The Protein Binding Partners of Copine A in *Dictyostelium discoideum*

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Abstract

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-Integrin A domain. Mutant studies of copines suggest that copines may be involved in signaling pathways and may play a significant role in cell differentiation, programmed cell death, and cell development. Copines need to be studied further to have a clear understanding of the function they play in organismal life processes. We are studying copine protein function in the model organism protozoan *Dictyostelium discoideum*. Previous research showed that the copine A (*cpnA*$^{-}$) knockout strain of *Dictyostelium* exhibited normal growth rates, a slight cytokinesis defect, a developmental defect, and a defect in contractile vacuole function. Furthermore, real-time reverse transcription-PCR data suggested that all of the copine genes except *cpnF* may be important regulators of *Dictyostelium* development. To fully understand the function of copines in *Dictyostelium*, it is critical to identify their target proteins. In this study we investigated the proteins that interact with Copine A by using the yeast two-hybrid system. The yeast two-hybrid system is a technique used to identify genes that code for proteins that are associated with a certain protein *in vivo*. We identified candidate genes *DAip1, corA, efaA1, capB, sun1,* and *rpl3* as coding for binding partners of the Copine A protein. The proteins DAip1, CorA, and EfaAl are associated with actin filament organization in *Dictyostelium* suggesting that CpnA may have a role in regulating the actin cytoskeleton. The mutant phenotype observed in the *cpnA*- cells is consistent with a defect in actin filament regulation.

Introduction

Copines make up a multigene family of calcium-dependent, phospholipid-binding proteins. Copine proteins were originally isolated from extracts of *Paramecium tetraurelia* **(**Creutz et al., 1998**).** Since then they have been found to be highly conserved in multicellular and unicellular eukaryotic organisms found in the kingdoms plantae, animalia, and protista **(**Creutz et al., 1998; Maitra et al., 2006; Damer et al., 2005**;** Yang et al., 2006). Copine proteins consists of two C2 domains at the N terminus followed by a domain often referred to as the "A domain" **(**Creutz et al., 1998**)**. Copines may play a significant role in cell differentiation, programmed cell death, and development and need to be studied further to have a clear understanding of the function they play in organismal life processes (Damer et al., 2007; Yang et al. 2006).

The A domain of copines is similar in sequence to the von Willebrand-Integrin A domain found in cell adhesion and extracellular matrix proteins (Tuckwell, 1999). The VWA domain of integrins mediates interactions with extracellular ligands**.** Therefore, it is reasonable to conclude that the A domain of copines may be involved in protein–protein interactions **(**Whittaker et al., 2002**).** Using the yeast two hybrid system, Tomsig's et al. (2003) found that the A domain of human copines I, II and IV interact with several signaling proteins including MEK1, protein phosphatase 5, and the CDC42-regulated kinase (Tomsig et al., 2003).

The C2 domains of copines bind to phospholipids in a calcium-dependent manner (Creutz et al., 1998; Damer et al, 2005). The domain structure of copines suggests that these proteins may be involved in signaling pathways by binding target proteins through their A domain and then recruiting the proteins to a membrane in response to calcium through the action of their C2 domains (Tomsig et al., 2003). Tomsig et al. (2004) demonstrated that a signaling pathway from the tumor necrosis factor-α receptor in human embryological kidney 293 cells is regulated by

copine I, which provides evidence that copines function in intracellular signaling pathways (Tomsig et al., 2004).

Copine proteins' roles in intracellular signaling pathways make them essential to many cellular processes (Yang et al, 2006). Research with copine mutants in *Arabidopsis* indicates that copines play a role in growth and development, and repressing apoptosis (Yang et al., 2006). Mammals have a specific copine expressed only in the brain that is thought to be involved in postsynaptic events and synaptic plasticity (Nakayama et al., 1999b). In addition, a human copine has been linked to interactions with a gene called OS-9 named by its amplification in osteosarcoma (Nakayama et al., 1999a). Therefore, copines may be involved in signaling pathways important to normal brain function and the development of cancer. Although, copines are important to many cellular processes in eukaryotic organisms, the functions of these proteins remain largely unexplored.

To better understand the function of copines, we are studying copine function in the unicellular eukaryote *Dictyostelium discoideum*. *Dictyostelium* is simple to grow and reproduce in a laboratory allowing research to be conducted over generations in a relatively short period of time. The *Dictyostelium* genome is sequenced and can be genetically manipulated. Researchers use *Dictyostelium* to gain a better understanding of cellular growth and development, cytokinesis, cell motility, membrane trafficking, cell differentiation, and cell signaling. Learning the fundamental basis of unicellular life like cell locomotion and phagocytosis is essential to understanding how cells function collectively in a complex multicellular organism. This is especially true for *Dictyostelium* because although each amoeba is an independent unicellular organism, when the *Dictyostelium* cells are grown under starvation conditions they interact and aggregate forming a multicellular stalk and spore structure. This survival mechanism is unique to

this amoeba organism and aids scientists in studying chemotaxis and cell-to-cell communication (dictyBase, 2010).

Previous research identified six copine genes (*cpnA*-*cpnF*) in *Dictyostelium* (Damer et al., 2005). To investigate the function of one of these proteins, CpnA, a *cpnA*$^{-}$ knockout strain of *Dictyostelium* was created. The *cpnA*- mutant strain exhibited normal growth rates, a slight cytokinesis defect, developmental defects (where they failed to culminate into fruiting bodies), and a defect in contractile vacuole function. Furthermore, real-time reverse transcription-PCR studies demonstrated that all of the copine genes except *cpnF* showed an upregulation of mRNA expression at one or two developmental transitions, suggesting that copines may be important regulators of *Dictyostelium* development (Damer et al., 2007).

Researchers are continuing to investigate how copines function and how they may play an important role in cellular growth and development. To fully understand the function of copines in *Dictyostelium,* it is critical to identify their target proteins. In this study we are searching for the proteins that interact with Copine A by using the yeast two-hybrid system. The yeast two-hybrid system is a technique used to identify genes that code for proteins that are associated with a certain protein *in vivo*. This method is based on a yeast (*Saccharomyces cerevisiae*) genetic assay using a protein called the GAL4 transcription factor. The GAL4 transcription factor is split in half and only when the two halves are close together do they cause the transcription of a reporter protein. One half of the GAL4 transcription factor is fused to the CpnA bait protein and the other half of the GAL4 transcription factor is fused with the cDNA library prey protein. We obtained the *Dictyostelium* developmental and vegetative cDNA libraries from Dr. Adam Kuspa, Department of Biochemistry at Baylor College of Medicine. Only if the bait protein and the prey protein interact will the GAL4 transcription factor be able to

activate transcription of the reporter gene (Figure 1). The interaction between the bait protein and the prey protein will result in the expression of the *lacZ* reporter gene, which will cause the yeast cells to turn blue. Thousands of yeast cells are screened for those that are making the reporter protein. The prey plasmid from these cells will be isolated and sequenced to identify the prey protein (Bartel and Fields, 1997; Damer, 2009).

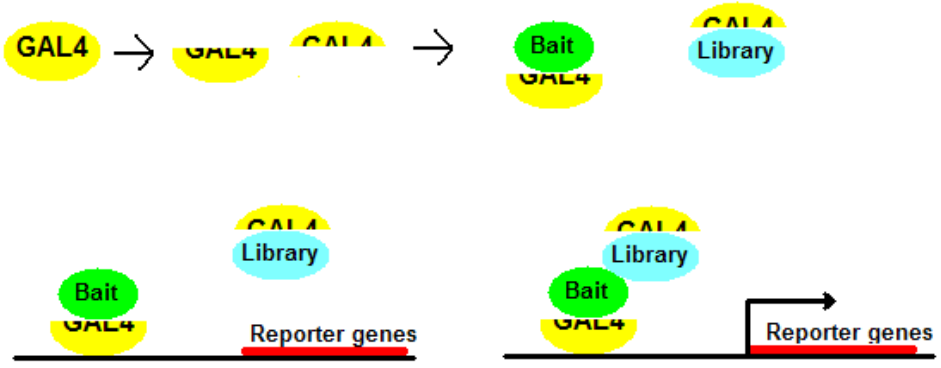

Figure 1. Principle of the yeast two-hybrid system. The GAL4 protein is split in half and one half is fused to the bait protein, while the other is fused to the prey. If the bait and prey proteins do interact, the reporter gene is transcribed and the reporter protein made.

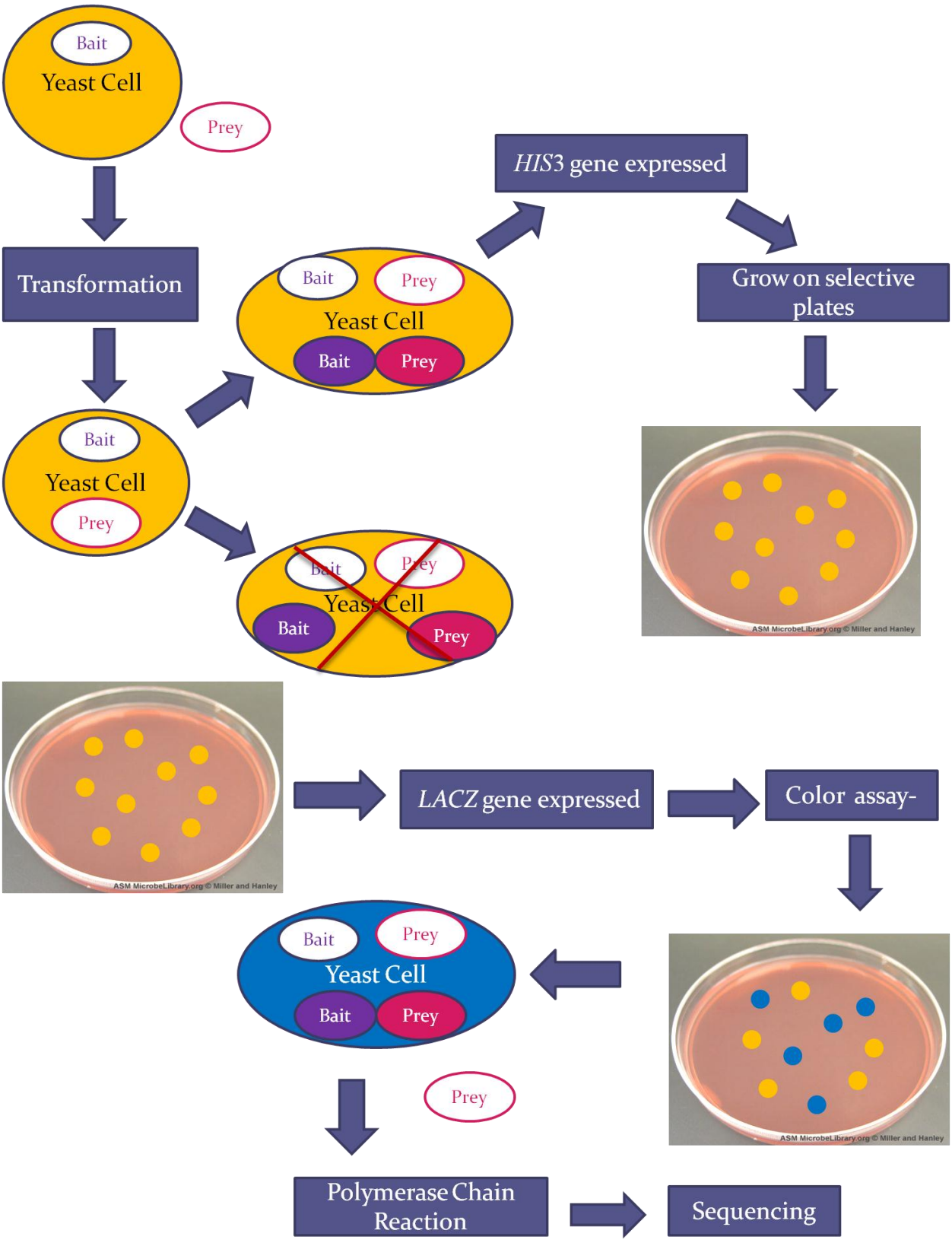

Figure 2: Materials and Methods (created by Helen Mao and Helena Lucente)

Material and Methods

Lithium Acetate Transformation of Yeast

To transform the pAS-2 plasmid containing the A domain of Copine A (pAS-2/Ado) into Y190 yeast cells, the yeast cells were grown in 10ml YPD media (20g/L peptone from meat, 5g/L yeast extract, 20g/L Dextrose, pH 6.5) incubated at 30°C on a shaking platform at 225rpm. Then the cells were diluted into 40ml of YPD media and incubated at 30°C for 4 hours shaking at 225rpm until late log phase (A_{600} = 0.5-1.0). The cells were centrifuged at 2,300rpm for 5 minutes at room temperature in a tabletop Beckman centrifuge, washed with 10ml dH_2O, and swirled to resuspend. The cells were centrifuged again at 2,300rpm for 5 minutes at room temperature. The cell pellet was resuspended in 10ml 0.1M lithium acetate solution and incubated at 30°C for 30 minutes on a shaking platform at 225rpm. After the incubation period, the cells were centrifuged at 2,300rpm for 5 minutes at room temperature. The pellet was resuspended in 0.40mL of 0.1M lithium acetate and then 50µL were mixed together with 6µL of salmon sperm carrier DNA (10 µg/µL denatured by boiling for 5 minutes and put on ice; Invitrogen, cat no: 15632-011), and 2µg (0.5 ng/µL) of the pAS-2/Ado plasmid. The mixture was incubated at 30°C for 30 minutes and then 0.6ml of 0.1M lithium acetate with 40% PEG 3350 was added, and the tube was swirled to resuspend. The cells were incubated at 30°C for 1 hour and then heat shocked for 10 minutes in a 42°C water bath. Then the cells were centrifuged at 2,000rpm for 30 seconds and the supernatant was discarded. The cell pellet was resuspended in 200µL ddH_2O and the cells were plated onto 100mm SD-Tryptophan plates (46.7g/L Minimal SD Agar Base 0.74g/L DO Supplement –Trp, pH 5.8) and incubated at 30°C.

In the second transformation, the pACT II developmental and vegetative cDNA libraries from *Dictyostelium discoideum,* obtained from Dr. Adam Kuspa, Department of Biochemistry at Baylor College of Medicine, were transformed into the pAS-2/Ado plasmid transformed yeast cells. The pAS-2/Ado transformed yeast cells were grown in 10ml SD-Tryptophan media (46.7g/L Minimal SD Base Agar, 0.74g/L DO Supplement -Trp, pH 5.8) and diluted to 50ml with SD-Tryptophan media and incubated at 30°C with shaking at 225rpm until cells were in late log phase (A_{600} = 0.5-1.0). The cells were pelleted at 2,300rpm for 5 minutes at room temperature and then washed with 50ml sterile dH_2O and then washed with 50ml LiTE (100mM LiAC, 10mM Tris pH8.0, 1mM EDTA). The cells were centrifuged again at 2,300rpm for 5 minutes and resuspended in 12.5mL LiSORB (100mM LiAC, 10mM Tris pH 8.0, 1mM EDTA, 1M Sorbitol). The resuspended cells were incubated at 30˚C for 1 hour shaking at 120rpm. After the incubation period, the cells were pelleted at 2,300rpm for 5 minutes at room temperature and then resuspended in 0.25ml Salmon Sperm Carrier DNA (10mg/ml denatured by boiling for 5 minutes and then put on ice; Invitrogen, cat no: 15632-011). Then 30µL of either the vegetative or developmental cDNA library (1.12 µg/µL) was added to the yeast cell mixture and incubated at 30°C for 30 minutes. For every 0.1ml carrier DNA added, 0.9ml of 40% PEG 3350 in LiTE was added and the mixture was incubated at 30°C for 20 minutes. Then the mixture was heat shocked for 15 minutes in a 42°C water bath, plated on selective SD-tryptophan-leucine-histidine (46.7g/L Minimal SD Agar Base, 0.62g/L DO Supplement –Trp/-Leu/-His, pH 5.8) + 3 Amino-1, 2, 4- triazole (3AT) plates (100mM) and incubated at 30°C for 5-7 days.

X-gal β-galactosidase Assay

The transformed yeast colonies were subjectively chosen and restreaked on fresh SD-tryptophan -leucine-histidine + 3AT plates that were divided into a grid so that one square was

equal to one colony. The plates were then incubated at 30°C over a growing period of several days. Once the colonies grew they were extracted from the plates by laying a 12.5cm clean VWR filter paper across each plate and allowing the colonies to adhere to the filter paper surface for 2 minutes. Then the filter paper was removed from the plates and put into an aluminum boat (colonies face up) and floated on top of liquid nitrogen for 10 seconds. During this time a fresh 12.5cm filter paper was put in a clean 150mm Petri dish and saturated with 5ml z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$) containing 1mg/ml X-Gal. The frozen filter paper was thawed at room temperature and placed into the plate with the z-buffer. The petri dish was wrapped in parafilm, incubated at 30°C, and checked periodically for 24 hours to observe which colonies turned blue.

Polymerase Chain Reaction

Yeast colonies that grew on the on SD-Trp/-Leu/-His + 3AT (100mM) plates were restreaked onto the new plates and single colonies were inoculated in 30µl of 0.02% SDS, vortexed for 15 seconds, and incubated at 90°C for 5 minutes. The DNA was then extracted by pelleting the cell membranes at 13,000rpm for 1 minute and collecting the supernatant containing the DNA in 1.5ml microcentrifuge tube to be stored at -20°C. The DNA was amplified using a Mastercycler PCR thermocycler (Eppendorf AG, Hamburg, Germany). The PCR temperature program was as follows: initial denaturation 30 seconds at 98˚C, denaturation 30 seconds at 98˚C, annealing 30 seconds at 65˚C, extension 60 seconds at 72˚C for 35 cycles, and final extension 10 minutes at 98˚C. The reaction mixture included Phire Hot StartII DNA polymerase, (20 µM) Reverse Primer (PACT2-PCR R: 5-CTTGCGGGTTTTTCAGTATCTACG-3) (20 µM) Forward Primer (PACT2-PCR-F: 5-CCCATACGATGTTCCAGATTAC-3), and ddH_2O. The PCR

products were analyzed using gel electrophoresis. The loading mixture was loaded into a 1% agarose gel with ethidium bromide (0.5 μg/mL) for UV visualization of the PCR Product.

Gel Extraction

The PCR products were extracted after gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen/Cat no: 28704 Note: RNase is added). The DNA was quantified using the Nano-Drop.

Sequencing of DNA

The PCR products were prepared to be sent to Michigan State University Sequencing Facility by adding 1μL of 30.0 pmol/μL R-primer to 6.5μL PCR products. The MSU Sequencing Facility sequenced the cDNA in the prey plasmid and sent us the sequences. BLAST was used to identify the genes in the *Dictyostelium* sequence databases.

*Note: All protocols have been adopted and modified from Matchker ^{tm} GAL 4 two-hybrid system 3 protocol manual.

Results

Lithium Acetate Transformation of Yeast

To discover the proteins that interact with Copine A we used the yeast two-hybrid system. First we conducted a lithium acetate transformation where we transformed the Y190 yeast cells with the pAS-2 domain plasmid (bait plasmid) containing the A domain of Copine A. Then we transformed the yeast cells containing the bait plasmid with the pACT II developmental and vegetative cDNA libraries from *Dictyostelium discoideum* (prey plasmids). The GAL4 transcription factor in the Y190 yeast cell is the basis of the yeast two-hybrid system. In essence, one half of the GAL4 transcription factor is fused to the A domain of the CpnA on the bait plasmid and the other half is fused to the cDNA on the *Dictyostelium* library prey plasmid. Therefore, if the A domain of Copine A and the protein expressed by the prey plasmid interact in the yeast cell, the two halves of the GAL4 transcription factor will interact causing the transcription of a reporter gene that can be observed. The Y190 yeast strain that we chose to use has two reporter genes, the bacterial gene *lac*Z and the biosynthetic yeast gene *HIS3*.

To check the effectiveness of the transformation and select yeast cells that only show a true bait and prey interaction, we plated the newly transformed yeast cells on SD-tryptophan-leucine-histidine + 3 Amino- 1, 2, 4- triazole (3AT) selective plates. Only yeast cells where the reporter gene *HIS*3 is expressed grow successfully. The vegetative library transformed yeast cells were spread onto twenty plates and incubated at 30°C for a week. Then we subjectively chose 111 of the biggest colonies to restreak on fresh, gridded SD-tryptophan-leucine-histidine + 3 Amino- 1, 2, 4- triazole (3AT) selective plates as shown in Figure 1. The developmental library transformed yeast cells were also spread on twenty plates and incubated at 30°C for two weeks

and 74 colonies were restreaked on a fresh gridded SD-tryptophan-leucine-histidine + 3 Amino-1, 2, 4- triazole (3AT) selective plates.

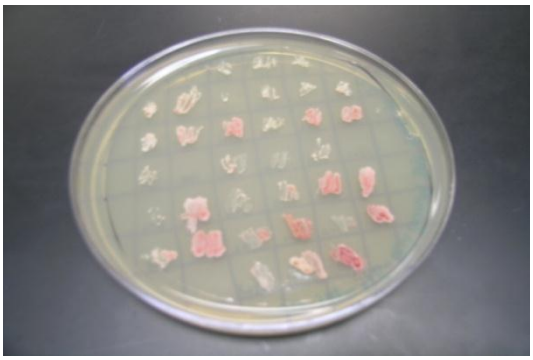

Figure 1: Gridded selective plates show *HIS*3 gene expression. The pACT II developmental and vegetative cDNA libraries from *Dictyostelium discoideum* were transformed into the pAS-2/Ado plasmid transformed yeast cells. The yeast cells were restreaked on selective SD-tryptophan-leucine-histidine + 3 Amino- 1, 2, 4- triazole (3AT) (100mM) gridded plates.

X-gal β-galactosidase Assay

To further validate that the yeast colonies on the gridded selective plates did in fact have a true bait and prey protein interaction, we conducted an X-gal β-galactosidase assay. The interaction between the proteins was recognized by the expression of the *lac*Z gene, which caused the yeast cells to turn blue after the X-gal β-galactosidase assay was performed (Figure 2). Out of the 111 colonies restreaked on the gridded plate from the vegetative library, 106 turned blue indicating the expression of the *lac*Z gene. Of the 74 colonies from the developmental library transformed cells on the gridded plates, 57 turned blue indicating a true bait and prey interaction.

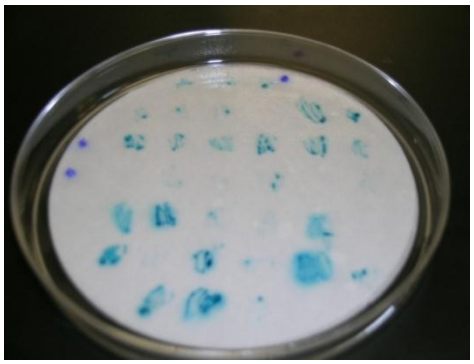

Figure 2: Gridded selective plates show *lac*Z gene expression after X-gal β-galactosidase Assay was performed. The yeast colonies with the *Dictyostelium* developmental library grew on the SD-tryptophan-leucine-histidine + 3 Amino- 1, 2, 4- triazole (3AT) (100mM) gridded plates and turned blue after incubation in 1mg/ml X-Gal at 30°C for 1-2 hours. These blue colonies were considered positive for bait and prey protein interaction.

Polymerase Chain Reaction

We used polymerase chain reaction (PCR) to amplify the cDNA fragments from the positive developmental colonies. The cDNA insert in the pACTII plasmid was amplified using primers that anneal to the plasmid. After amplification, the PCR products were analyzed by gel electrophoresis. According to the agarose gel (Figure 4), most of the fragments amplified were around 1.6-2 kb. The PCR products were purified from the agarose gel using the QIAquick Gel Extraction Kit (Company: Qiagen/Cat no: 28704).

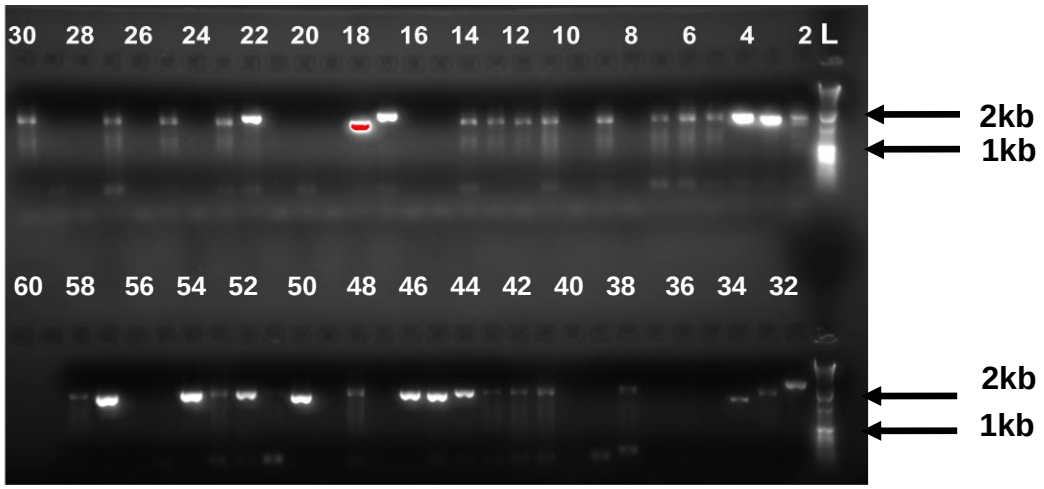

Figure 3: 1% Agarose gel analysis of target cDNA fragments amplified from DNA extracted from positive colonies. Bait and prey plasmids were extracted from yeast cells with 0.02% SDS. The target fragments were amplified with Phire Hot StartII DNA polymerase. Trackit 1kb DNA ladder from Invitrogen was loaded into Lane L. 57 samples were loaded on the gel.

Sequencing of DNA

The yeast cells with the developmental library that successfully grew on the SD-tryptophan-leucine-histidine + 3 Amino- 1, 2, 4- triazole (3AT) selective plates and turned blue after the X-gal β-galactosidase Assay were selected for DNA sequencing. We amplified the target cDNA fragments through PCR and then sequenced the PCR product. We used the reverse primer used in the PCR as the sequencing primer and sent the PCR products to the Michigan State University Sequencing Facility for sequencing. We received only six sequences from the 32 samples we sent and used BLAST to search the *Dictyostelium* genome database to identify the candidate genes. The six sequences that came from plasmids in the developmental library are shown in Table 1 below.

Table 1: Identities of candidate genes from DNA Sequences of the Prey Plasmid of Developmental Library

Candidate Genes	Biological Processes Involved
DAip1	Chemotaxis, pinocytosis, phagocytosis, cell proliferation, actin filament depolymerization
corA	Actin filament organization, Cytokinesis
efaAl	Actin filament bundle formation, translational elongation, translation, response to bacterium
capB	Response to stress, cAMP binding protein
sun1	Nucleus organization, mitotic sister chromatid segregation, centrosome localization, centromere complex assembly, centrosome organization
rpl3	Large ribosomal subunit protein

Discussion

Previous research showed that CpnA has roles in vegetative cells and in the developmental stages of *Dictyostelium.* Cells lacking *cpnA* exhibited defects in cytokinesis and contractile vacuole function. In addition, *cpnA*- cells were arrested prior to culmination and differentiation of cells failed to take place (Damer et al., 2007). Furthermore, real-time reverse transcription-PCR concluded that all of the copine genes except *cpnF* showed an upregulation of mRNA expression at one or two developmental transitions, suggesting that copines may be important regulators of *Dictyostelium* development (Damer et al., 2007).

We performed the yeast two-hybrid system to identify protein-protein interactions. According to the method we genetically engineered the yeast to produce CpnA (the bait protein) and then transformed these cells with a *Dictyostelium* vegetative or developmental library (the prey protein). Consequently, if the prey protein interacts with CpnA bait protein, the yeast colony will express *lac*Z and *HIS*3 reporter genes and be easily observed. Once identification and verification of the yeast cells took place through a series of selective plates and assays the prey plasmid was removed and sequenced to determine the identity of the gene in the plasmid. When the developmental library was sequenced six candidates were recognized as genes that support our previous research and conclusions of the possible functions of CpnA. We identified candidate genes *DAip1*, *corA, efaA1*, *capB, sun1*, and *rpl3* as binding partners of the copine A protein. The proteins DAip1, CorA, and EfaAl are associated with actin filament organization in *Dictyostelium*. ABP-50 protein otherwise known as the Elongation Factor 1A (EfaA1) in *Dictyostelium* is an actin filament bundling protein (Demma et al., 1990). According to Dr. Yang et al. (1990), the identification of ABP-50 as EfaA1 is the first indication that an apparatus known for protein synthesis binds to the actin cytoskeleton. In addition, the discovery may

present a mechanism for the regulation of protein synthesis within the cell (Yang et al., 1990). It can be hypothesized that without Copine A the EfaA1 protein may not function properly and cause actin cytoskeletal and protein synthesis defects in the *cpnA*- mutants.

Dictyostelium Actin-interacting protein (DAip1) and Coronin (CorA) have also been associated with actin filament function and *Dictyostelium* development. Research performed by Ishikawa-Ankerhold et al. (2010) provided evidence that both proteins work together to balance the actin polymerization and depolymerization in *Dictyostelium* cells. *Dictyostelium* mutants that failed to express both proteins displayed a delay in early growth and development and failed to form fruiting bodies. In addition, the mutant had more actin filaments. Consequently, the cells failed to perform essential functions like cell motility, cytokinesis, and endocytosis properly because those functions depended on the cells' ability to reorganize actin filaments accordingly (Ishikawa-Ankerhold et al., 2010). These results directly correlate with the phenotypes of our *cpnA*-mutants. Therefore without CpnA, DAip1 and Coronin proteins may not be regulated properly in the *Dictyostelium* cells, which might explain why the cells failed to develop properly, differentiate into fruiting bodies, and perform functions like cell motility and cytokinesis.

Actin filaments alone may not be the sole cause of the *Dictyostelium* cells stunted development and defects in cytokinesis, deficiencies in the centrosomes performance could also be partly to blame. The centrosome plays a role in cell-cycle progression, checkpoint control, and formation of primary cilium, flagella, and cilia. During mitosis the centrosomes also function in spindle orientation, spindle-associated ubiquitin-mediated degradation, actin deposition, and cytoplasmic organization necessary for cytokinesis (Doxsey, 2001). The research of Shultz et al. (2009) on the Sun1 protein in *Dictyostelium* indicated that Sun1is important for centrosomes to

function properly during mitosis and as a result, if Sun1 is defective in our mutant cells that could explain centrosomal defects associated with mitosis and cytokinesis we observed.

Another gene candidate that we identified as a possible binding protein of CpnA is cAMP-binding protein (CapB). According to Bain and Tsang's article (1991), when two genes related to CapB were disrupted the mutant cells grew slower than the wild-type control and had a delayed development that took up to three hours more to develop. These results indicate that CapB plays a role in growth and development. CpnA may be needed for the proper function of the CapB protein. This could explain why our *cpn*A- mutant cells display delayed growth and development.

Another protein that was identified as a possible binding protein of CpnA was ribosomal protein L3 (Rpl3). The levels of Rpl3 protein are high during the aggregation stage and then decrease during cell differentiation (Steel et al., 1995). With this knowledge we can hypothesize that CpnA may be a factor in controlling the regulation of ribosomal protein produced and therefore if the protein is upregulated during the aggregation stage in our mutants but does not decrease that could be one of the factors preventing culmination and cell differentiation.

In summary, we have identified candidate genes *Daip1*, *corA*, *efaA1*, *capB*, *sun1*, and *rpl3* as coding for possible binding partners of CpnA. Reviewing the functions of each of these proteins suggests to us that they all may be true binding partners of CpnA. However, Efa1 and Rpl3 are very highly expressed proteins that are commonly found in yeast two-hybrid screens as false positives. Therefore, in the future, we plan to further investigate the interaction between CpnA and the remaining four candidate proteins: Aip1, CorA, Cap1, and Sun1.

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