rotator, and incubated at room temperature for 30 minutes. The beads

were washed three times in PBS by pelleting at 3000rpm for 5 minutes at 4°C. The beads were resuspended in 150µl of PBS to make a 50% bead slurry. The cells in lysis buffer were spun in a microfuge at 14000rpm for 5 minutes at 4°C. The bead slurry was added to the cell lysate supernatant and placed on the rotator at room temperature for 30 minutes. The beads were washed by pelleting at 3000 rpm for 5 min at 4°C four times in PBS, and two times using lysis buffer. The beads were washed with 5ml 10mM Tris (pH 8.0), then incubated for 10 min on a rotator at room temperature in elution buffer (10mM reduced glutathione in 50mM Tris-HCl pH 8.0). The beads were washed three times with elution buffer and the supernatants were kept frozen at-20°C.

F-actin Binding Assay

Eluted samples of the GST-CpnA were thawed and then spun at 150,000xg (50,000rpm) for 1 hour at 4°C. The supernatant was removed and kept on ice. The F-actin binding assay kit from Cytoskeleton, Inc. was used. An F-actin stock was prepared as described in the published protocol (Cytoskeleton, Inc.). Six samples were prepared for the F-actin binding assay: 1) an F-actin control sample made with mixing 40µl F-actin stock and 10µl of elution buffer, 2) a protein control made with mixing 10µl GST-CpnA protein and 40µl F-actin buffer,3) an alpha-actinin control made with mixing 40µl F-actin buffer and 10µl alpha actinin,4) an alpha-actinin control sample made with mixing 40µl of F-actin stock with 10ul alpha-actinin, 5) a negative control made with mixing 2µl BSA, 8µl Milli-Q water and 40µl F-actin stock, and 6) a

test sample made with mixing 40μl F-actin stock and 10μl GST-CpnA protein. All the samples were spun at 50,000rpm for 1.5 hour at 24^{o}C. The supernatants were removed and 10μl 5X sample buffer was added into each sample. The pellets were resuspended with 30μl 2X sample buffer. All the samples were run on a 12% polyacrylamide gel and then stained with Coomassie Blue. A Western Blot was performed for the supernatants and pellets from the samples that included GST-CpnA. A 1:2000 dilution of the CpnA antibody (polyclonal rabbit antisera against a recombinant fragment consisting of the first C2 domain of CpnA) was added to the membrane as the primary antibody. A 1:5000 dilution of a horseradish peroxidase-conjugated anti-rabbit antibody was added to the membrane as the secondary antibody.

Triton Cytoskeleton Isolation

Dictyostelium cells expressing GFP, GFP-Adomain, or GFP-CpnA were harvested in HL-5 media and counted by using a hemocytometer. The cells were pelleted at 1500rpm for 5 minutes at room temperature and resuspended in buffer A containing either EGTA or calcium (0.1M MES pH 6.8, 5mM $MgCl_2$, 0.5mM ATP and 2mM calcium chloride or 2.5mM EGTA) to a concentration of 5×10^6cells/ml. Cells (1.5×10^6 cells) were transferred into a new tube and then pelleted at 2300rpm for 5 minutes at room temperature. The supernatant (150μl) was removed and 150μl of Buffer B (Buffer A with 0.5% Triton X-100 and protease inhibitors) was added into the tube. All the tubes were vortexed for 5 seconds at medium speed and immediately

spun down at 14,000rpm in a microfuge for 1 minute at 4^{o}C. The supernatants were transferred to tubes with pre-chilled 700µl acetone, briefly mixed, and kept on ice for 15 minutes. Acetone-precipitated proteins were spun down at 14,000rpm for 15 minutes at room temperature. The supernatant was discarded and the pellet was resuspended with 30µl 2X sample buffer and saved as supernatant samples for Western Blot analysis. The pellets containing the insoluble cytoskeleton were resuspended with 30µl 2X sample buffer and saved as pellet samples for Western Blot analysis. All the samples were heated for 1 minute at 95^{o}C before loading on a 12% SDS polyacrylamide gel. For Western blotting, a monoclonal antibody to GFP was used at a 1:500 dilution and an anti-mouse horseradish peroxidase-conjugated secondary antibody was used at a 1:15000 dilution. An ECL chemiluminescence kit along with Gel Logic 2200 was used for imaging the blot.

Immunoprecipitations

Dictyostelium cells expressing GFP, GFP-Adomain, or GFP-CpnA were harvested in HL-5 media and counted by using a hemocytometer. Cells (2×10^{8} cells) were pelleted at 1500rpm for 5 minutes at room temperature. The cells were washed once with 5ml of 4^{o}C pre-chilled 50mM HEPES (pH7.4) buffer and spun again. Cells were resuspended in 1.5ml of lysis buffer (50mM HEPES pH7.4, 100mM NaCl, 2mM $CaCl_2$, 10% w/v sucrose, 0.3% v/v NP-40 and 1X protease inhibitor) and incubated for 10 minutes on a rotator at room temperature. The lysate was spun down at 14,000rpm for 10 minutes at room temperature.$CaCl_2$ was added to the lysate to

bring it to 5mM. Dynabeads (50µl of 30mg/ml) in phosphate buffered saline (pH7.4), with 0.01% Tween-20 (PBS-T) and 0.09% sodium azide was transferred to three tubes separately. The beads were washed with 50mM HEPES (pH7.4) one time and incubated with 200µl 50mM HEPES for 30 minutes. The tubes were placed on the magnet to separate the beads from the solution, and the supernatants were removed. The rabbit polyclonal antibody to GFP (10µg) was added to each tube and incubated at room temperature for 10 minutes on a rotator. The tubes were placed on the magnet again and the supernatant was removed. The beads-antibody complex was resuspended with 200µl PBS-T by gentle pipetting. The tubes were placed on the magnet to remove the PBS-T and the beads were resuspended in cell lysates and incubated for 10 minutes at room temperature. The beads were washed three times with 200µl 50mM HEPES, pH7.4 using the magnet. After the final wash, the beads were resuspended with 100µl 50mM HEPES, pH7.4 and transferred to new tubes. Then 20µl F-actin or G-actin and 20µl polymerization buffer was added to the tubes and incubated with the beads-antibody complex for 30 minutes to allow the F-actin to bind. The tubes were placed on the magnet to separate the bead complex and the solution. The proteins were eluted from the beads using 40µl 2X sample buffer. The tubes were heated for 10 minutes at 70^{o}C and placed on the magnet to separate the beads from the proteins in the sample buffer. Eluted samples were analyzed using SDS-PAGE and Western Blot using a monoclonal anti-actin antibody.

CHAPTER III

RESULTS

GFP-CpnA Associates with the Triton X-100 Insoluble Cytoskeletal Fraction in the Presence of Calcium

Dictyostelium cells expressing GFP, GFP-CpnA, and the A domain of CpnA tagged with GFP (GFP-Ado) were treated with Triton X-100 and the insoluble cytoskeletal fraction was isolated by centrifugation. Both the pellets and the supernatants were analyzed by Western blotting with an antibody to GFP. In the presence of calcium, most of the GFP-CpnA was found in the pellet with the cytoskeleton. However, in the absence of calcium, none of the GFP-CpnA was in the pellet. In contrast, a small amount of GFP-Ado was found in the pellet with the cytoskeleton in both the presence and absence of calcium. The GFP, which served as a control, was found only in the supernatant and did not pellet with the cytoskeleton with or without calcium (Figure 1). These experiments suggest that GFP-CpnA interacts with the actin cytoskeleton in a calcium-dependent manner, but it does not tell us whether CpnA is able to interact with actin directly.

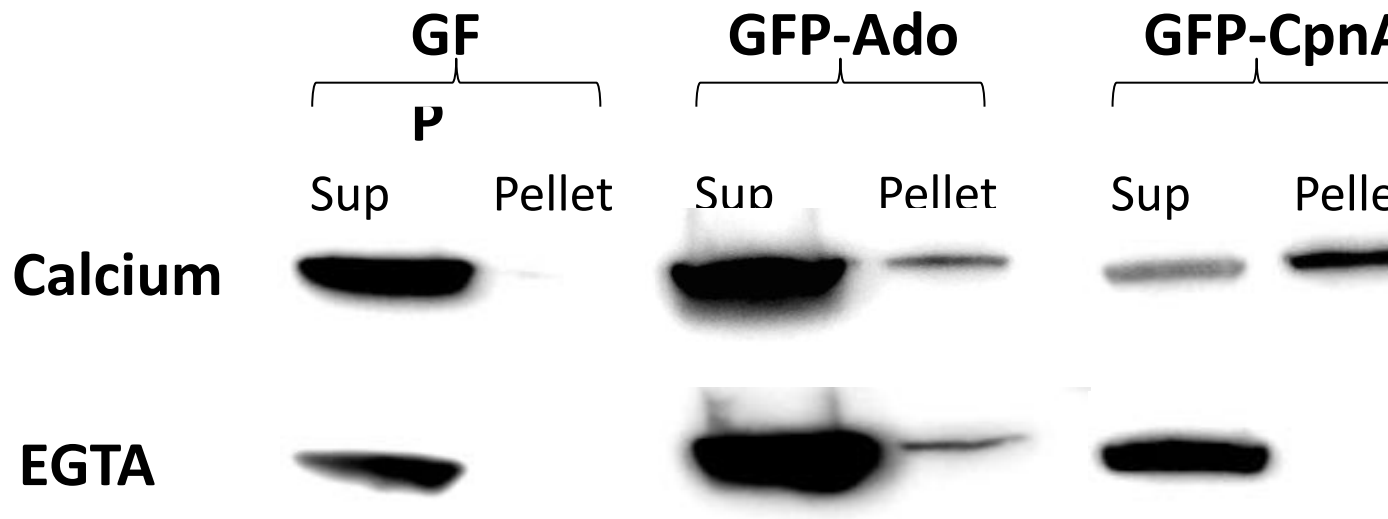

Figure 1. GFP-CpnA Pellets with the Actin Cytoskeleton in a Calcium-dependent Manner.
Wild-type *Dictyostelium* cells expressing GFP, GFP-Ado, or GFP-CpnA were treated with Triton X-100 and centrifuged. Both the pellets and supernatants (Sup) were analyzed by Western blot using a polyclonal antibody to GFP.

Construction of the pDXA-GST/*cpnA* Plasmid and the Transformation of *Dictyostelium* Cells with New Plasmid

In order to determine if CpnA can bind directly to actin, purified CpnA protein was needed. A GST-tagged CpnA protein was chosen for the purification. The first step was to construct the plasmid to express the GST-CpnA. An expression plasmid (pDXA-GST) to create GST-fusion proteins was obtained from the Dicty Stock Center. The *cpnA* cDNA was ligated into the *Kpn*I site within the multicloning site of the pDXA-GST plasmid. The ligations were transformed into bacterial cells and colonies were screened for plasmids with the *cpnA* cDNA inserted in the correct direction. Isolated plasmids were digested with *EcoR*V first. *EcoR*V cleaves the plasmid at one site and the *cpnA* cDNA at one site to create two fragments of sizes 2.6 kb and 6 kb if the insert is in the correct orientation (Figure 2). The plasmids were

also confirmed with a double digest with *BamH*I-HF and *Sal*I-HF. Each enzyme cuts the plasmid once creating two fragments, 2.3 kb and 6.3kb (Figure 3).The newly made plasmid was transformed into *Dictyostelium* cells and expression was tested with a Western Blot using an antibody to CpnA (Figure 4).

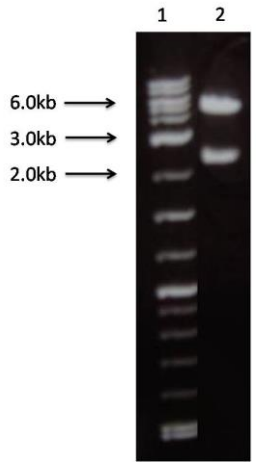

Figure 2. pDXA-GST/*cpnA* Plasmid Digested with *EcoRV*.
The agarose gel is 1% and was run at 110V for 1 hour. Lane 1: 5μl of Tri-Dye log-2 DNA ladder. Lane 2:1μl of 10X tracking dye and 10μl of DNA plasmid digested with *EcoRV*. This gel picture was taken using Kodak gel imaging system.

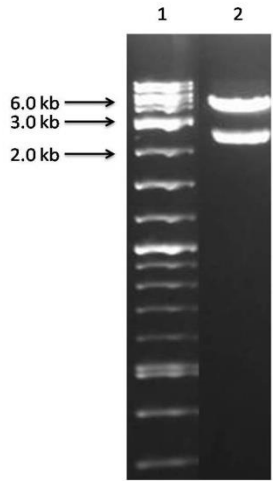

Figure 3. pDXA-GST/*cpnA* Plasmid Digested with *BamH*I-HF and *Sal*I-HF.
The agarose gel is 1% and was run at 110V for 1 hour. Lane 1: 5μl of Tri-Dye log-2 DNA ladder. Lane 2:1μl of 10X tracking dye and 10μl of DNA plasmid digested with *Bam HI-HF* and *Sal I-HF*. This gel picture was taken using Kodak gel imaging system.

In lane 2, two fragments were shown, one is about 2.3 kb and the other one is about 6.3 kb.

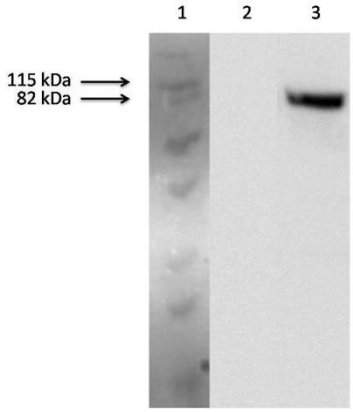

Figure 4. Western Blot Analysis of *Dictyostelium* Cells Expressing GST-CpnA Using a CpnA Antibody.
Sample buffer was added to both wild-type and plasmid transformed cells to make cell lysates. Lysates from 2 $X10^6$ cells were run on a 12% polyacrylamide gel at 200V for 30 minutes. Lane 1:5μl of Precise Protein Plus standard. Lane 2: Wild-type cells. Lane 3: GST-CpnA transformed cells. All the proteins were transferred from the gel to PVDF membranes at 100V for 1 hour. The membrane was incubated overnight in the cold room with primary antibody anti-CpnA (1:2000) and incubated with the secondary antibody, anti-rabbit HRP conjugated (1:5000) for 1 hour.

Purification of GST-CpnA Protein

Once we verified that GST-CpnA was overexpressed, we used glutathione agarose chromatography to purify the GST-CpnA protein from the transformed *Dictyostelium* cells (Figure 5).The GST-CpnA fusion proteins bound to the glutathione agarose beads and were separated from the other proteins by centrifugation. The fusion protein was eluted off the agarose beads using reduced glutathione. A small amount of the fusion protein was eluted from the beads during the washes; however, most of the fusion protein was eluted from the beads with glutathione.

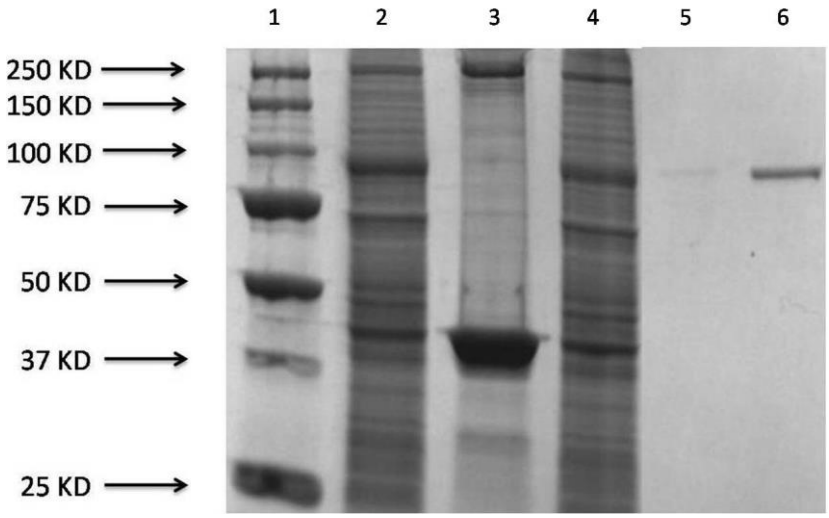

Figure 5. Purification of GST-CpnA from *Dictyostelium* Cells by Glutathione Agarose Chromatography.

A GST-tagged version of CpnA was expressed in wild-type *Dictyostelium* and the GST-CpnA was purified by glutathione agarose chromatography. Gel samples were taken at each step of the purification and analyzed by SDS-PAGE. Lane1: 5μl of Precise Protein Plus standard. Lane 2: Supernatant after the cell lysis and centrifugation. Lane 3: Pellet after the cell lysate centrifugation. Lane 4: Cell lysate after incubation with the glutathione agarose beads. Lane 5: Last wash of the glutathione agarose beads with 10mM Tris pH 8.0. Lane 6: Elution of the GST-CpnA from the glutathione agarose beads with glutathione.

CpnA Binds to F-actin and Causes Depolymerization in a Calcium-dependent Manner

To determine if GST-CpnA is able to directly bind to actin, we performed F-actin binding assays. G-actin was polymerized into F-actin and then incubated with GST-CpnA for 30 minutes. The F-actin was pelleted by ultracentrifugation and both the pellets and supernatants were analyzed by SDS-PAGE and Coomassie staining (Figure 6 and 7).Although we only added a small amount of GST-CpnA, we found that it was able to cause depolymerization of almost half of the F-actin in the assay. In addition to incubating the F-actin with GST-CpnA, we performed several control assays. Alpha-actinin was used as a positive F-actin binding control and BSA was used as a negative F-actin binding control. Alpha-actinin was shown to pellet with

F-actin, while BSA did not. This result indicates that GST-CpnA can interact with F-actin directly and also it is able to depolymerize F-actin into G-actin. However, it did not show if this interaction is calcium-dependent. Therefore, we performed these assays again, but instead of adding calcium to the buffer, we added EGTA (Figure 8). Without calcium, the GST-CpnA did not cause the depolymerization of F-actin. This result indicates that the depolymerizing activity of CpnA is dependent on calcium. However, these assays did not tell us whether GST-CpnA was actually binding to the F-actin since we did not add enough GST-CpnA to be able to see it on the Coomassie stained gel. Therefore, we used a Western blot with an antibody to CpnA to determine whether the CpnA binds to F-actin and/or G-actin (Figure 9).The Western blot revealed that GST-CpnA does not bind to the G-actin and pellets with the F-actin only in the presence of calcium.

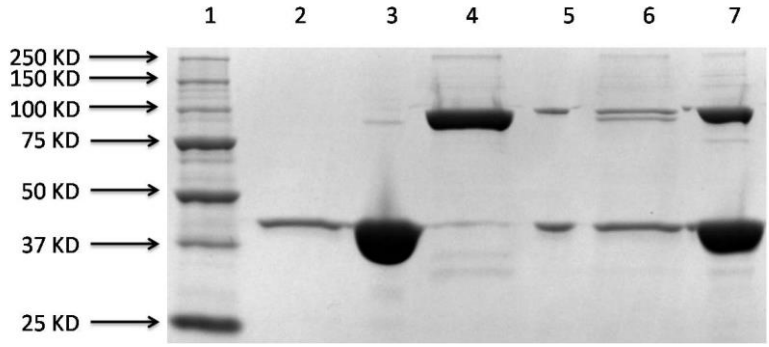

Figure 6. F-actin Binding Assays. All the samples were prepared by ultracentrifugation to separate the F-actin from G-actin. The supernatants and pellets were analyzed by SDS-PAGE. Lane1: 5μl of Precise Protein Plus standard. Lane 2-3: the supernatant and pellet of F-actin control. Lane 4-5: the supernatant and pellet of α-actinin control. Lane 6-7: the supernatant and pellet of α-actinin and F-actin control.

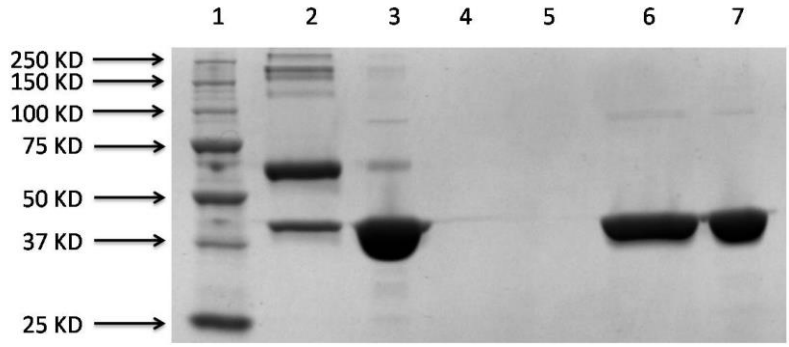

Figure 7. F-actin Binding Assays Shows Purified GST-CpnA Depolymerizes F-actin. All the samples were prepared by ultracentrifugation to separate the F-actin from G-actin. The supernatants and pellets were analyzed by SDS-PAGE. Lane1: 5μl of Precise Protein Plus standard. Lane 2-3: the supernatant and pellet of BSA and F-actin control. Lane 4-5: the supernatant and pellet of GST-CpnA fusion protein sample. Lane 6-7: the supernatant and pellet of GST-CpnA fusion protein and F-actin.

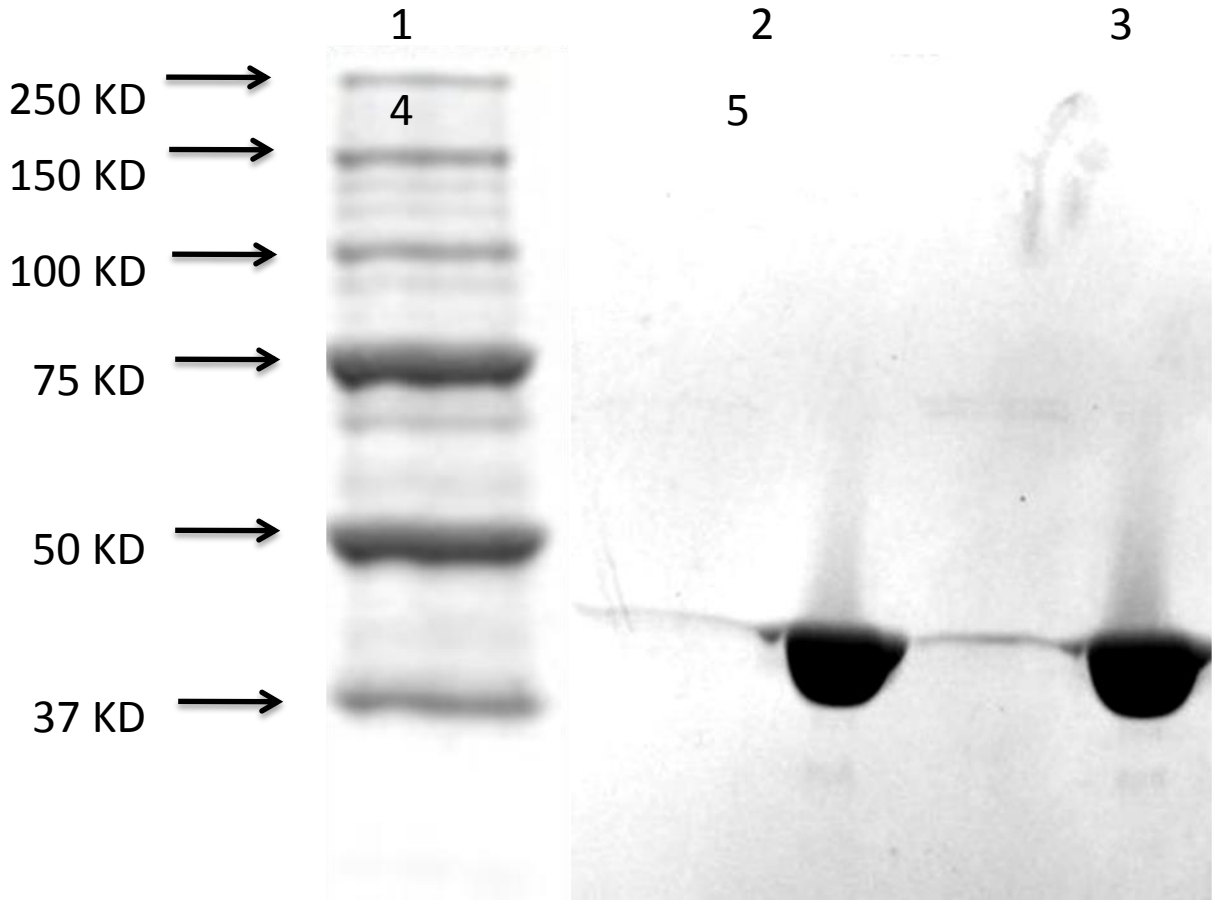

Figure 8. F-actin Binding in the Absence of Calcium.

Lane1: 5μl of Precise Protein Plus standard. Lane 2-3: the supernatant and pellet of F-actin control. Lane 4-5: the supernatant and pellet of GST-CpnA fusion protein and F-actin. EGTA was used to create an absence of calcium environment.

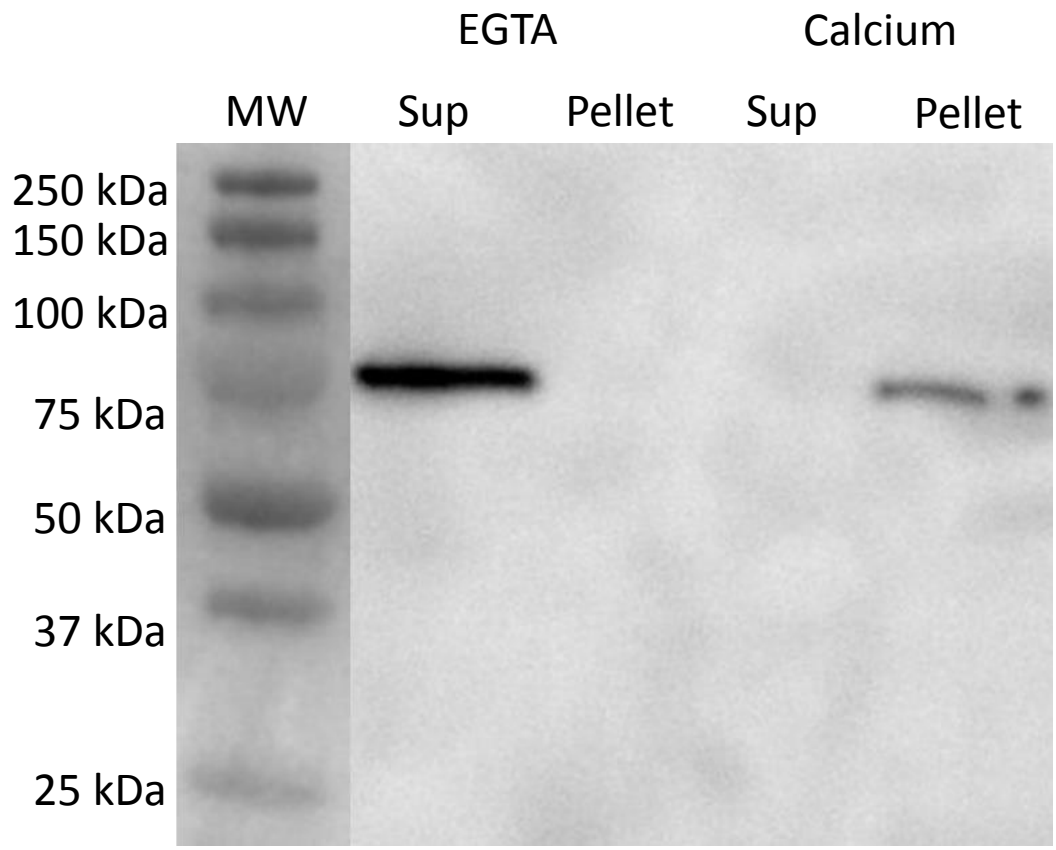

Figure 9. Western Blot of the F-actin Binding Assay Using CpnA Antibody.
The sample of GST-CpnA and F-actin mixture was pelleted in the presence and absence of calcium (EGTA). The 20μl supernatant was mixed with 2X sample buffer and the pellet was resuspended with 40μl 2X sample buffer. The proteins were separated on a 12% polyacrylamide gel at 200V for 30 minutes. Lane 1:5μl of Precise Protein Plus standard. Lane 2: Supernatant with EGTA. Lane 3: Pellet with EGTA Lane 4: Supernatant with calcium. Lane 5: Pellet with calcium. All the proteins were transferred from the gel to a PVDF membrane at 100V for 1 hour. The membrane was incubated overnight in the cold room with primary antibody anti-CpnA (1:2000) and incubated with the secondary antibody, anti-rabbit HRP conjugated (1:5000) for 1 hour.

F-actin Binds to Immunoprecipitated GFP-Ado and GFP-CpnA

To determine whether the A domain of CpnA is binding actin and if the two C2 domains confer calcium-dependent binding, we went back to our IP experiments, but

this time isolated GFP,GFP-CpnA and GFP-Ado from cells using an antibody to GFP and then added F-actin to the IP. IPs were performed using GFP, GPF-Ado and GFP-CpnA *Dictyostelium* cells with calcium present. The F-actin co-precipitated with the GFP-CpnA and the GFP-Ado in the presence of calcium (Figure 10).

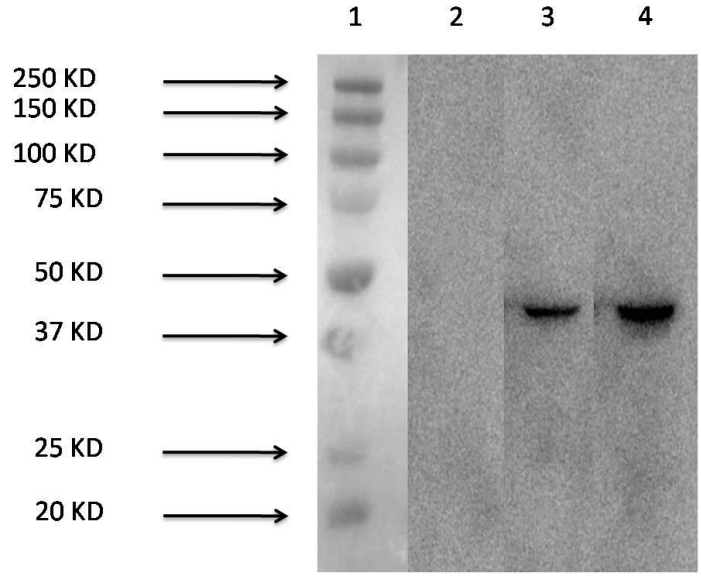

Figure 10. Western Blot of the Immunoprecipitation of GFP, GFP-A Domain and GFP-CpnA Cells Using a GFP-antibody.
The GFP and GFP-tagged proteins were pulled out by using a polyclonal GFP-antibody binding to Dynabeads. F-actin was added to the dynabeads/antibody/GFP-fusion protein complex. The proteins were eluted using 30μl 2X sample buffer. The proteins were separated on a 12% polyacrylamide gel at 200V for 30 minutes. Lane 1: 5μl of Precise Protein Plus standard. Lane 2: GFP elution sample. Lane 3: l GFP-Ado elution sample. Lane 4: GFP-CpnA elution sample. All the proteins were transferred from the gel to a PVDF membrane at 100V for 1 hour. The membrane was incubated overnight in the cold room with primary monoclonal antibody to actin (1:300) and incubated with the secondary antibody, anti-mouse (1:15000) for 1 hour.

CHAPTER IV

DISCUSSION

One of the ways to uncover the function of a protein is to discover its binding partners. Therefore, we used a variety of techniques to identify candidate binding partners of CpnA in *Dictyostelium*. We used affinity chromatography and immunoprecipitation (IP) along with mass spectrometry and identified actin as a potential binding partner for CpnA (unpublished data).Because actin is an extremely abundant protein in eukaryotic cells, we would not be surprised to find actin contaminating elution or IP samples. However, several of the phenotypes observed in *cpnA*- cells were consistent with a defect in the actin cytoskeleton. These phenotypes include defects in cytokinesis, contractile vacuole function, aggregation, slug phototaxis and thermotaxis, and fruiting body formation (Damer *et al.,* 2007; Smith *et al.,* 2010; Flegel*et al.* 2011).This result led us to look at actin filaments in *cpnA*-cells. We stained *cpnA*- cells and wild-type cells with rhodamine phalloidin and found that *cpnA*- cells had 2.5 times more staining for actin filaments than wild-type cells. These data suggested that CpnA may have a role in regulating actin filament disassembly (unpublished data).

To determine if CpnA associates with the actin cytoskeleton, we treated cells with Triton X-100, pelleted the insoluble actin cytoskeleton, and used Western blot analysis to determine if CpnA was in the pellet or supernatant (Figure 1).Previously in the lab we had created cells overexpressing a GFP-tagged version of CpnA that

contained only the A domain of CpnA and cells overexpressing a GFP-tagged full length CpnA. We also used cells overexpressing GFP as a control. We found that GFP does not pellet with the actin cytoskeleton in the presence or absence of calcium, while a similar small portion of the GFP-tagged A domain pellets with the actin cytoskeleton in the presence and absence of calcium. In contrast, GFP-CpnA does not pellet with the actin cytoskeleton in the presence of EGTA, while a large portion of the GFP-CpnA does pellet with the actin cytoskeleton in the presence of calcium. Taken together, these data suggest that the A domain of CpnA is able to bind to the actin cytoskeleton in a calcium-independent manner, while the full-length CpnA binds to the actin cytoskeleton in a calcium-dependent manner (Damer *et al.,* 2005).Therefore, the C2 domains, which are absent in the GFP-tagged A domain protein, confer calcium-dependent binding activity to CpnA. These experiments suggest that CpnA is binding the insoluble cytoskeleton, but they do not tell us to what CpnA may be directly binding. In addition, GFP-CpnA has previously been shown to bind membranes in a calcium-dependent manner. Therefore, although the cells were treated with Triton X-100 that disrupts membranes, it is possible that the GFP-CpnA was binding to membranes in a calcium-dependent manner and not the cytoskeleton.

To determine whether CpnA is able to bind actin filaments directly, we decided to express a GST-tagged version of CpnA in *Dictyostelium* cells so that we could use purified CpnA in purified F-actin binding assays. However the purification of CpnA using the GST tag was wrought with problems. We were able to overexpress

GST-tagged CpnA in *Dictyostelium* cells, but had several problems with the glutathione agarose chromatography purification protocol. First, we tried cutting the CpnA from the GST with thrombin to purify just CpnA. Unfortunately, the CpnA remained on the beads after cleavage from the GST. We then tried eluting the full fusion protein from the beads with glutathione, but again the GST-CpnA remained on the beads. Next, we tried adding 1mM dithiothreitol (DTT), a reducing agent, to the lysis buffer, washing the beads several times in lysis buffer, and then eluting the GST-CpnA. With the addition of the DTT, we were able to elute the GST-CpnA from the glutathione beads.

The F-actin binding assay showed that the GST-CpnA could depolymerize F-actin into G-actin (Figures 6 and 7).To determine if the GST-CpnA was pelleting with the F-actin and/or bound to G-actin, we did a Western blot on the pellets and supernatants of the F-actin binding assay. We did this four times and found that the GST-CpnA was found only in the pellet in the presence of calcium and only in the supernatant in the absence of calcium suggesting that GST-CpnA only binds F-actin and not G-actin twice (Figure 8). However, two other times we found the GST-CpnA in the both the supernatant and pellet in the absence and presence of calcium suggesting GST-CpnA can bind F-actin and G-actin. We will need to repeat these experiments several times to determine the correct results. The results could indicate that CpnA may interact with F-actin to depolymerize it, but remain bound to short fragments of F-actin that are not pelleted.

To determine if GST-CpnA can bind to G-actin, we performed immunoprecipitations with GFP, GFP-A domain, and GFP-CpnA expressing cells. We then added F-actin to the IPs and found that F-actin binds to GFP-A domain and GFP-CpnA in the presence of calcium (Figure 10). We have also does these same experiments with G-actin and found that none of the constructs are able to bind G-actin in the presence and absence of calcium.

Other Actin Depolymerization Proteins

There are many actin-binding proteins and some of them have the ability to depolymerize actin, while, any of them can regulate the assembly of actin. Both assembly and disassembly of actin filaments are important for all actin-based functions. A protein family called ADF/cofilin family plays an important role in disassembly of actin. This family has many members such as ADF (Actin depolymerizing factor), cofilin, actophorin, depactin and destrin (Maciver *et al.,* 1998; Bamburg *et al.,* 1999). Actin filament could be shorten in the presence of ADF proteins, which could interact with actin in different ways, such as, severing actin filaments to create more G-actin (Maciver *et al.,* 1991) or increasing the G-actin loss from filament ends (Theriot *et al.,* 1997).

Future Experiments

We know that GST-CpnA could depolymerize F-actin *in vitro*. However, the mechanism is still not clear. The GST-CpnA could cap the actin filament at the plus

end causing disassembly from the minus end. To determine if CpnA is a capping protein, we plant to stain purified actin filaments with rhodamine phalloidin and add cell lysate containing GFP-, GFP-Ado, or GFP-CpnA and using a fluorescence microscope. We should be able to tell where the GFP-CpnA binds to actin filaments.

In addition, to F-actin binding assays, we could do actin polymerization/depolymerization assays using pyrene-labeled G-actin and a fluorimeter. We could add GST-CpnA to G-actin and activate polymerization to see how it may affect polymerization rates. We could also add GST-CpnA to F-actin to look at the kinetics of depolymerization. In addition, to determine if the A-domain can cause depolymerization, we could also express a GST-tagged *A domain* and use it in F-actin binding and polymerization/depolymerization assays.

Because the other copines in Dictyostelium have similar domains, we could also do the same experiments with the other copines. We have obtained cDNAs of *cpnB* and *cpnE* and have recently made cDNAS of *cpnD* and *cpnF*. It's possible that all copines regulated the actin cytoskeleton in some way.

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