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Functional characterization of human CAP2.

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FUNCTIONAL CHARACTERIZATION OF HUMAN CAP2

by

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ABSTRACT

Essential cellular processes such as cell motility, cell migration and endocytosis/exocytosis require a dynamic actin cytoskeleton. Actin filaments (F-actin) polymerize and depolymerize into monomeric actin (G-actin) in response to signals from the environment. Several different signaling pathways lead to changes in the actin cytoskeleton (e.g. Rac, RhoA, Cdc42). Cyclase Associated Proteins (CAPs) interact with monomeric actin and are conserved in many species. Humans have two CAP genes, CAP1 and CAP2. Previous studies have demonstrated a role for CAPs in regulating the actin cytoskeleton and a role in endocytosis/exocytosis through sequestration of actin monomers. The objective of this study was to determine the localization and regulation of human CAP2. Subcellular fractionation of transfected human embryonic kidney cells (HEK293) indicated CAP2 is a cytosolic protein with some perinuclear localization. Immunocytochemistry confirmed that endogenous CAP2 is cytosolic in rat embryo fibroblasts (REF52). Cell wounding assays in REF52 cells demonstrated that endogenous CAP2 is mobilized to the leading edge of cell migration where the actin cytoskeleton is being remodeled. An *in vivo* phosphorylation assay in HEK293 cells revealed that CAP2 is phosphorylated. Deletion and site directed mutagenesis mutants were constructed and immunoprecipitation reactions were performed with wild type and mutant HA epitope tagged CAP2. Results revealed that phosphorylation of two conserved serines of CAP2 are important for the interaction between CAP2 and monomeric actin. However, phosphorylation was not abolished in a CAP2AA mutant containing alanine residues substituted for the conserved serine doublet. Therefore it can be postulated that CAP2 is phosphorylated on multiple residues. Two-dimensional gel

electrophoresis suggests multiple phosphorylated forms of CAP2 exist. A mutant of CAP2 (CAP2N433) missing part of the second dimerization domain is hyper-phosphorylated, suggesting CAP2 phosphorylation may be regulated by autoinhibition. Treatment of serum starved HEK293 cells with PMA, a PKC activator, caused a large increase in CAP2 phosphorylation. Immunocytochemistry of PMA treated REF52s demonstrated a redistribution of CAP2 to F-actin-containing lamellipodial extensions. This suggests the PKC is one of the signaling molecules involved in the pathway leading to CAP2 phosphorylation. These results support the role of CAP2 providing a link between cell signaling and regulating actin dynamics.

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LIST OF ABBREVIATIONS

2D – Two-dimensional

$^{32}PO_4$ – orthophosphate / radiolabelled phosphate

α – greek letter alpha

Abp1 – Actin binding protein 1

AC – adenylyl cyclase

ATP – adenosine triphosphate

Ark1 – Actin regulating kinase 1

Arp2/3 – Actin related protein 2/3 complex

Ca^{2+} - calcium ion

cAMP - cyclic Adenosine Monophosphate

CAP – Cyclase Associated Protein

cap$^-$ - CAP deficient

Cdc42 – Cell division cycle 42

Da – Daltons

DAG - diaceylglycerol

δ – greek letter delta

EDTA - ethylenediaminetetraaceticacid

Elf-1α – elongation factor 1 alpha

End4 – Endocytosis 4

ε – greek letter epsilon

F-actin – Filamentous actin

FBS – Fetal Bovine Serum

G-actin – Globular or monomeric actin

GST – Glutathione-S-Transferase

HA – Hemagglutinin

HEK293 – Human Endothelial Kidney 293

ι – greek letter iota

IP – Immunoprecipitation

λ – greek letter lamba

LIMK – LIM kinase

MALDI-TOF – Matrix Adsorption Laser Desorption Ion – Time of Flight

MARCKS – Myristoylated Alanine Rich C-Kinase Substrate

MS – Mass Spectroscopy

Munc18 – Mammalian unc18 protein

NECAB1 – Neuronal Calcium Binding Protein 1

PAK – p21 Activated Kinase

PBS – Phosphate Buffered Saline

PDGF – Platlet-derived Growth Factor

PIP2 – Phosphatidylinositol 4,5-bisphosphate

PKA – Protein Kinase A

PKC – Protein Kinase C

PMA – phorbol 12-myristate 13-acetate

Prk1 – Protein C Related Kinase 1

Ras – Rat sarcoma

REF52 – Rat Embryonic Fibroblast 52

Rho – Ras Homology

RNAi – RNA interference

ROCK – Rho Kinase

RpL3 – ribosomal protein 3

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Ser - Serine

SH3 – Src Homology 3

Sla2 – Synthetically lethal with Abp1 2

SNC1 – suppressor of null allele of CAP

Srv2 – Supressor of ras valine 2

TBS – Tris Buffered Saline

WASp – Wiskcott Aldrich Syndrome protein

CHAPTER ONE

Introduction

The goal of cytoskeletal research is to identify molecules that link the external signals a cell receives to the cytoskeletal changes that result. Essential cellular processes such as cell motility, division, exocytosis and endocytosis are carried out by rearrangements of the cellular cytoskeleton. The cytoskeleton is a complex of several scaffolding proteins including tubulin, intermediate filaments, and actin. Actin is present in the cell in both a monomeric form, called G-actin, and a filamentous form, called F-actin. A large number of proteins can interact with actin, some bind G-actin while others interact with F-actin filaments. A family of proteins that interact with G-actin are cyclase associated proteins (CAPs). Actin interacting proteins have a variety of functions including inhibiting or promoting F-actin formation from G-actin (actin polymerization), altering the rate of actin monomer addition or loss and sequestering monomeric actin to decrease the concentration of monomer at ends of the filament (Pollard and Cooper, 1986). They can also control the affinity of the monomer for ATP/ADP (ATP monomers are more easily polymerized), capping or severing and crosslinking of actin filaments (dos Remedios et al., 2003). In addition, actin-associated proteins are working simultaneously and may interact with each other or with other regulatory/signaling molecules. CAP is a monomeric actin binding protein that has a negative effect on actin polymerization (Freeman et al., 1995). The cytoskeleton is a common target of dangerous bacterial pathogens like *Yersinia* and *Listeria* (Gruenheid and Finlay, 2003). Further understanding of cytoskeletal proteins like CAP may provide a new defense against this type of

infection. Research on cytoskeletal diseases such as Wiscott Aldrich Syndrome may also benefit from such discoveries. Understanding the role of CAP2 in cytoskeletal signaling will provide a basis for studying all signaling proteins that cause cytoskeletal changes.

Yeast CAP

CAP was originally identified by two separate groups of scientists using different methods. A 70kDa protein that complexed with adenylyl cyclase in the yeast *Saccharomyces cerevisiae* was discovered and named CAP (Field et al., 1988). At the same time CAP was identified as a protein that suppressed the heat-shock sensitivity of cells containing an activated $Ras2^{Val-19}$ allele in the same yeast. Yeast CAP was also termed Srv2 for suppressor of Ras valine (Fedor-Chaiken et al., 1990). In yeast, the Ras2 signaling pathway activates adenylyl cyclase (AC), which generates cyclic AMP (cAMP). It was originally thought that CAP was regulating this pathway by interacting with adenylyl cyclase or with Ras itself. Therefore the earliest theory of CAP function implied CAP was a signaling molecule. Phenotypes found in CAP deficient (*cap*$^-$) yeast cells included an inability to grow on rich media, temperature sensitivity, sensitivity to nitrogen starvation and abnormal cell morphology (Field et al., 1990). These phenotypes suggested CAP may be involved in other processes than signaling, such as cytoskeletal structure. In 1991, the idea that CAP is a bifunctional protein was proposed by assigning the phenotypes described above to specific regions of CAP (Gerst et al., 1991). The carboxyl (C) terminus is able to suppress morphological abnormality, temperature sensitivity, nitrogen starvation sensitivity and an inability to grow on rich media, while the amino (N)

terminus is required to suppress the heat shock sensitivity (Gerst et al., 1991; Goldschmidt-Clermont and Janmey, 1991).

In order to determine the precise function of the C terminus of CAP, this region was deleted and a search for other genes that could complement its function was performed. Profilin was capable of suppressing C-terminal deletion of CAP (Vojtek et al., 1991). Profilin binds G-actin and decreases its ability to polymerize into F-actin by decreasing its affinity for ATP. This research suggested that CAP is not just a regulator of AC in yeast but perhaps also a regulator of cytoskeletal events. SNC1, a yeast homolog of a mammalian endocytic protein, synaptobrevin, was also found to suppress loss of C-terminal CAP functions (Gerst et al., 1992). In mammals synaptobrevins are associated with trafficking of synaptic vesicles, a process that involves cytoskeletal rearrangement. This research implied that CAP may be playing a role in endocytosis, perhaps by modifying the cytoskeleton. It appears that CAP is a multi-functional protein with roles in cytoskeletal events, AC signaling, and in endocytosis.

In 1995 it was demonstrated that CAP was able to bind monomeric actin and that this function required the C terminus (Freeman et al., 1995). Since actin is an essential cytoskeletal protein, this result reinforced the theory that CAP is involved in regulating cell shape. It is thought that CAP prevents actin polymerization by sequestering actin monomers.

When CAP was first sequenced, a centrally located stretch of proline residues was noted (Fedor-Chaiken et al., 1990). A purpose for the poly-proline region was identified when Src-homology domains were understood. This region of repeating proline residues is homologous to the c-Src proto-oncogene and is a binding site for

proteins containing an SH3 (Src homology 3) domain. It has been proposed that the SH3 binding domain could be a docking site for a protein that regulates CAPs' ability to bind actin. Actin-binding protein 1 (Abp1p) was a strong candidate for this regulatory role since it was shown that an interaction between the SH3 domain of Abp1p and CAP occurred *in vitro* (Freeman et al., 1996; Lila and Drubin, 1997). However, an interaction between full length Abp1 and CAP has never been shown. It was also determined that the SH3 binding domain is required for localizing CAP to cortical actin patches (Lila and Drubin, 1997). A region in the N terminus was identified as being able to regulate binding of SH3 proteins to the SH3 binding domain (Yu et al., 1999). Perhaps this SH3 interaction is triggered by a signaling cascade to regulate CAP localization or its ability to bind actin.

Yeast CAP contains two poly-proline regions denoted P1 and P2. P1 is well conserved in CAPs of other species while P2 is not (Hubberstey and Mottillo, 2002). Fragments of CAP smaller than sixty amino acids in length, containing P1 and/or P2 were used in a GST pull-down assay to identify interacting proteins (Yanagihara et al., 1997). Elongation factor 1α (Elf-1α) and ribosomal protein L3 (RpL3) bound to residues 308-368 of CAP (contains P2) but not to full length CAP missing the P2 region. No interaction between Elf-1α or RpL3 and wild type CAP was shown to verify this interaction. It is unlikely that those results will provide useful insight into the function of CAPs as the P2 region is not well conserved among CAPs.

Non-Yeast CAPs

Seventeen CAPs have been sequenced in fourteen different species. The majority of research performed has focused on yeast CAP, Srv2, due to the simplicity

of the yeast system. Yeast molecular work has provided an excellent basis for understanding CAPs of other species as the central and N terminal domains are well conserved through different species.

CAP homologues have been identified in two plant species, *Gossypium hirsutum* (cotton) and *Arabidopsis* (a member of the mustard family) (Barrero et al., 2002; Kawai et al., 1998). Cotton fibers begin life as single cells that elongate over 1000 times their diameter very early in development. Cotton CAP (GhCAP) was found to be expressed mainly in these young fibers undergoing elongation (Kawai et al., 1998). When expressed in yeast, *Arabidopsis* CAP1 (AtCAP1) was able to rescue the abnormal cell morphology and random budding phenotype from *cap*$^-$ cells. AtCAP1 was able to bind actin both *in vitro* and *in vivo*. Overexpression of AtCAP1 in transgenic plants lead to a reduction in cell size and number causing growth abnormalities. Tobacco suspension culture cells expressing AtCAP1 displayed abnormal F-actin and an inability to undergo mitosis (Barrero et al., 2002). This research suggests CAP's role in plants is associated with cell elongation and division. Similar to AtCAP, mushroom CAP (*Lentinus edodes*) can complement C terminal CAP deletions and can bind *S. pombe* actin in a yeast two hybrid test (Zhou et al., 1998).

In *Dictyostelium discoideum,* CAP binds G-actin in a calcium independant manner (Gottwald et al., 1996). The C terminal of *Dictyostelium* CAP (DdCAP) can sequester twice as much actin as full length DdCAP (Gottwald et al., 1996). Interestingly, phosphatidylinositol-4,5-bisphosphate (PIP2) can prevent the interaction between CAP and actin in this species (Gottwald et al., 1996; Noegel et al., 1999). This form of regulation has not been shown with any other CAP. DdCAP

is a cytoplasmic protein but localizes to the leading edge of migrating cells (Gottwald et al., 1996).

Chlorohydra viridissima CAP homologue, CvCAP, is thought to be a signaling protein. In *Chlorohydra* there is a signaling pathway that effects nerve-cell determination and differentiation. When the proper agonist binds a transmembrane receptor called head activator (HA), cAMP is generated. CAP antisense oligonucleotides were able to block this pathway upstream of cAMP production (Fenger et al., 1994). This suggests CvCAP is playing a role in transducing a signal from HA to adenylyl cyclase (AC is the enzyme that generates cAMP). It is not known if CAP binds HA or AC in this cascade. In fact CAP has not been shown to interact with AC in any species other than yeast.

Porcine CAP, Actin Sequestering Protein 56 (ASP-56), was the first non-yeast homolog discovered. ASP-56 has been shown to bind actin *in vitro* with 1:1 stoichiometry and is unable to generate sites of actin nucleation (Gieselmann and Mann, 1992). This paper was the first to show CAPs' ability to bind actin, which was essential to learning more about CAP function with respect to the cytoskeleton.

CAP has recently been identified in *Xenopus laevis*, the African frog, where XCAP1 expression is developmentally regulated (KhosrowShahian et al., 2002). XCAP1 is highly expressed in the developing eye, specifically in the lens where actin dynamics are essential for proper development. Also, XCAP2 has been isolated (unpublished results). Capulet or Act up, the *Drosophila* homolog of CAP (DmCAP) is also expressed in the eye (Benlali et al., 2000). During development, within the eye disc, cells rapidly and drastically alter their shape prior to neuronal differentiation. DmCAP is essential for this shape changing process in *Drosphila*

(Benlali et al., 2000). DmCAP inhibits actin polymerization within the eye disc to prevent F-actin accumulation. At the same time, profilin, another actin-binding protein, is acting antagonistically and encouraging actin polymerization. This suggests that actin binding proteins, including CAP, are essential to cellular processes that require shape changes, such as development. At the same time another group of scientists discovered DmCAP to be involved in cell polarity (Baum et al., 2000). Mutants deficient in CAP were unable to establish proper anterior-posterior and dorsal-ventral axes during oocyte development. This process involves dynamic actin and microtubule cytoskeletons. This research suggests that CAP plays a role in generation of polarity through its relationship with the actin cytoskeleton.

Wills et. al. discovered that DmCAP collaborates with the proto oncogene c-abl in *Drosophila* S3 cells (Wills et al., 2002). This collaboration was generated using full length DmCAP and small fragment of c-abl containing only its SH3 domain. Without demonstration of an *in vivo* interaction between full length c-abl and CAP, it is still premature to conclusively correlate a role for CAP in signaling with c-abl.

Recently, an RNA interference (RNAi) experiment was performed on over 90 proteins thought to be involved in actin dynamics (Rogers et al., 2003). *Drosophila* S2 cells were treated with RNAi for 7 days, then plated on glass coverslips coated with concavalin A. Wild type cells will attach and spread within thirty minutes. Cells treated with DmCAP1 RNAi did not form lamellipodia and therefore failed to spread. Within these cells, F-actin was diffusely localized which was altered from the pattern of stress fibers present in wild type cells. Cofilin RNAi treatment presented the identical morphology. This is interesting because cofilin is an actin binding

protein thought to act in concert with CAP (Moriyama and Yahara, 2002). In addition, inhibition of each of these proteins also resulted in cytokinesis defects (Rogers et al., 2003). Since CAP is thought to play a role in cell migration and actin dynamics these results are not surprising but it would be interesting to see if similar results are achieved in mammalian cells with two CAP homologs present.

In both mouse and rat, two homologues of CAP have been sequenced (Vojtek and Cooper, 1993; Zelicof et al., 1993). CAP1 appears to be expressed in all tissue types at varying levels while CAP2 has more restricted expression (Swiston et al., 1995; Vojtek and Cooper, 1993; Zelicof et al., 1993). Perhaps these differences in expression represent different functional roles of CAP1 and CAP2. Mouse CAP1 has been seen to localize to the leading edge of growing 10T1/2 mouse fibroblasts, supporting a role for CAP1 in cytoskeletal rearrangement (Vojtek and Cooper, 1993). The presence of two CAP homologues in all vertebrates studied suggests that these two proteins may have evolved different functions in response to increasing developmental complexity.

Signaling and the Actin Cytoskeleton

In the yeast *S.cerevisiae*, Ras activation induces cytoskeletal changes through activation of adenylyl cyclase (AC). Yeast CAP, Srv2, binds AC as a contributing member of this pathway (Fedor-Chaiken et al., 1990; Field et al., 1988). Although the AC binding domain is partially conserved in all CAPs, this particular signaling function has not been demonstrated in higher vertebrates. Perhaps mammalian CAPs are involved in a pathway that does not include AC.

Three members of the Rho family of small GTPases, Rac, RhoA, and Cdc42, have been implicated in signaling pathways leading to cytoskeletal changes in a variety of mammalian cell types (for review see Hall, 1998). In Swiss 3T3 fibroblasts each of these GTPases has a very specific effect. Upon activation by platelet-derived growth factor (PDGF), Rac causes the assembly of lamellipodia. Lamellipodia are pleat shaped protrusions at the cell periphery (Symons, 1996). Rho can be activated by lysophosphatitic acid and this leads to actin stress fiber and focal adhesion formation. Filopodia, or thin finger-like extensions at the cell membrane, are generated by Cdc42 activation. There is crosstalk between the three GTPases, by which they can activate or suppress each other. Allen et. al. examined macrophage chemotaxis and determined only Rho and Rac are required for cell migration, suggesting Cdc42 is required for direction of migration but not force (Allen et al., 1998). Rac, Rho, and Cdc42 are the most commonly studied cytoskeletal regulators but it is likely that there are additional signaling molecules regulating cytoskeletal dynamics.

Role of CAP in cell elongation, development and cell migration

Cell Elongation

Cell elongation is a process that is essential for motility in single cell organisms like yeast. In multicellular organisms cell elongation is involved not only in mitosis but in growth, development, and differentiation. *Xenopus* CAP1 is highly expressed in the developing eye (KhosrowShahian et al., 2002). Verbrate eye differentiation requires rapid cytoskeletal changes that CAP-assisted actin polymerization may provide. In order to form the lens, cells at the posterior of the

eye lose their nuclei and elongate to create the primary lens fibers (KhosrowShahian et al., 2002). Since CAP1 is expressed in the eye during this stage of *Xenopus* development, it has been suggested that CAP1 plays a role in cell elongation.

The yeast *Canadida albicans*, is able to change form between budding and filamentous or hyphal growth, during its life cycle. *C. albicans* can form both a pseudohyphal and a true hyphal form. Pseudohyphae are cells that continue to bud but never separate, a type of cell elongation. Transitions between these forms is controlled by various signaling pathways and cAMP levels (Bahn and Sundstrom, 2001). A *C. albicans* strain deficient in CAP1 displayed decreased cAMP levels and was unable to perform bud-hyphal transitions or filamentous growth (Bahn and Sundstrom, 2001). CaCAP1 was able to regulate these bud hyphal transitions perhaps by controlling intracellular cAMP levels through its relationship with adenylyl cyclase. This experiment suggests CAP1 plays a functional role in cell elongation in yeast.

When cotton CAP, GhCAP, was first identified it was noted that GhCAP was mainly expressed in young fibers (Kawai et al., 1998). A cotton fiber consists of a single cell that has elongated immensely during development, but has never divided. This result suggests CAP is present in young fibers because it assists in the process of cell elongation, which is performed extensively in young fibers.

Recently, *Arabidopsis* CAP1 (AtCAP1) was overexpressed in transgenic plants and this led to phenotypes that could imply a defect in cell elongation (Barrero et al., 2002). These plants had smaller leaves and stems but normal length of the main root. Further analysis revealed that cell number was reduced. AtCAP1 overexpression in tobacco suspension cells caused inhibition of mitosis and loss of

actin filaments. Both mitosis and leaf growth require cell elongation using actin polymerization. Abberant expression of AtCAP1 causes defects in cell elongation. Therefore AtCAP1 may play a role in the actin dynamics associated with cell elongation.

Development

CAPs role in development is not well understood but a few studies have attempted to clarify its function. In *Xenopus,* CAP1 expression is upregulated before gastrulation and it localizes specifically to head mesenchyme, lens, otic vesicle and trunk mesoderm (KhosrowShahian et al., 2002). XCAP1 is a developmentally regulated gene as its expression levels vary at different stages of *Xenopus* development. XCAP1 expression in the developing eye is similar to *Drosophila* CAP, which is expressed in the eye disc (Benlali et al., 2000). During *Drosophila* development, as well as in most vertebrates, anterior-posterior and dorsal-ventral axes are generated within the oocyte (Baum et al., 2000). These axes create polarity for the developing organism. DmCAP accumulates within the oocyte to inhibit actin polymerization in order to assist in generating poles. CAP deficient mutants do not establish correct polarity (Baum et al., 2000). Areas within organisms that are undergoing early development experience rapid and drastic cell shape changes. The cellular cytoskeleton is activated via signaling pathways to produce specific cytoskeletal dynamics. Since CAP is present in these specific developing areas in *Xenopus* and *Drosphila*, perhaps CAP aids in developmental cytoskeletal dynamics or signaling in other species.

Cell Migration

When scientists began to examine mammalian CAPs, a new role for CAP was discovered. Mouse CAP1 (mCAP1) transfected into mouse fibroblasts was localized to the leading edge of migrating cells (Vojtek and Cooper, 1993). Although this was not endogenous mCAP1 expression, it was not alarming to see a known actin binding cytoskeletal protein present at a site of actin remodeling. The result could not be obtained in yeast as they are unicellular organisms. Mammalian cell migration is similar to the movement of cells during development, yeast cells cannot initiate this type of movement. *Drosophila* S2 cells were treated with DmCAP antisense for seven days then examined for morphological defects (Rogers et al., 2003). S2 cells deficient in CAP were unable to migrate and therefore failed to spread after plating and had defects in cytokinesis. This research reinforces a role for CAP in cell migration and cell division.

Mammalian CAPs and Protein Interactions

Mammals have two homologs of CAP with very similar domain structure (**Figure 1**) but different patterns of expression. In rat, CAP1 is expressed highly in spleen, testes, and lung while CAP2 is only highly expressed in testes, low in lung and undetectable in spleen (Swiston et al., 1995). mCAP1 has similar expression patterns as rat CAP1 (Swiston et al., 1995; Vojtek and Cooper, 1993) but CAP2 expression in mouse and CAP1 and CAP2 expression in humans has not been published. Human, mouse and rat CAP1 have over 90% amino acid identity but have

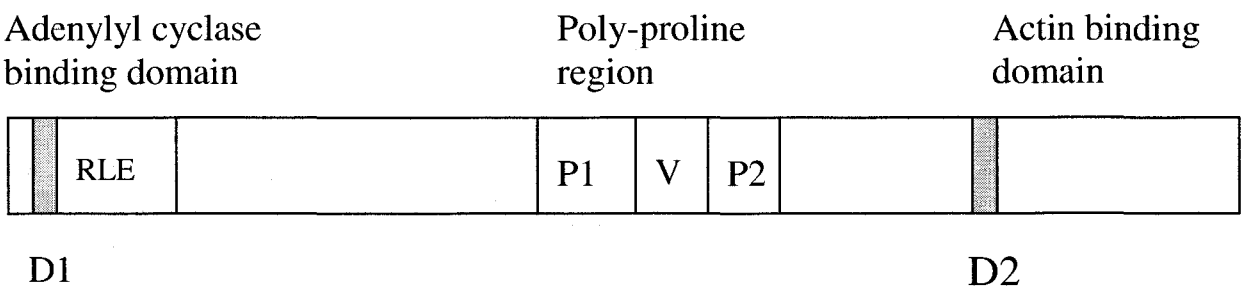


Figure 1: Common Domains of Cyclase Associated Proteins. This diagram is representative of the functional domains of CAP. Yeast CAP is used here as an example. CAPs contain several domains that have been conserved from yeast to mammals with the exception of the second poly-proline region (P2), which is not well conserved. A verprolin homology region is present between the two poly-proline regions. D1 and D2 represent the N and C terminal dimerization domains, respectively. The N terminal Adenylyl cyclase binding domain contains both a dimerization domain (D1) and the RLE motif. The C terminal actin binding domain encompasses D2.

less than 40% identity with yeast CAP, Srv2 (for review see Hubberstey and Mottillo 2002). Human CAP1 and CAP2 have 64% amino acid identity with greater homology in the central and C terminal domains (Yu et al., 1994). The distinctive expression patterns of rCAP1 and rCAP2 and the decrease in amino acid identity from 97% between hCAP1 and mCAP1 to 64% between hCAP1 and hCAP2, suggests that these two homologues may play different roles in mammals (Hubberstey and Mottillo, 2002).

CAPs contain several interactive domains including two dimerization domains, one or two poly-proline regions, and an actin binding domain. The N terminal dimerization domain is also called the RLE or CAP motif. This motif contains the amino acids RLE repeated twice and is thought to form an amphipathic helix which may participate in protein-protein interactions (Cohen and Parry, 1990). Recently, the N terminus of *Dictyostelium* CAP was crystallized (Ksiazek et al., 2003). The overall structure is an alpha helix composed of six antiparallel helices which may serve to form multimers with other CAPs. rCAP1 has been shown to dimerize via a yeast two hybrid assay, and this interaction did not require P1 or P2 (Zelicof et al., 1996). mCAP1 interacts with itself and its N or C terminal deletions (Hubberstey et al., 1996). An interaction between the N and C terminal mCAP1 fragments demonstrates the use of both dimerization domains (Hubberstey et al., 1996). mCAP1 has been shown to interact with its homologue mCAP2 and BAT3, a nuclear protein via yeast two-hybrid and *in vitro* immunoprecipitations of epitope tagged proteins (Hubberstey et al., 1996; Manchen and Hubberstey, 2001; Thress et al., 1998). The functional significance of the interaction between CAP and BAT3 is still unknown. mCAP1 and hCAP2 interact with endogenous actin from yeast extract

or NIH 3T3 cell lysate (Hubberstey et al., 1996). This plethora of interaction information indicates CAP's inclination for oligomerization. Perhaps CAP can form large complexes with itself and/or other interacting protein to sequester monomeric actin.

Throughout the vast literature pertaining to human CAP and its homologues, the names of actin, cofilin, actin binding protein (Abp1) and others are heard frequently. While CAPs' interaction with actin is well accepted, it relation to cofilin is non-physical. Cofilin is able to enhance the treadmilling of actin filaments to aid in motility (Carlier et al., 1997). Actin based motility relies on the elongation of actin filaments in a single direction. Actin monomers are released from the pointed end of the F-actin filament and cofilin aids in their rapid release. When the actin-cofilin complex was purified from cell extracts, CAP1 and Aip1 (actin interacting protein 1) were found in high abundance (Moriyama and Yahara, 2002). Moriyama theorizes that Aip1 helps cofilin bind F-actin while CAP1 assists in the removal of actin monomers. This is quite possible considering CAPs ability to bind G-actin and its presence at sites of actin rearrangement in *Dictyostelium* and mouse (Gottwald et al., 1996; Vojtek and Cooper, 1993). In addition, it was found that CAP1 was able to increase nucleotide exchange on G-actin. G-actin is released from F-actin in its inactive ADP-actin form and polymerized in its active ATP-actin form. Another actin-binding protein, profilin, promotes nucleotide exchange from ADP to ATP on freshly released actin monomers (Goldschmidt-Clermont and Janmey, 1991; Vinson et al., 1998). It is interesting to note that profilin can compensate for C terminal

deletion of CAP in yeast (Vojtek et al., 1991), suggesting a functional linkage between these two proteins.

While cofilin and profilin link CAP to cytoskeletal events involving actin, Abp1 suggests a role for CAP in SH3 binding interaction and potential signaling activities. Although CAPs are clearly multifunctional proteins, the regulation of CAPs, especially mammalian CAPs, is virtually unknown.

The role of CAP in endocytosis

It is practical that the actin cytoskeleton is involved in a process like endocytosis. Endocytosis involves four steps: invagination of the cell membrane, formation of coated pit, associated of molecules with coated pit, and detachment of the vesicle (for review see (Qualmann et al., 2000). Each of these steps could be enhanced or inhibited by actin polymerization. Actin binding proteins could assist in actin remodelling to promote endocytosis.

The first indication that CAP may function in endocytosis came from experiments with yeast CAP. It was found that SNC1, a yeast synaptobrevin homologue, could suppress the morphological defects caused by the loss of the C terminal of CAP in *S.cerevisiae* (Gerst et al., 1992). Synaptobrevins are known to be involved in targeting and fusion of synaptic vesicles (Elferink et al., 1989; Sudhof et al., 1989). SNC1 deletion causes no phenotype in yeast, likely due to genetic redundancy. If synaptobrevin can complement CAP function than perhaps CAP plays a role in endocytosis. A yeast temperature sensitive endocytosis mutant was identified as having a defect in a gene called Sla2 (Wesp et al., 1997). Sla2 contains a coiled-coil domain that is required for its endocytic function. In addition, Sla2

deficient yeast cells are viable unless they are also deficient in Srv2 or Abp1 (synthetic lethal with Srv2, Abp1) (Wesp et al., 1997). A mutant of CAP defective for endocytosis called End4p was also identified in this study. This suggests that CAP performs a redundant role in endocytosis perhaps through a protein-protein interaction via its coiled-coil domain.

Endocytic function also has also been suggested for Abp1. Abp1 is able to bind to the SH3 domain of Rvs167p, a homolog of mammalian amphiphysin (Lila and Drubin, 1997). Amphiyphysin is a synaptic vesicle associated protein that has a role in endocytosis at the synapse (Wigge and McMahon, 1998). As mentioned, the poly-proline region of CAP has been shown to collaborate with the SH3 domain of Abp1 (Freeman et al., 1996). If CAP interacts with Abp1, an endocytic protein, CAP may be a regulator of endocytosis itself.

Recently, CAP2 was isolated in a complex with synaptotagmin VII and Abp1 (Fukuda et al., 2002). The family of synaptotagmin proteins are thought to regulate vesicle trafficking, an integral function of endocytosis (Marqueze et al., 2000). An actual interaction between full-length CAP2 and synaptotagmin or Abp1 was not shown. This implies that CAP2 like its homolog CAP1 may play a role in endocytosis by interacting with other endocytic proteins.

Protein Regulation

Little may be known about CAP regulation but other mammalian actin-interacting proteins, such as Wiscott Aldrich Syndrome protein (WASp), have been well explored. WASp is a regulator of actin cytoskeletal dynamics that is able to initiate and increase the rate of actin polymerization (Machesky and Insall, 1999).

When WASp is activated it creates an actin nucleation center by encouraging the formation and activation of a seven protein complex called the Arp2/3 complex. Once this complex is activated it becomes an actin nucleation center, a site of rapid actin polymerization (Machesky and Insall, 1999). Actin is rapidly polymerized or depolymerized when a cell needs to change shape or move (Hall, 1998). If WASp were constitutively active the cell would be extremely rigid and unable to move.

The ability of WASp to activate the Arp2/3 complex is controlled by several complex mechanisms (reviewed by(Kim et al., 2000; Zigmond, 2000). WASp contains interactive domains that allow the protein to fold over on itself creating an autoinhibitory loop. This inhibited or bent conformation cannot activate the bound Arp2/3 complex thus preventing actin polymerization. When the small GTPase, Cdc42, binds to the inhibited form of WASp, the protein can unfold and activate the Arp2/3 complex. Recent data has demonstrated that phosphorylation of WASp on tyrosine 291 by Hck (a Src family kinase) enhances the activation of the Arp2/3 complex, increasing actin polymerization (Cory et al., 2002). Essentially WASp uses both autoinhibition and phosphorylation to control its ability to activate actin polymerization. It is interesting to hypothetheize that CAP2 may be regulated in a similar fashion as WASp. Like WASp, CAP2 is a G-actin binding protein that contains two dimerization domains that interact with each other. It was unknown whether CAPs are phosphorylated however, most CAPs contain two serine residues at positions 433 and 434 (**Figure 2**) that may constitute sites of phosphorylation.

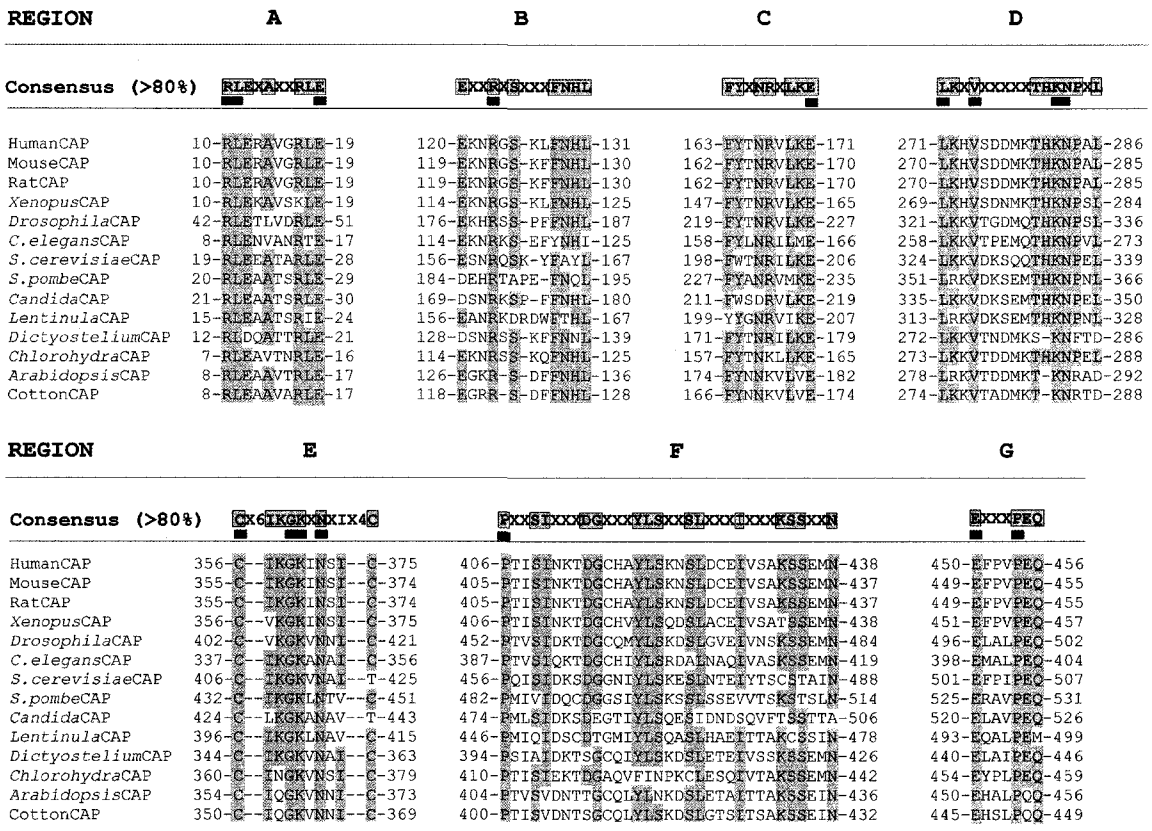


Figure 2: Conserved Residues in the CAPs. The two sequential serines in positions 433 and 434 (for hCAP2) are highly conserved throughout all CAPs. Eleven out of fourteen CAPs shown in this figure have both serines. Those that do not have a second serine contain a threonine residue in its position. *Lentinula*CAP has a cysteine residue but this is a likely sequencing error and may be a serine residue. (Figure was borrowed with permission from Hubberstey and Mottillo 2002)

Thesis Objectives

Very little is known about mammalian CAP2 as previous research has focused primarily on CAP1. Research on yeast CAP is more extensive than the work done with mammalian CAPs. It is thought that mammalian CAP1 is involved in cytoskeletal remodeling since mouse CAP1 was found to localize to the leading edge in migrating cells. The subcellular localization of human CAP2 is unknown. Little is known about the regulation of mammalian CAPs. It is known that yeast CAP is involved in a Ras signaling pathway via its interaction with adenylyl cyclase. Since mammalian CAPs do not interact with adenylyl cyclase it is unlikely that they are involved in a similar pathway. Regulatory mechanisms involving post-translational modifications of CAP have not been identified. Previous research indicates CAP2 is phosphorylated *in vivo.* Determining the functional significance of phosphorylation of human CAP2 is the underlying goal of this study. The following specific issues were addressed:

1. Determine the subcellular localization of human CAP2
2. Determine if CAP2 localization changes upon actin rearrangement
3. Confirm that CAP2 phosphorylated *in vivo*
4. Identify the phosphorylated residues of CAP2
5. Identify the pathway leading to phosphorylation of CAP2
6. Does phosphorylation of CAP2 alter actin binding?

CHAPTER TWO

Human CAP2 is phosphorylated *in vivo* in response to PKC activation

Introduction

Multifunctional proteins require strict regulatory mechanisms to ensure that the specific function is performed at the proper time. Proteins are often controlled by post-translational modifications like ubiquitination, glycosylation and most commonly phosphorylation. Phosphorylation is the reversible addition of a phosphate group (from ATP) to a serine, threonine or tyrosine residue performed by a protein kinase. Kinases and phosphatases are activated by an upstream signal that the cell receives from its external environment. The goal of a signaling cascade is to relay an external message to induce an internal response. In addition to post-translational modifications, proteins can be regulated by other mechanisms like autoinhibition.

Cytoskeletal changes are a type of internal response to an environmental cue. The presence of light or food will activate a receptor on the cell surface causing activation of a signaling cascade that results in actin remodeling to move the cell towards the stimulus. CAPs are thought to be involved in this complex process as mCAP1 is present at the leading edge in migrating cells (Vojtek and Cooper, 1993). Yeast CAP signaling is more clearly understood than vertebrate CAP signaling. In the yeast *S.cerevisiae*, signaling through Ras2 leads to activation of adenylyl cyclase (AC), generating cAMP. CAP binds AC in yeast and is thought to regulate this pathway. This pathway regulates cell growth through cytoskeletal changes. Vertebrate CAPs have not shown interaction with AC and therefore must be involved

in a different signaling pathway. Signaling in mammals may be more complex than in yeast and perhaps this is why there are two CAP homologs in mouse, rat and human.

CAP contains several domains that could relay signals from upstream effectors and cause cytoskeletal changes. The central poly-proline region could be utilized to interact with signaling proteins containing SH3 domains. The putative N terminal coiled coil domain may interact with an upstream regulator or downstream effector of CAP. Most important is the C terminal actin binding domain of CAP. This region may be responsible for actin sequestration or release according to specific environmental cues. CAPs also contain two highly conserved serine residues at position 433 and 434 (in human CAP2). Both of these serines are present in 14 of 17 CAPs, and those that do not have a second serine have a threonine residue in its place (Hubberstey and Mottillo, 2002). Since these potential phosphorylation sites are highly conserved, they suggest a regulatory mechanism that may be conserved from yeast to mammals.

This chapter explores the possible mechanisms of regulation of CAP2, revealing whether human CAPs are phosphorylated *in vivo*, and if so, what signaling pathway may be responsible. Results indicate that CAP2 is phosphorylated at several sites within the carboxyl terminus including two conserved serine residues (433, 434). The level of phosphorylation of CAP2 is increased in response to PKC activation by PMA, a phorbol ester. PKC is naturally activated by increasing concentrations of diaceyl glycerol (DAG). PMA is a DAG mimic. Increased phosphorylation upon treatment with the DAG mimic PMA, suggests that CAP2 is modified by part of a signal transduction pathway downstream PKC. Remembering that CAP2 is an actin-

binding protein, these results reinforce the theory that CAP2 is a protein that links signal transduction (i.e. PKC) to cytoskeletal dynamics (i.e. actin binding). Further evidence to support this role of CAP2 is that alteration of two conserved serine residues not only decreases phosphorylation but also reduces actin binding, a major function of CAP2. Perhaps the ability of CAP2 to bind actin or its affinity for actin is regulated through phosphorylation induced by PKC activation.

Material and Methods

Molecular Cloning

Full length human CAP2 in pCI-HA was used as template DNA to create the deletion mutants C232, N304, and N433 (**Figure 3A**). Polymerase Chain Reaction (PCR) using the appropriate primers (listed in Appendix A) was used to generate these deletion fragments which were then digested with EcoR1 and Xho1 and ligated into pCI-HA, an HA (influenza hemagglutin protein traffic marker) tagged mammalian expression vector. All vectors were transformed into *E.coli* strain DH5α competent cells. Clones were verified through diagnostic restriction digestion and sequence analysis. Serines 433 and 434 were replaced with alanine residues in the CAP2AA construct. CAP2AA was generated using a two-step PCR-mutagenesis method (**Figure 3B**). Briefly, in separate PCR reactions primers A and B and primers C and D were used to generate a large (AB) and a small (CD) fragment of CAP2 containing the alanine residues in place of serines 433 and 434 (primer sequences in Appendix

Figure 3A: CAP2 Mutant Constructs. Various CAP2 deletion mutants were constructed in order to isolate regions of CAP2 phosphorylation. The CAP2N304 fragment was generated via PCR using primers 31 and 109. CAP2C232 was generated using primers 108 and 32 while CAP2N433 was generated from primers 31 and 161. Each fragment was cut with EcoR1 and Xho1 and ligated into pCI-HA, an epitope tagged mammalian expression vector.

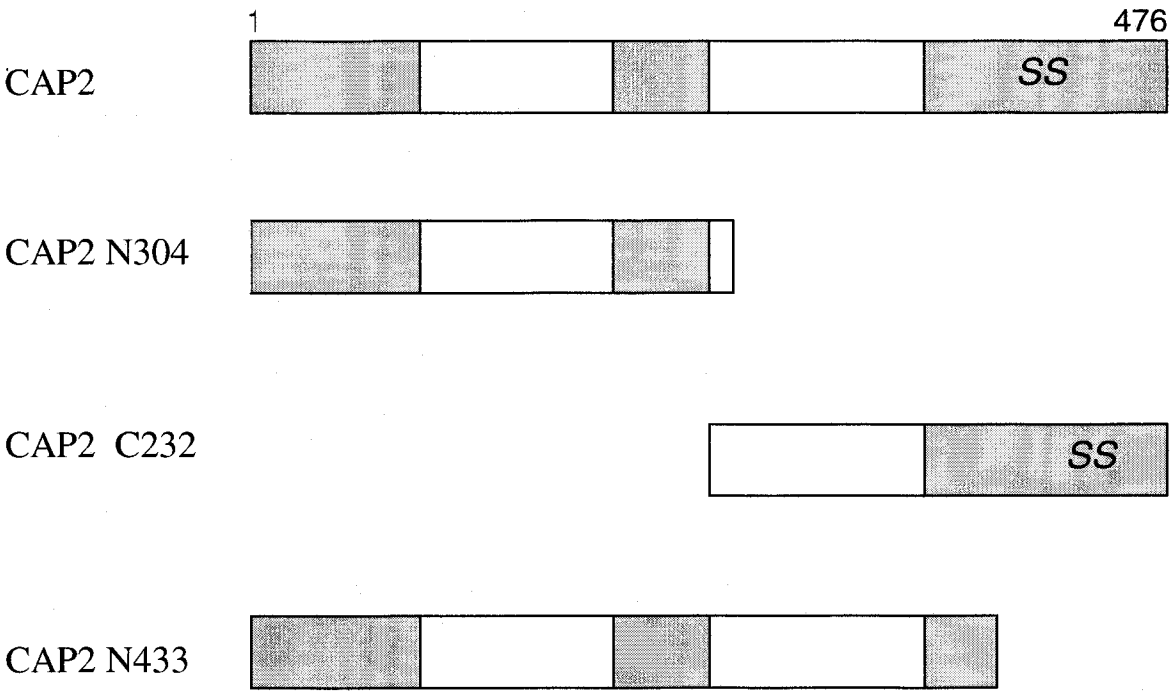


Figure 3A.

Figure 3B: Construction of CAP2AA Mutant. The CAP2AA mutant was generated to create a mutant of CAP2 lacking two highly conserved serine residues thought to be potential phosphorylation sites. This was performed using a two-step PCR process using wild type CAP2 primers A and D (primers 31 and 32) and larger forward and reverse primers (157 and 158) containing altered nucleotides to create alanine residues in place of serine residues. The alteration in the nucleotides also generated a new Hsp90I site that was used as a diagnostic tool.

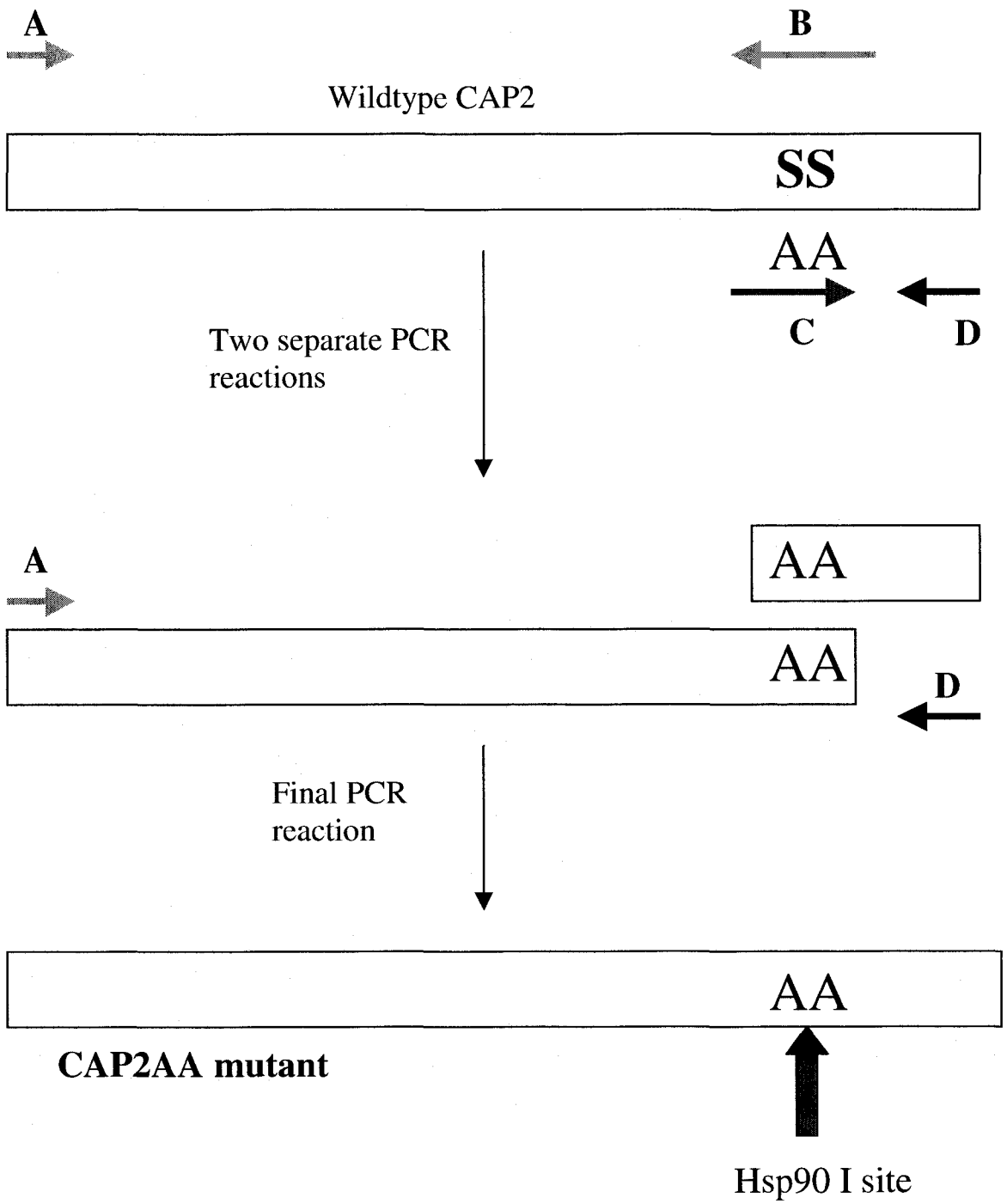


Figure 3B.

A). Primers B and C were designed to generate a new Hsp90I restriction site within the altered nucleotides. The large and small fragments were combined with the forward and reverse CAP2 primers (primers A and B) to create the full length C2AA DNA fragment. Once the CAP2AA fragment was cloned into pCI-HA it was digested with Hsp90I and sequenced to verify the serine to alanine switch.

Cell Culture

Human embryonic kidney cells (HEK293s) were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 100 units/ml penicillin/streptomycin (Invitrogen/Life Technologies). Cells were incubated in a humidified 37°C incubator with 5% CO_2.

In Vivo Phosphorylation Assay

HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen) in 60mm dishes using 4μg of each DNA construct, pCI-HACAP2, CAP2C232, CAP2N304, CAP2N433, or CAP2AA and 6μl Lipofectamine 2000, as per manufacturers instructions (Invitrogen). 24h post-transfection, cells were washed twice with 2ml phosphate free DMEM. Media was then replaced with 1ml phosphate free DMEM + 10%FBS and 0.3μCi/ml orthophosphate (Amersham). Cells were incubated for 3h at 37°C with gentle agitation every 15m to prevent desiccation of the cells. Media was removed and cells were washed twice with sterile 1X PBS. Cells were extracted in ice-cold 300μl RIPA lysis buffer [20mM TrisOH pH7.5, 150mM NaCl, 10mM KCl, 1mM $NaVO_4$, 50mM NaF_2, 1% IGEPAL CA-630 (NP-40), 10% glycerol, and protease inhibitor (Complete Mini, Roche)]. Cell extracts were briefly sonicated then

centrifuged at 15000rpm for 10m at 4°C. Supernatants were used for immunoprecipitations and protein extract samples.

For the sodium vanadate control 100μM $NaVO_4$ was added to radiolabelled media for the final hour of incubation. $NaVO_4$ is a phosphatase inhibitor that was present in the cell lysis buffer that will prevent dephosphorylation of CAP2 by phosphatases within the cell extract (Heffetz et al., 1992).

In the case of the alkaline phosphatase treated samples, the washed beads were resuspended in a total volume of 100μl with 10X Calf Intestinal Alkaline Phosphatase buffer containing 20 units of Calf Intestinal Alkaline Phosphatase (CIAP) and incubated at 30°C for 2h. After incubation, beads were washed twice with 500μl RIPA buffer. CIAP will remove phosphate groups from phosphorylated CAP2 while the protein is attached to the protein A bead.

In the calcium dependency assay, 3mM $CaCl_2$ and 3mM EDTA together or 3mM $CaCl_2$ alone was given to transfected HEK293s for 16h prior to *in vivo* phosphorylation assay.

For the phosphorylation assays involving drug treatment, transfected cells were serum starved for 16h prior to the addition of radioactive phosphate. The radiolabelled media used was also serum free. Drugs were added to the radiolabelled media when 1h of incubation remained. Drugs were added in the following final concentrations 2μM PMA, 10μM forskolin, or 1μM staurosporine. In the PMA dosing experiment the following final concentrations of PMA were used: 0.5μM, 1μM, 3μM, and 5μM.

Antibody-linked Beads

1.5mL of protein A agarose beads (Sigma) and 300µl 12CA5 monoclonal antibody was resuspended in 1X PBS to a final volume of 10mL in a 15mL conical tube. The tube was gently rocked for 1.5h at room temperature. The beads were spun at 3400rpm for 30s. The supernatant was discarded and replaced with 10mL 0.2M sodium borate pH 9. Beads were washed with 10mL 0.2M NaBo twice before adding 20mM dimethylpimelimidate. The tube was rocked for 1h at room temperature then spun at 3400rpm for 30s. Supernatant was discarded and beads were washed once with 0.2 M ethanolamine and then rocked for 2h. Beads were spun at 3400rpm for 30s and supernatant was discarded. Beads were washed twice with 10mL 1X PBS then resuspended to a final volume of 1.5mL of bead slurry.

Immunoprecipitation and Western Immunoblotting

Equivalent amounts of radiolabelled protein extracts were mixed with 25µl protein A agarose beads (Sigma) for 30min in a total volume of 500µl at 4°C. Extracts were centrifuged at 2500rpm for 30s. Supernatant was combined with 30µl protein A beads chemically coupled to monoclonal anti-HA antibody (12CA5) and mixed for 2h at 4°C. Beads were then washed 5x 10m each with 500µl RIPA without glycerol or protease inhibitors at 4°C. The beads were then boiled in 1X sample buffer and proteins were analyzed via western immunoblot. Protein extract samples and immunoprecipitated proteins were resolved on 10% SDS PAGE gels then transferred to nitrocellulose membranes. Membranes were blocked in 2% skim milk in TTBS overnight. For the detection of HA-tagged proteins monoclonal 12CA5 antibody and

secondary horseradish peroxidase conjugated sheep anti-mouse antibodies were used at dilutions of 1:10,000 and 1:3,000 respectively. For the detection of actin, mouse anti-actin antibody was used at a dilution of 1:2,000. Protein bands were visualized using a Lumi Light Chemiluminescence kit (Roche) as per manufacturers instructions. If required, blots were stripped using 20ml Western Restore Buffer (Pierce) for 1h at 37°C with gentle agitation, then reblocked and probed again. Nitrocellulose membranes containing potentially radiolabelled proteins were rinsed in distilled water then covered in plastic wrap. Membranes were mounted into an autoradiography cassette beneath a sheet of X-ray film (Kodak) and an intensifying screen (Kodak). The membranes were exposed for a short exposure (24-48h) and a long exposure (5-7d) at –80°C.

Antibodies

The N-terminal 304 residues of CAP2 were conjugated to GST to create a GST fusion protein. The purified protein was used to raise a rabbit polyclonal antibody against CAP2 by Covance Inc. CAP2 antibodies were affinity purified from whole sera using a CNBr-activated Sepharose 4B column (Ambersham Biosciences) using CAP2 N304 as an antigen. The anti-HA (12CA5) mouse monoclonal antibody was a kind gift from Dr. Dallan Young (University of Calgary). Monoclonal mouse anti-actin antibodies were purchased from Chemicon.

Two-dimensional Gel Electrophoresis

HEK293 cells were transfected with HA-tagged CAP plasmids using Lipofectamine 2000 as per manufacturers instructions. Two 100mm plates of cells were used per

immunoprecipitation. Cells were collected in a total volume of 500µl RIPA lysis buffer for each immunoprecipitation. Immunoprecipitations were carried out in the same manner as previously described. After washing, beads were resuspended in 140µl of rehydration buffer (Biorad) and incubated at 37°C for 15m. This buffer contains urea and detergent that will dissociate the proteins from the beads and denature the proteins. Beads were then centrifuged briefly and the supernatant was pipetted into separate channels on a focusing tray. Immobilized pH gradient (IPTG) strips of pH range 5-8 (Biorad) were placed gel side down onto the protein samples. The strips were overlayed with 1ml of mineral oil (Biorad) then incubated for 16h at room temperature. The proteins, now absorbed into the strips, were focused using Biorad Isoelectric Focus (IEF) power supply to 10,000-volt hours (approximately 5.5h). Once the proteins were focused on the strips the strips were incubated with two equilibration buffers (Biorad) for 15m each with gentle agitation. The strips were inserted gel side forward between two glass plates containing 7% polyacrylamide gels. Strips were overlayed with prewarmed agarose to prevent air bubbles. Gels were run at 150V for 45m. Proteins were transferred from gels onto nitrocellulose membranes using a starting current of 200mA for 1hour at 4°C. Western immunoblotting was performed as described previously. HA-CAP2 proteins were visualized using the anti-CAP2 antibody and the anti-HA antibody.

Results

CAP2 is phosphorylated in vivo

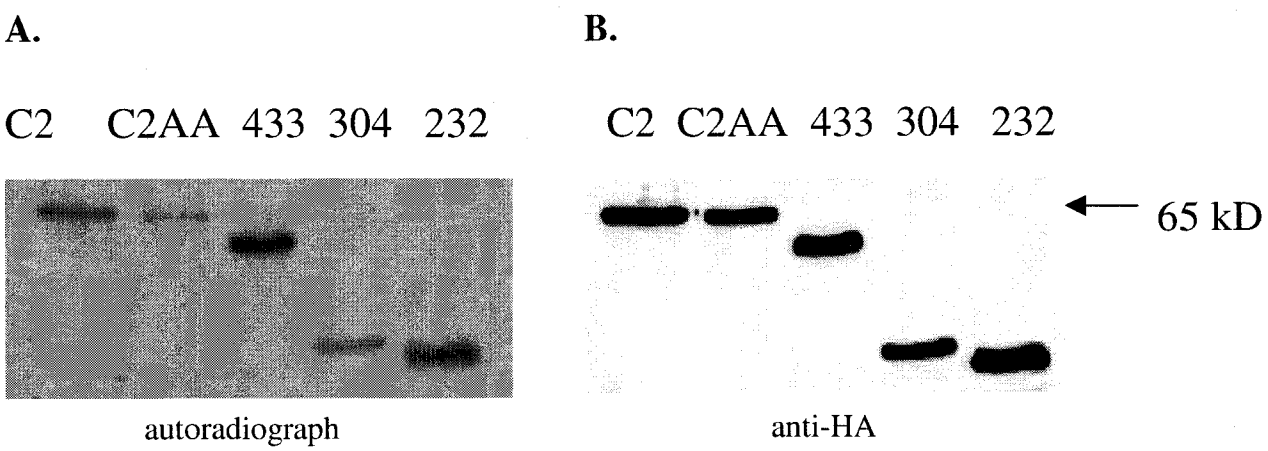
The fact that several actin binding proteins (e.g. WASp, cofilin) are phosphorylated led to our hypothesis that CAPs may also be post-translationally modified. Therefore, HA-tagged CAP2 was overexpressed in HEK293 cells and the phosphorylation status of CAP2 was determined. The results of an *in vivo* phosphorylation assay are shown in **Figure 4A.** HA-CAP2 was found to be phosphorylated. To examine which regions of the protein contained phosphorylated residues, several HA-CAP2 mutants were constructed. The CAP2AA mutant has two alanine residues in place of two highly conserved serine residues at positions 433 and 434. The CAP2N433 mutant is missing the two conserved serines and the C terminal 41 amino acids. CAP2C232 contains an N terminal deletion of 242 amino acids while CAP2N304 is missing 217 amino acids of the C terminus. The results indicate that HA-CAP2AA is less phosphorylated than full length CAP2 however, CAP2N433 was more phosphorylated than wildtype CAP2 (**Figure 4A**). Since both CAP2N433 and CAP2AA are missing the potential phosphorylation sites (Ser 433, 434), less phosphorylation than wild type CAP2 was expected. Perhaps since CAP2N433 is missing part of the C terminal dimerization domain, this protein is unable to fold properly and more residues are available for phosphorylation. By comparing the amount of phosphorylation of CAP2N304 and CAP2C232, the C terminal of CAP2 is more phosphorylated than the N terminal. The anti-HA immunoblot indicates that equal amounts of protein were present in each lane (**Figure 4B**). Controls for the

phosphorylation assay include sodium vanadate ($NaVO_4$) treatment (**4C,D**) and calf intestinal alkaline phosphatase (CIAP) treatment (**4E,F**) of CAP2 transfected HEK293 cells. The 5d exposure autoradiograph in **Figure 4C** shows that treatment with $NaVO_4$ increases the amount of phosphorylation, while treatment with CIAP (**Figure 4E**) decreases the overall level of phosphorylation. Anti-HA blots in **Figure 4D** and **4F** indicate that similar amounts of protein were present in each lane.

Conserved serines are important for actin binding

Post-translational modifications such as phosphorylation are a common mechanism used to regulate protein function. In order to determine if phosphorylation of CAP2 regulates its actin binding ability, co-immunoprecipitations were performed with wild type and deletion mutants of CAP2. HEK293 cells were transfected with HA-tagged CAP2 constructs, and protein extracts were subjected to immunoprecipitation using anti-HA antibody chemically coupled to protein A beads. Actin binding ability was determined through western blot analysis with anti-actin and anti-HA antibodies. CAP2N433 was unable to bind actin while CAP2AA bound significantly less actin in comparison with wild type CAP2 (**Figure 5**). Both of these mutants are missing the two conserved Ser 433 and Ser 434. CAP2N433 is additionally missing part of the carboxyl terminal actin-binding domain. The anti- HA blot (**Figure 5B**) indicates that all proteins were immunoprecipitated in comparable amounts.

Figure 4: CAP2 is a Phosphoprotein. A: Autoradiograph of radiolabelled immunoprecipitated CAP2 constructs from transfected HEK293s. Band intensity indicates CAP2 wild type, CAP2N433, and CAP2C232 are highly phosphorylated while CAP2AA and CAP2N304 are minimally phosphorylated. B: Anti-HA blot of immunoprecipitation shows that equal amounts of CAP2 were loaded into each lane. C: In a separate *in vivo* phosphorylation assay, HEK293 cells transfected with HA-CAP2 were incubated with sodium vanadate for the final hour of the incubation with labeled media or left untreated. Untreated CAP2 is less phosphorylated than sodium vanadate treated CAP2. D: The anti-HA blot of the autoradiograph of 4C demonstrates that equal amounts of protein were present in each lane. E: immunoprecipitated radiolabelled CAP2 protein was treated with calf intestinal phosphatase (CIAP) for 2h at 37°C. A control was incubated without enzyme. The sample containing CAP2 treated with CIAP displayed significantly less phosphorylation than the control. F: The anti-HA blot of the autoradiograph in 4E demonstrates that equal amounts of protein were present in each lane.



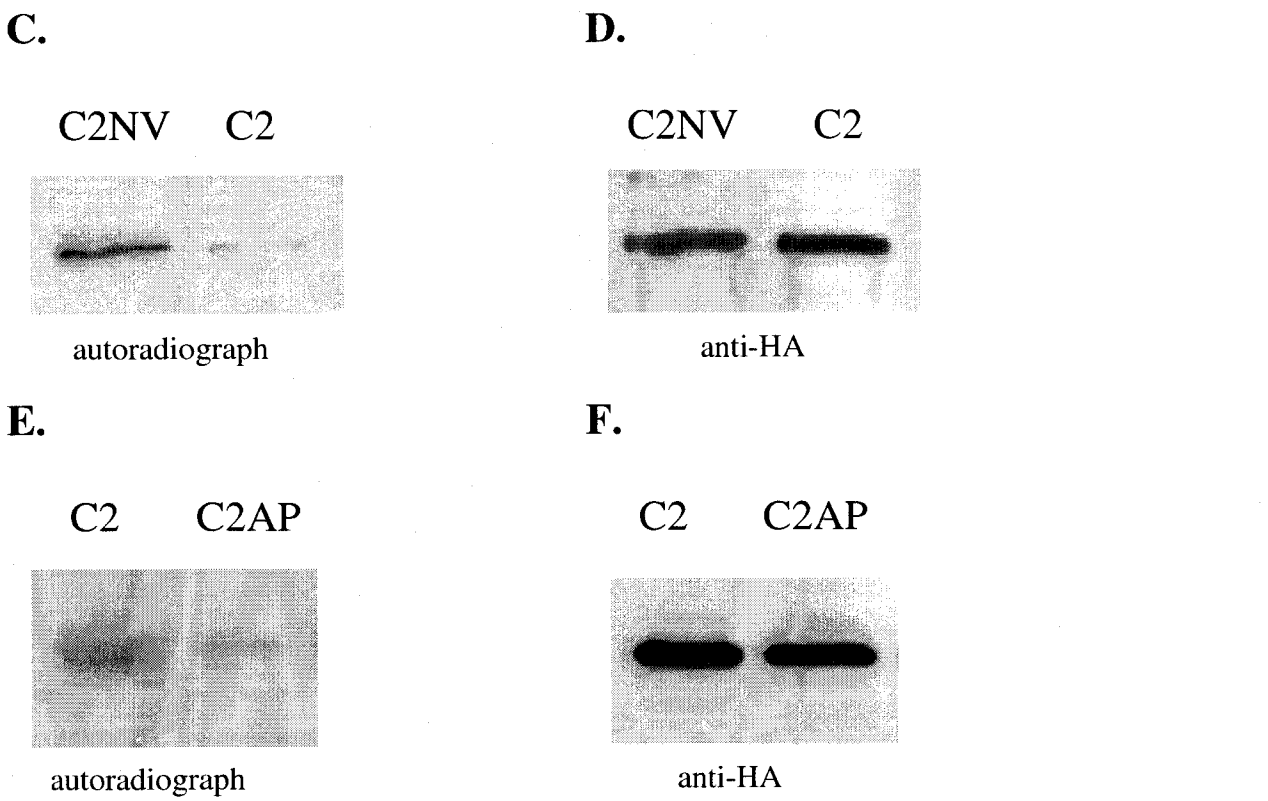


Figure 4.

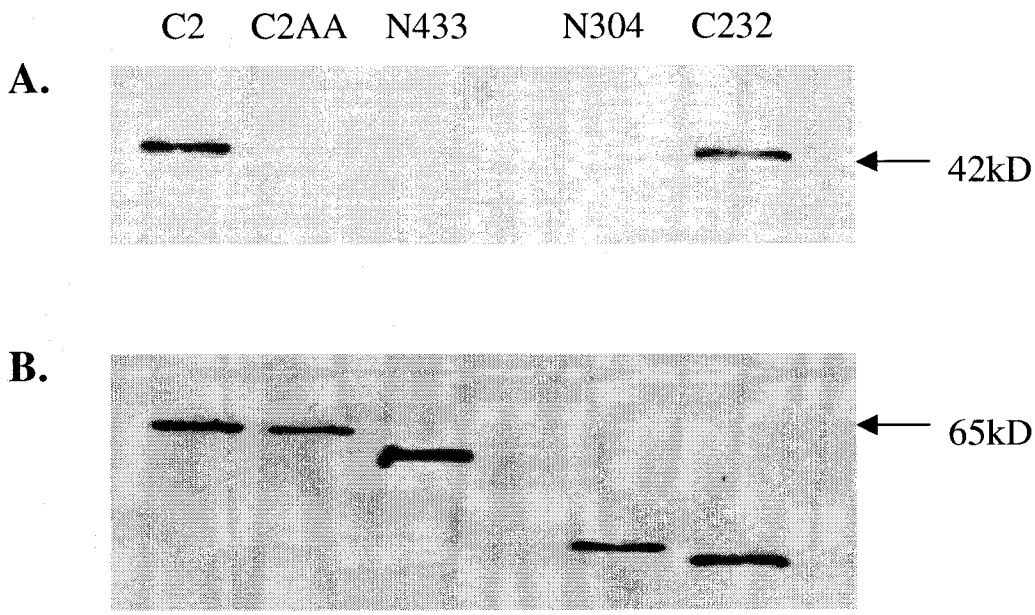


Figure 5: Effect of Phosphorylation of CAP2 on Actin Binding. In a duplicate trial of the phosphorylation assay shown in Figure 4, the blot containing the radiolabelled proteins was probed for the presence of actin. CAP2 has a strong interaction with actin while CAP2AA and CAP2C232 are binding minimal actin (A). No actin binding is seen with CAP2N304. This mutant is lacking the actin binding domain. Actin binding is not seen with CAP2N433, a mutant that is missing most of the actin binding domain, serines 433 and 434, and part of the second dimerization domain. The anti-HA blot that demonstrates that similar amounts of protein were present in each lane (B).

CAP2 Phosphorylation is Calcium Independent

Calcium is required for many biological processes such as synaptic transmission and by several signaling molecules like kinases and phosphatases. An *in vivo* phosphorylation assay was performed in the absence and presence of a divalent cation chelator EDTA to determine if CAP2 phosphorylation is calcium dependant. The autoradiograph indicates that there is no significant change in the amount of CAP2 phosphorylation in the presence or absence of calcium (**Figure 6A**). The anti-HA immunoblot indicates that equal amounts of protein were present in each lane (**Figure 6B**). Interestingly the results indicate that actin binding increased was in the presence of a calcium chelating agent, while the presence of calcium had no effect on actin binding (**Figure 6C**).

Figure 6: Phosphorylation of CAP2 is Calcium Independent. HEK293 cells transfected with CAP2 were subjected to an *in vivo* phosphorylation assay. To determine the effect of calcium on CAP2 phosphorylation, cells were treated with $CaCl_2$ alone (Ca +) or $CaCl_2$ with EDTA (Ca-). No significant difference in CAP2 phosphorylation was seen in the presence or absence of calcium (A). The anti-HA blot indicates that equal amounts of protein were present in each lane (B). The anti-HA blot was stripped and reprobed for actin. Actin binding was increased in the presence of $CaCl_2$ and EDTA (C).

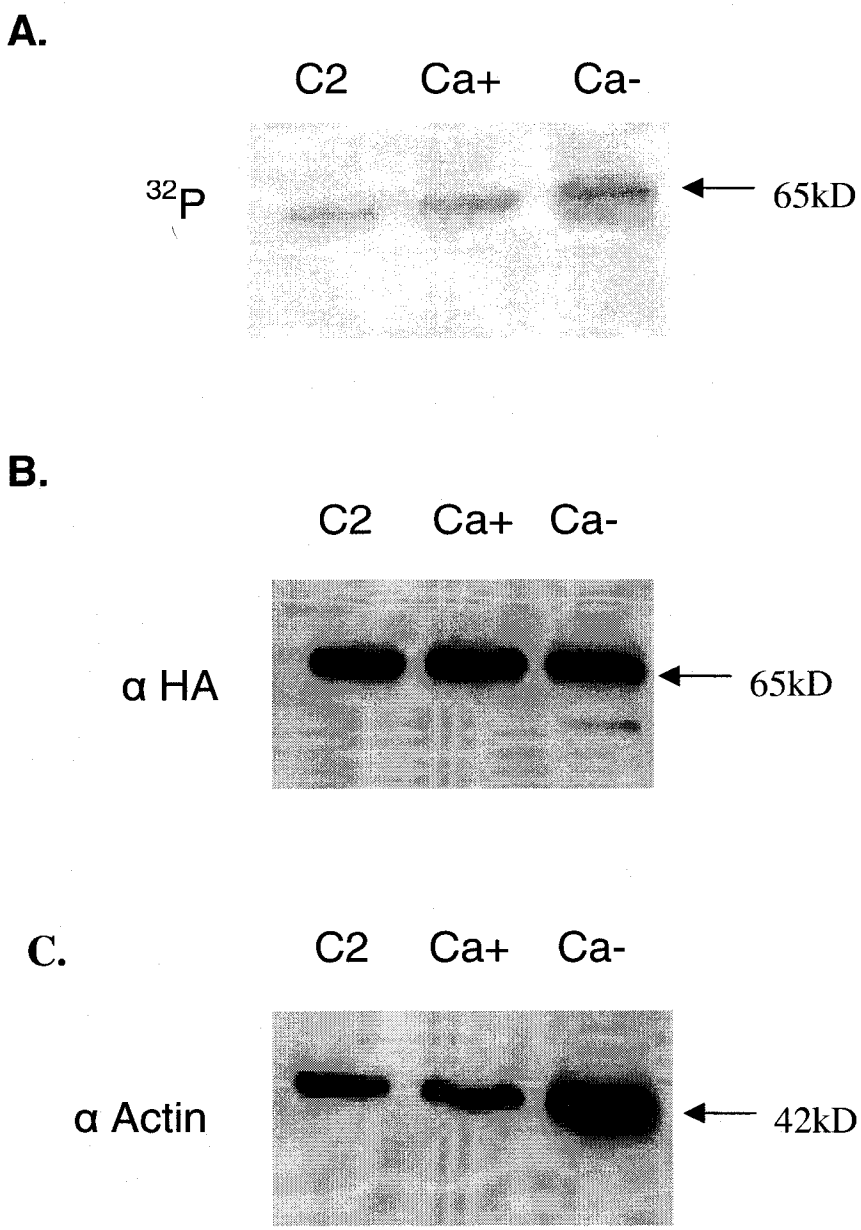


Figure 6.

PKC enhances CAP2 phosphorylation

Kinases have the ability to phosphorylate target proteins. Kinases are often activated as a part of a signal transduction pathway. Protein Kinase C (PKC) is a very common kinase that is involved in various signal cascades leading to different intracellular responses. Protein Kinase A (PKA) is activated downstream of Ras in a pathway involving CAP in yeast. Rac is a protein that is known to induce changes in the actin cytoskeleton (Hall, 1998). In order to determine which pathway may lead to phosphorylation of CAP2, chemical activators of PKA, Rac, and PKC were utilized (platelet derived growth factor (PDGF), forskolin, and PMA respectively). Serum starved HEK293 cells expressing CAP2 were incubated with media containing radiolabelled phosphate for three hours. The chemical activators were added to the media when one hour of incubation remained. The autoradiograph of the immunoprecipitated protein is shown in **Figure 7A.** Activators PDGF and forskolin caused no significant change in CAP2 phosphorylation. Treatment with PMA dramatically increased the level of CAP2 phosphorylation. The anti-HA blot indicates that equal amounts of CAP2 protein was present in each lane (**Figure 7B**).

Effect of PKC activation on Actin Binding

Since PKC activation increased CAP2 phosphorylation, and loss of phosphorylation sites Ser 433 and 434 decreased actin binding, it was questioned whether or not PKC activation affects CAP2 actin binding. Serum starved HEK293 cells transfected with HA-CAP2 were exposed to increasing concentrations of the PKC activator PMA during the incubation with labeled orthophosphate. An increase in phosphorylation of CAP2 was seen in the PMA treated cells versus untreated cells (**Figure 8A**). CAP2

phosphorylation did not increase with increasing concentrations of PKC activator, PMA. The anti-actin blot indicates that the level of actin binding did not change significantly with increasing levels of PMA (**Figure 8B**). The anti-HA blot of equal amounts of protein extract indicates that similar amounts of CAP2 were used for immunoprecipation (**Figure 8C**).

Figure 7: Effect of Chemical Activators on CAP2 Phosphorylation.

A: Autoradiograph of phosphorylated CAP2. Transfected HEK293s were serum starved for 16h prior to 3h incubation with media containing radiolabelled phosphate. Lane 1 is CAP2 from untreated cells and lane 2 is from serum starved cells. The following chemical activators were added to the media for the final hour of incubation: 10μM forskolin (lane 3), 3μM PDGF (lane 4), 2μM PMA (lane5). Proteins were immunoprecipitated with anti-HA coupled protein A beads, resolved on an SDS PAGE gel and then transferred to a nitrocellulose membrane to perform autoradiography. B: Anti-HA immunoblot of the same membrane from 6A.

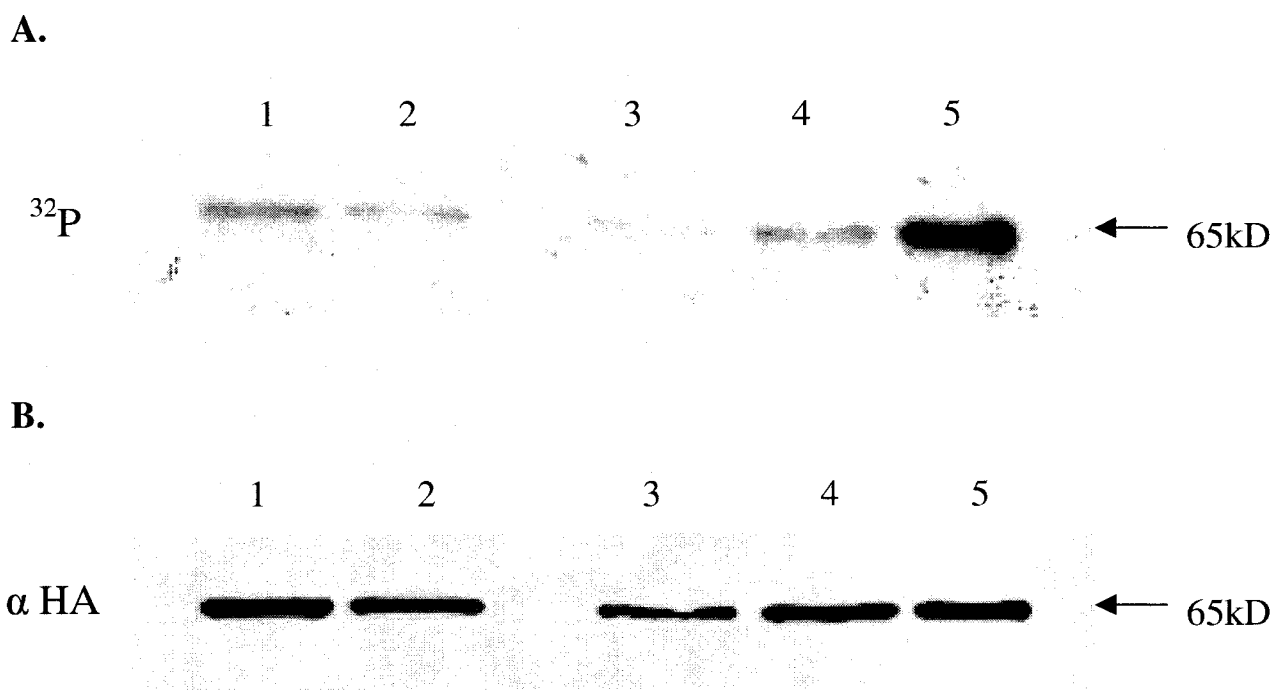


Figure 7.

Figure 8: Effect of PKC Activation on Actin Binding. HEK293 cells transfected with HA-CAP2 exposed to orthophosphate-containing media were incubated with increasing amounts of PMA (5μM, 3μM, 1μM, 0.5μM, 0) in lanes 1-5. Lane 6 is an untransfected control. The autoradiograph in figure 8A shows an increase in phosphorylation from wildtype in lane 5 and PMA treated in lane 4. Increasing concentrations of PMA do not produce a dose-dependant increase in CAP2 phsphorylation. No significant change in actin binding ability of CAP2 is seen (8B). The anti-HA blot indicates that similar amounts of CAP2 were used in each immunoprecipitation (8C).

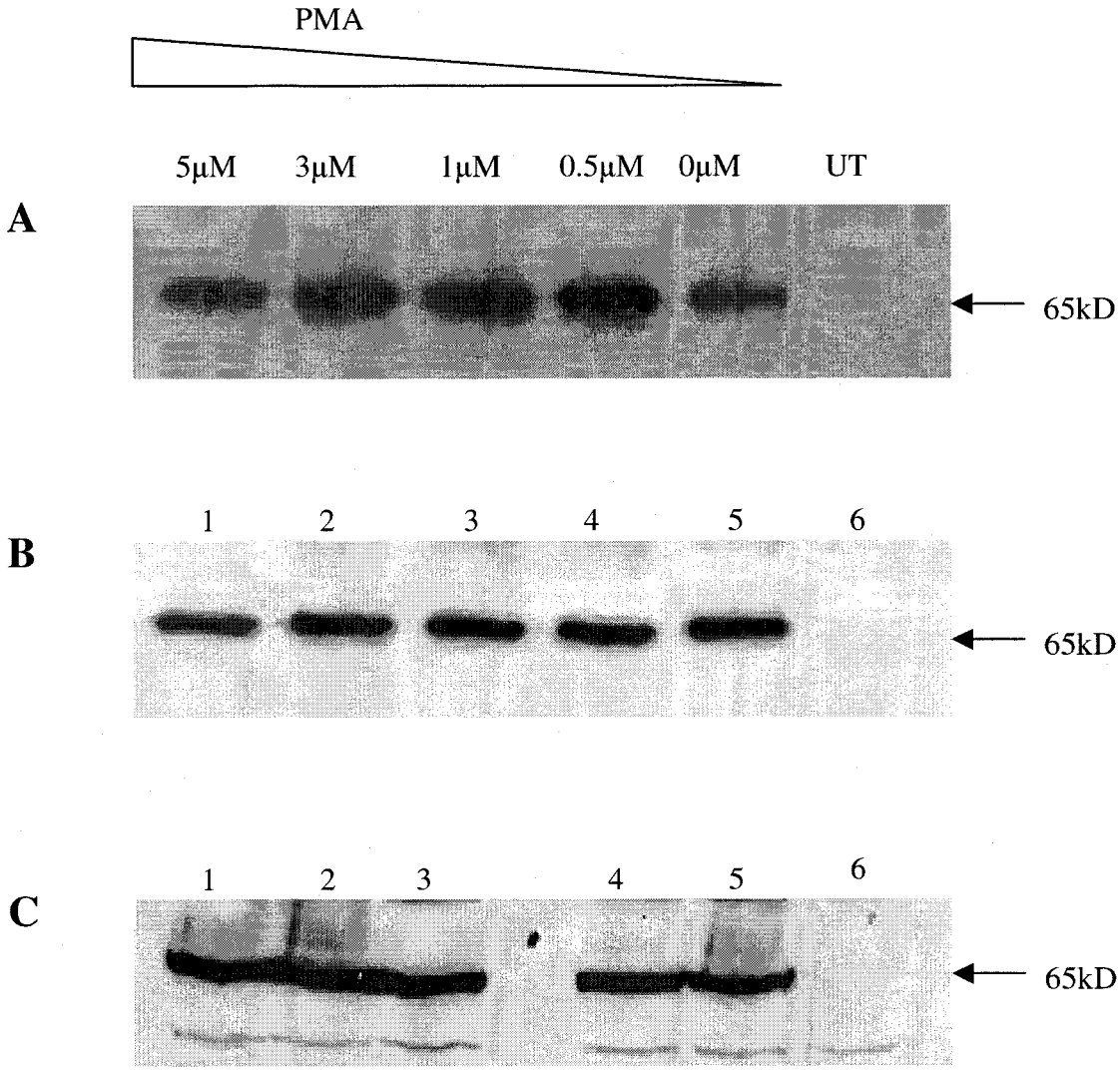


Figure 8.

Further Evidence of Multiple Phosphorylation Sites

It was clear from the initial phosphorylation assays that CAP2 is phosphorylated on multiple residues. In order to understand the mechanism of regulation behind CAP2 phosphorylation, the sites of phosphorylation must be determined. Two-dimensional gel electrophoresis is used to visualize small differences in protein mobility due to slight changes in isoelectric point (for review (Mann and Jensen, 2003). The movement of the protein is based on the overall charge of its side chains and post-translational modifications. Small changes in isoelectric point can be caused by phosphorylation of one or more residues. A phosphate group is very negatively charged and alters the overall charge of the protein, therefore changing the mobility of the protein. Other post-translation modifications like glycosylation and ubiquitination are not charged groups are often detected by overall change in the mass of the protein.

A two-dimensional (2D) gel is performed in two steps. First, proteins are separated in one dimension according to their isolectric point and then separated in a second dimension according to their molecular weight. In this experiment an immunoprecipitation was used to isolate the proteins of interest, and 2D gel electrophoresis was performed to determine if the protein has multiple isoelectric points. Since all of the proteins are HA-tagged, CAP2 constructs were visualized with both anti-CAP2 antibodies and anti-HA antibodies to ensure that spots are indeed CAP2 protein. Results of 2D analysis showed that CAP2 has four different forms while CAP2AA appears to only have three **(Figure 9)**. CAP2N304 has four isoelectric points while CAP2N433 has seven. The untransfected control gels have no distinct spots, indicating that there were no non-specific proteins binding to the

protein A beads in the immunoprecipitation. This experiment was performed twice with identical results.

Figure 9: Two-dimensional Electrophoresis. Two-dimensional electrophoresis was used to visualize small differences in isoelectric point of CAP2 protein. Immunoprecipitated HA-tagged CAP2 constructs (CAP2, CAP2AA, CAP2N433) were subjected to isoelectric focusing followed by SDS-PAGE gel electrophoresis. Gels were transferred to nitrocellulose membranes and immunoblotted with anti-HA (left) and anti-CAP2 (right) antibodies. Untransfected HEK293 cells were used as a control (UT).

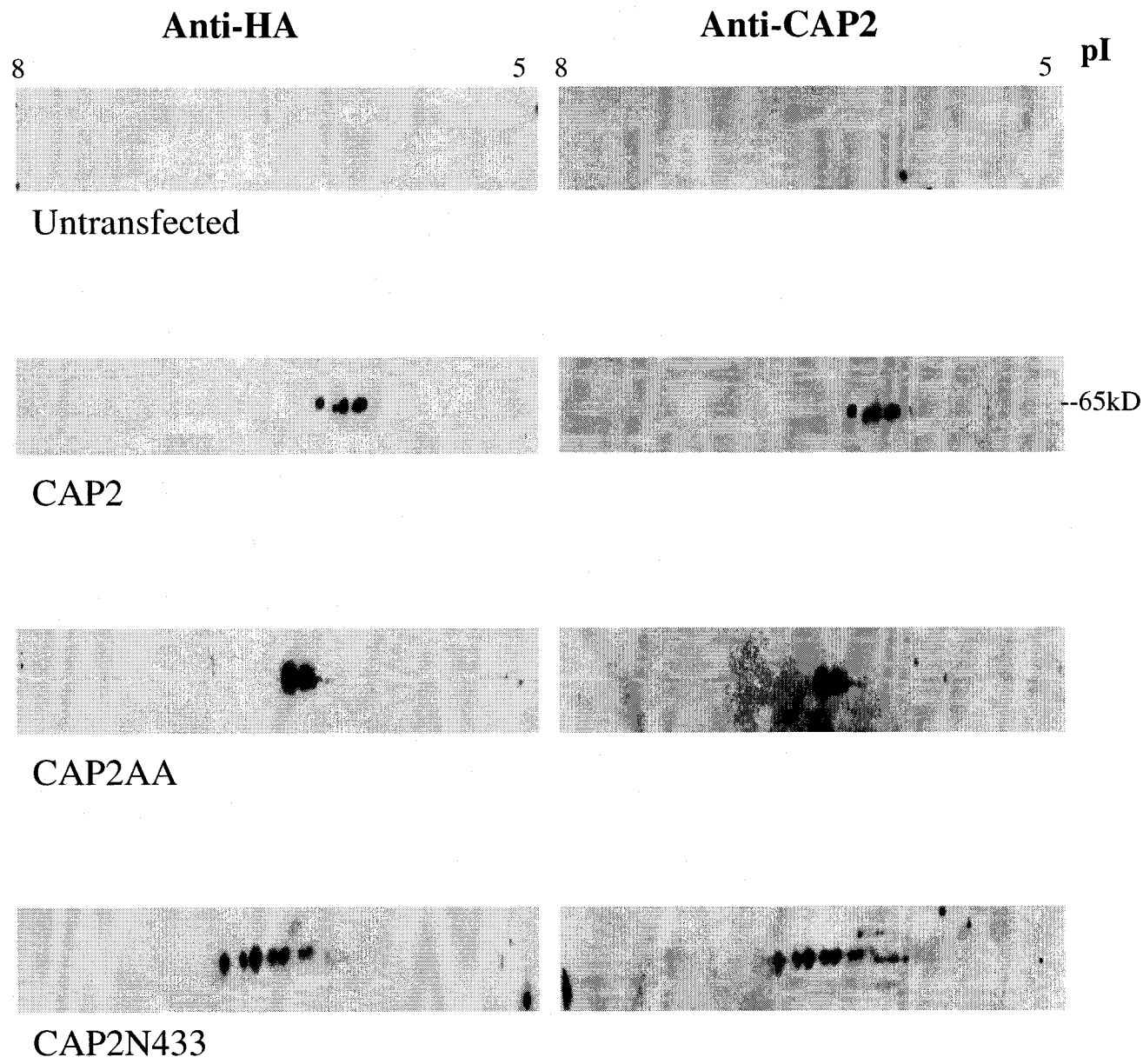


Figure 9.

Discussion

It is known that other actin binding proteins (i.e. WASp) are regulated by phosphorylation, therefore this suggests that perhaps CAP2 is regulated in a similar fashion. First, it had to be determined whether CAP2 is phosphorylated *in vivo*. *In vivo* phosphorylation assays revealed that human CAP2 is phosphorylated on serine(s) 433 and/or 434. These residues are highly conserved in all CAPs identified to date (see **Figure 2**). In fact, CAPs that do not have these two serines have a threonine residue in place of the serine, so essentially all CAPs have two residues available for phosphorylation at this site. From this information, it seems possible that all CAPs are phosphoproteins.

One of the interesting results involved CAP2N433; the mutant is lacking the two conserved serine residues, the actin binding domain and part of the second dimerization domain. It is unclear why CAP2N433 would be more phosphorylated than wild type CAP2 if it were missing the two phosphorylation sites. There are several explanations for this result. Perhaps CAP2N433 is folded into a different conformation because it is truncated and this shape allows more residues to be phosphorylated. CAP2N433 may not be able to form dimers as well as wild type CAP2 and this may leave the protein open / unfolded for more residues to become phosphorylated. Actin binding is decreased in this mutant of CAP2 and perhaps actin binding decreases phosphorylation. It is very clear that CAP2 is phosphorylated at multiple sites because altering the two conserved serines reduced, but did not abolish phosphorylation of the protein. Comparing the amount of phosphorylation of CAP2-N304 with CAP2-C232 indicates additional phosphorylation sites are more likely to

be in the carboxyl terminus than the amino terminus. As mentioned earlier, phosphorylation is often used to regulate proteins involved in signaling cascades. Phosphorylation by an activated kinase may be activating CAP2 in order to cause cytoskeletal changes.

Once it was established that CAP2 was phosphorylated, the functional role of phosphorylation was examined. A two amino acid alteration (CAP2AA) caused a significant decrease in both phosphorylation and actin binding of CAP2. This suggests that this post-translational modification regulates the interaction between CAP2 and actin. CAP2N433 is further impaired in its ability to bind actin but this is more likely do to the truncation of its actin-binding domain.

Perhaps CAP2 is phosphorylated in response to a signal to regulate the protein itself. It was mentioned earlier that CAP shows similarities with WASp, a protein that is regulated by auto inhibition. WASp contains two self-interacting domains, as does human CAP1 and CAP2, that allow for the formation of an auto inhibited loop (Hubberstey et al., 1996; Pufall and Graves, 2002). Perhaps full length CAP2 is present in a looped conformation that blocks phosphorylation sites, but has enhanced affinity for actin. This suggests that phosphorylation regulates conformation of the CAP2 protein and then conformation regulates actin-binding function.

The 2D gel blot of CAP2N433 show several more spots than wild type CAP2. In addition, the CAP2N433 construct was more phosphorylated than wild type CAP2 in the *in vivo* phosphorylation assay. Perhaps CAP2 is usually in a folded conformation that inhibits phosphorylation of several residues but upon truncation of the actin binding domain the protein is unable to fold properly and several more sites are phosphorylated.

Another important question to be answered is which signaling pathways are responsible for the phosphorylation of CAP2. Several different signaling pathways result in changes in the actin cytoskeleton. Several potential pathways that could lead to phosphorylation of CAP2 were examined. In yeast, *S. cerevisiae*, CAP is involved in the RAS2 pathway. Upon activation of the small G protein, RAS2, adenylyl cyclase (AC) is activated, generating cAMP, which causes a response in cell growth regulation. CAP is a binding partner of AC and is thought to regulate this pathway. Protein Kinase A (PKA) is activated downstream of AC in the RAS1 pathway leading to cytoskeletal changes (Hubberstey and Mottillo, 2002). The effect of PKA activation on CAP2 phosphorylation was examined even though mammalian CAPs have not been shown to be involved in an activated RAS pathway. PKA activation is known to have a negative affect on axonal transport, a process that requires actin rearrangement (Hiruma et al., 1999). Another small GTPase, Rac, is known to induce changes in the actin cytoskeleton and thus was an adequate suspect upstream CAP signaling protein (Hall, 1998).

In an attempt to examine the potential pathway leading to CAP2 phosphorylation a discovery was made. Treatment of transfected HEK293 cells with the phorbol ester PMA significantly increased the amount of CAP2 phosphorylation. These results suggest that a cellular signal causes activation of PKC (activated by PMA) that leads to direct or indirect phosphorylation of CAP2, which may regulate its actin binding ability at a site(s) where actin rearrangement is required. This evidence supports a role for CAP2 as a link between signaling pathway(s) through PKC and actin rearrangement.

Calcium is an essential ion within the cells. Calcium is required for many forms of signal transduction such as synaptic transmission. In the calcium dependency experiment (see Figure 6), EDTA was used to deplete intracellular calcium. Levels of phosphorylation of CAP2 did not change in the presence or absence of calcium. The results of this experiment suggest that phosphorylation of CAP2 is independent of calcium but interestingly, actin binding increased in the absence of calcium. EDTA is not specific to only calcium ions, it will bind any divalent cation. Therefore the increase in actin binding of CAP2 that was seen in the presence of EDTA may be due to a loss in magnesium or another divalent cation. Depletion of intracellular calcium will have many different effects within a cell such as reduction in enzyme activity of those enzymes that require calcium in their active site. A decrease in calcium could have a negative affect on actin polymerization by destabilizing F-actin. In the presence of a huge excess of monomeric actin, CAP2 may bind more actin than it typically would. The ratio of one CAP2 proteins binding one actin monomer will not change but the proportion of bound CAP2 versus unbound CAP2 could increase. This could explain why CAP2 bound significantly more actin in the presence of EDTA.

There are three classes of PKCs: classical, novel and atypical. Each isoform has different expression patterns in different tissues of the body. Since this study indicates that the phosphorylation of CAP2 is calcium independent, the classical isoforms can be ruled out due to their calcium dependency. The result that CAP2 phosphorylation is calcium independent corroborates previous studies that have shown that actin binding in *Dictyostelium* CAP is calcium independent (Gottwald et al., 1996).

As mentioned earlier, CAP is a bifunctional protein that has been theorized to play a role in actin dynamics and endocytosis. The process of endocytosis requires actin rearrangement. Yeast synaptobrevin homologue, SNC1, is thought to be involved in targeting the fusion of synaptic vesicles. SNC1 can suppress C-terminal deletions of CAP in yeast (Gerst et al., 1991). This suggests a potential role for CAP in the endocytic process. Munc18, a syntaxin binding protein, is phosphorylated by PKC. Upon modification, syntaxin binding to Munc18 is reduced. This allows syntaxin to form the snare complex essential to endocytosis/vesicle fusion (Barclay et al., 2003). As mentioned earlier, PKC has been shown to play a role in actin remodeling in nerve cell and macrophages (Baron et al., 1998; Larsen et al., 2002). Perhaps upon activation by phosphorylation by PKC, CAP2 is delivering actin monomers to sites of actin rearrangement. PKC also phosphorylates SNAP 25 and synaptotagmin I, other endocytic proteins (Hilfiker et al., 1999; Iwasaki et al., 2000).

Further studies must be performed to elucidate the pathway through which PKC is activating CAP2 phosphorylation. Auto inhibition of CAP, through its dimerization domains, should be examined with respect to phosphorylation and actin binding. This study sheds some light on the potential regulatory mechanisms that control CAP2 function, but more data is required to fully elucidate the role of phosphorylation of CAP2. The fact that CAP2 is phosphorylated and that PKC is playing a role, reinforces the theory that CAP2 links actin dynamics to signaling events.

CHAPTER THREE

Human CAP2 Localizes to Areas of Actin Rearrangement

Introduction

One of the main objectives of this thesis was to examine the localization of human CAP2 in mammalian cells. Mouse fibroblasts transiently transfected with MYC-tagged mouse CAP1 (mCAP1) indicate that mCAP1 is a cytosolic protein that can localize to the leading edge of migrating cells (Vojtek and Cooper, 1993). Endogenous rat CAP1 shows a similar expression pattern (Zelicof et al., 1996). Dictyostelium CAP is cytosolic with distinct membrane localization that requires the N terminal 102 residues (Noegel et al., 1999). Human CAP1 was transiently transfected into Swiss 3T3 cells and was shown to localize to F-actin stress fibers and lamellipodia (Freeman and Field, 2000). The amino terminal domain of hCAP1 was required for this specific expression. In the absence of the amino terminus, hCAP1 is diffusely cytoplasmic. Recently, endogenous mCAP1 was found to colocalize with actin and cofilin at the leading edge of migrating mouse C3H-2K fibroblasts (Moriyama and Yahara, 2002). Cofilin is involved in cytoskeletal dynamics through its ability to depolymerize F-actin (Carlier et al., 1997; Rosenblatt et al., 1997). Human CAP1 and CAP2 protein may not exhibit the same localization within mammalian cells due to their differential mRNA expression patterns in rat cells (Swiston et al., 1995).

The previous chapter demonstrated PKC may play a role in CAP2 phosphorylation. This result may lead to a better understanding of CAP2 regulation. If phosphorylation regulates a function of CAP2, then perhaps PKC activation will affect CAP2 localization or its activity with the actin cytoskeleton. Activation of PKC, by

PMA, in primary rat brain cells caused phosphorylation of MARCKS (myristoylated alanine rich C-kinase substrate) and caused cytoskeletal rearrangement (Baron et al., 1999). PKC is naturally activated when an agonist binds a cellular receptor that causes increased production of diacylglycerol. PMA is able to activate PKC by being a DAG mimic. PMA is a more stable compound than the membrane permeable DAGs, therefore PMA is a more potent PKC activator (Nishizuka, 1992). PKC also plays a role in the rearrangement of F-actin during phagocytosis and phagosomal maturation (Holm et al., 2003). Another PKC substrate called Clone 72, relocalizes to lamellipodia upon phosphorylation, suggesting that PKC is involved in cytoskeletal remodeling (Chapline et al., 1998). PKC activation via PMA in rat oligodendrocyte progenitor cells causes cytoskeletal rearrangement that prevent differentiation (Baron et al., 1998). In macrophages PKCε overexpression can enhance actin polymerization during phagocytosis (Larsen et al., 2002).

Subcellular fractionation of HEK293 cells transfected with CAP2 displays that this protein is mainly cytosolic with some membrane localization. This result was verified in REF52 cells that displayed endogenous CAP2 as a cytosolic protein with some perinuclear localization. Wound assays were performed on REF52 cells to create sites of actin remodeling. Upon wounding, CAP2 localizes to the leading edge of REF52s where actin is being rearranged. In a separate experiment REF52 cells were treated with PMA, a PKC activator previously shown to increase CAP2 phosphorylation. Upon PKC activation, cytoskeletal abnormalities appeared and CAP2 was localized to these F-actin containing structures. This confirms that PKC activation by PMA can result in actin rearrangement and suggests that CAP2 is playing a role in this process.

Material and Methods

Subcellular Fractionation

HEK293 cells on 100mm plates were transfected with 8μg pCI-HA-Scythe-313, pCI-HA-CAP2, or no vector, using 10μl Lipofectamine 2000 (Invitrogen/Life Technologies), according to manufacturers directions. At 48h post-transfection, cells were collected in 500μl RIPA buffer [20mM Tris pH 7.5, 150mM NaCl, 10mM KCl, 1% IGEPAL CA-630 (NP-40), 10% glycerol, and protease inhibitor cocktail (complete Mini, Roche)]. Prior to differential centrifugation, cells were homogenized on ice in a 1mL Dounce homogenizer with 50 strokes. The method used was modified from Shi et. al. 1996. Briefly, cell extracts were spun at 1500g for 8m to obtain the nuclear pellet and the first supernatant (S1). S1 was spun at 100,000g for 90m at 4°C to obtain the membrane pellet and the second supernatant is the cytosolic fraction. Nuclear and membrane pellets were resuspended in 500μl STE buffer [0.25M sucrose, 10mM Tris pH 7.5, 1mM EDTA pH 7.4, protease inhibitor cocktail]. Equivalent amounts of each protein fraction were electrophoresed on a SDS-PAGE gel and transferred to a nitrocellulose membrane for immunoblotting. After blocking over-night in 2% skim milk in TBS the blot was probed with 12CA5 (anti-HA) antibodies followed by anti-mouse secondary antibodies conjugated with horseradish peroxidase. Bands were visualized using Lumi Light Chemiluminesence kit (Roche).

Immunocytochemistry

To visualize endogenous CAP2, confluent REF52 cells on glass coverslips were fixed in 3.7% formaldehyde, permeabilized in 0.5% Trition X-100 and probed with rabbit

polyclonal anti-CAP2 antibodies (1:40) followed by fluorophore labeled secondary antibody goat anti-rabbit IgG Alexa 488 (1:10,000).

For wound assays, REF52 cells were grown on glass coverslips until 90% confluent. Media was removed and cells were scored with a rubber policeman. Coverslips were fixed and processed for CAP2 and actin expression at 0m, 10m, 30m, 2h, 3h, and 6h post-wounding. Cells were probed with anti-CAP2 rabbit polyclonal antibody, fluorophore labeled goat anti-rabbit IgG Alexa 488 (Molecular Probes), and Alexa 568 phalloidin (1:10000)(Molecular Probes). Cells were viewed with a Bio-Rad 1024 confocal microscope; images were collected using Biorad software, then processed using Confocal Assistant and Corel Draw 9.

For chemical effect assays, REF52 cells were grown on coverslips, serum starved for 16h, then treated with PMA at increasing concentrations for different time intervals. Coverslips were removed at various time intervals and processed for CAP2 or actin expression as described above.

Results

CAP2 is a cytosolic protein

CAPs have been previously demonstrated to be cytoplasmic proteins that can bind monomeric actin, however the localization of human CAP2 has never been described (Freeman and Field, 2000; Freeman et al., 1996; Gottwald et al., 1996; Vojtek and Cooper, 1993). REF52 fibroblasts were stained with an antibody raised against human CAP2. Localization of CAP2 was diffusely cytoplasmic with some perinuclear

localization (**Figure 10A**). This expression pattern supports the role of CAP2 in actin cytoskeletal dynamics as both forms of actin are known to be found within the cytoplasm.

Since human CAP1 has been implicated in vesicle trafficking and endocytosis, the subcellular localization of CAP2 was examined. HEK293 cells overexpressing CAP2 were separated into cytoplasmic, nuclear and membrane fractions. CAP2 was found within the cytoplasmic fraction but some was found in the membrane fraction (**Figure 10B**).

CAP2 is localized to areas of actin rearrangement

CAP1 localizes to stress fibers in REF52 fibroblasts and lamellipodia in Swiss 3T3 fibroblasts (Freeman and Field, 2000). Mouse actin-binding protein 1 (mAbp1) also localizes to sites of cell migration and has been suggested as a potential binding partner of yeast CAP (Srv2) (Freeman et al., 1996; Lila and Drubin, 1997). This interaction suggests that CAP2 may have a role in regulating actin treadmilling in mammalian cells. In order to determine the legitimacy for this role, wounding assays were performed on REF52 fibroblasts. REF52 cells were chosen because they express a large amount of endogenous CAP2 (**Figure 10A**). A confluent monolayer of REF52 cells was cut and cells were allowed to migrate into the wound. At the edge of this cut the cells are actively moving into the wound to repair the wound. At the migrating edge actin is being remodeled and monomeric actin is being delivered at a rapid rate that cannot be explained by simple diffusion (Zicha et al., 2003). As early as 30 minutes after wounding, CAP2 expression is intensified at the cut edge of REF52 fibroblasts (**Figure 11B**). CAP2 remains localized to the leading edge up to 4 hours post wounding (**Figure 11C-E**). This phenomenon dissipates by 6 hours after wounding when the cells are nearly finished

closing the wound (**Figure 11F**). It was noticed that filamentous actin disappears from the edge of cells once they began migrating, while CAP2 expression increased at the cell edge (compare Figure 11A and B). This suggests that F-actin is being depolymerized into G-actin (G-actin is not stained by phalloidin) so that the cytoskeleton can be remodeled to migrate into the wound. Actin treadmilling involves removal of actin monomers from the growing end of the actin filament and addition of actin monomers to the opposite end of the filament to push the filament forward to generate the locomotion of the leading edge of the cell. Perhaps CAP2 is releasing actin monomers at this site of actin rearrangement to encourage forward outgrowth. It was recently shown in tobacco suspension culture cells that overexpresion of AtCAP1 (*Arabadopsis*) causes decreased leaf size due to loss of F-actin (Barrero et al., 2003). This result reinforces the theory that CAP2 is involved in actin dynamics.

Figure 10 : CAP2 localization. REF52 cells were plated onto glass coverslips and grown to 80% confluency. Cells were fixed and then probed with rabbit anti-CAP2 polyclonal antibodies. Secondary fluorophore labeled goat anti-rabbit IgG antibodies were used to visualize endogenous CAP2 protein. CAP2 is a cytosolic protein with some perinuclear localization (A). Subcellular fractionation of transfected HEK293 cells was performed to verify CAP2 localization. Nuclear (N), cytosolic (C), and membrane (M) fractions were collect via differential centrifugation. CAP2 is present mainly in the cytsolic fraction but also in the membrane fraction.

A

B

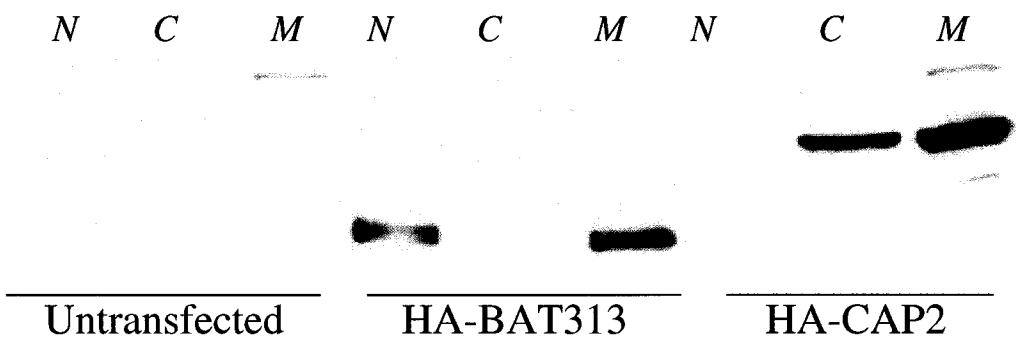


Figure 10.

Figure 11: CAP2 localizes to the leading edge of fibroblasts. REF52 fibroblasts expressing endogenous CAP2 were seeded onto glass cover slips and grown to 90% confluency. Cells were wounded and then allowed to heal. Cover slips were removed and processed for CAP2 and actin expression at 30m(B), 1h(C), 2h(D), 4h(E), 6h(F), or untreated (A). CAP2 localization is initially cytoplasmic on the unwounded cover slip (A). As soon as 30m after wound CAP2 relocalizes to the leading edge of the migrating cells (B-E). CAP2 expression is intense in the tips of the lamellipodial extensions (C, E). CAP2 localization returns to cytoplasmic 6h after wounding (F).

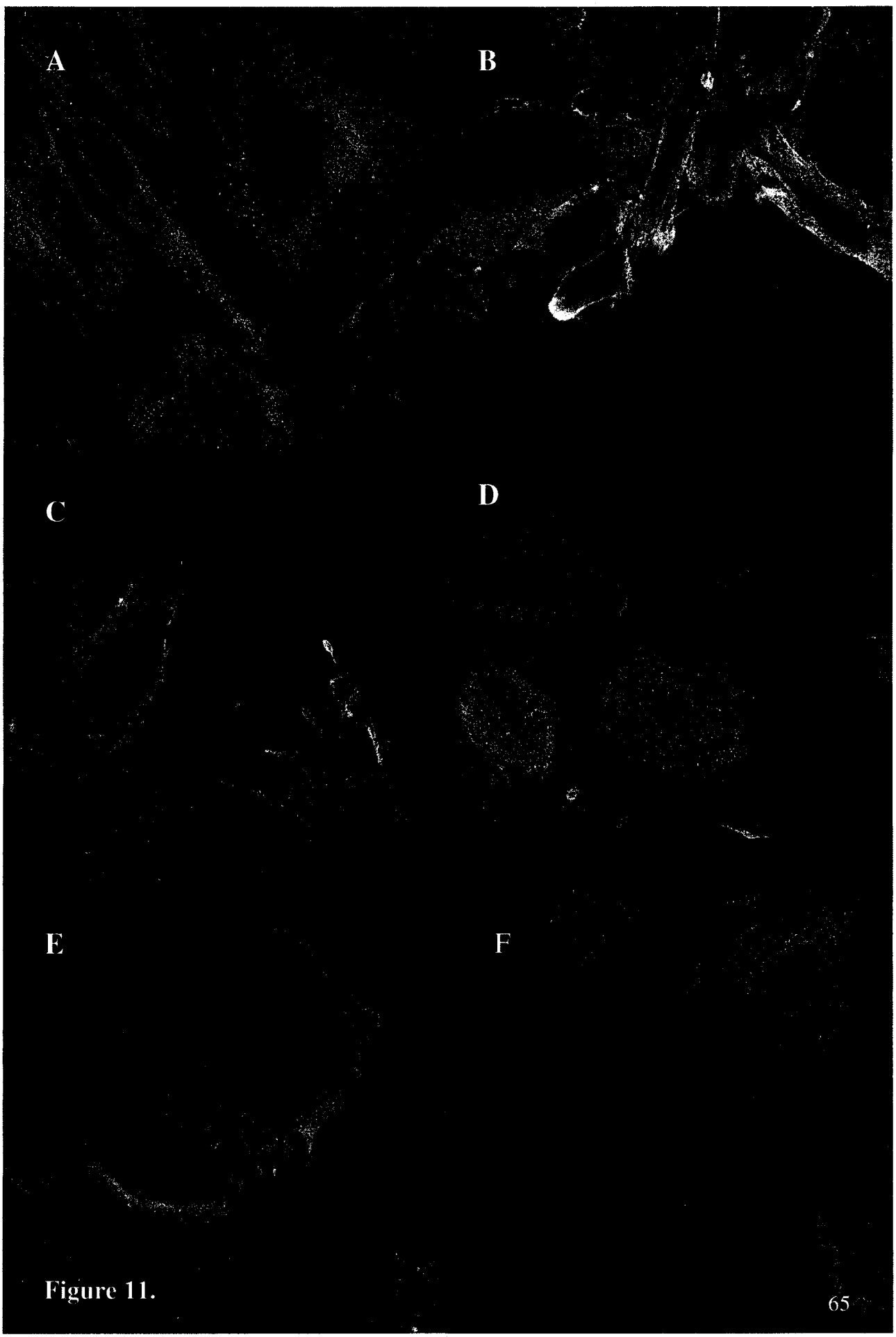


Figure 11.

PKC activation induces cytoskeletal changes and relocalization of CAP2

REF52 fibroblasts were treated with various chemical activators in order to identify the signaling pathway that may regulate CAP2 activity. Cells were serum starved to deplete the media of any growth factors that may interfere with or mimic the effect of the activating agents the cells were being incubated with post-starvation. The phorbol ester PMA, an activator of PKC, produced the most significant results while forskolin, staurosporine did not create any noticeable effect (data not shown). It is known that PMA treatment induces actin reorganization (Hedberg et al., 1994). PKC is required for several actin-dependant processes including proper axonal transport, neurite outgrowth, and repair (Hiruma et al., 1999). It is not surprising that PKC, a kinase with various cytoskeletal effects, causes changes in the localization of CAP2, a cytoskeletal regulator since PKCα is relocalized to the leading edge of cells migrating into a wound (Liao and Jaken, 1993).

Untreated REF52 cells contain CAP2 in cytosolic localization that was verified through subcellular fractionation (**Figure 10B**). Upon treatment of REF52s with PMA, cytoskeletal changes are visible. Lamellipodial extensions are seen at the edge of the cell after 1h incubation with 10μM PMA (**Figure 12B and C**). CAP2 expression is intensified within the tips of these lamellipodia. F-actin is present within these lamellipodial extensions that are generated upon PKC activation (**Figure 13B-D**). It seems that PKC activation induces cytoskeletal rearrangement at the cell's periphery, quite similar to the wounding response described earlier. Clearly, upon activation of PKC, CAP2 relocalizes to actin containing structures. This suggests that CAP2 is involved in actin remodeling and that PKC activation induces this response.

Figure 12: Effect of PKC activator, PMA, on endogenous CAP2. REF52 fibroblasts were serum starved for 16h prior to treatment with DMSO (A), 10μm PMA (B and C), or 20μM PMA (D). Cover slips were removed at time intervals and processed for endogenous CAP2 localization after 1 hour (A-C) or 6 hours (D) of treatment. Treatment with PKC activator PMA induces the formation of lamellipodial extensions that are rich in CAP2.

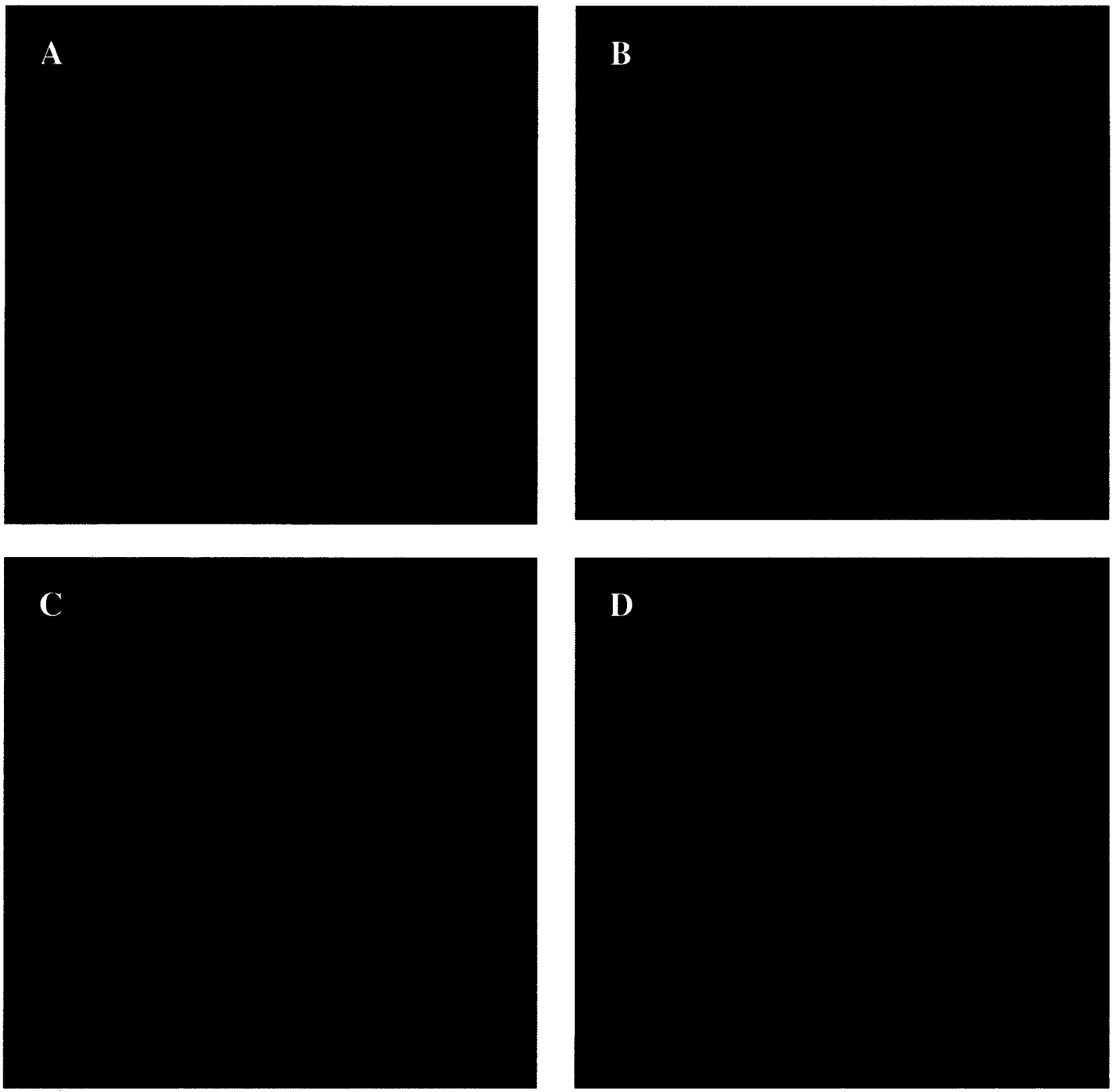


Figure 12.

Figure 13: F-actin is localized to lamellipodial extensions in response to PKC activation. Serum starved REF52 fibroblasts were treated with 0μM (A), 10μM (B,C) or 20μM (D) PMA for 1h (A,B) or 6h (C,D). Coverslips were fixed and stained with phalloidin to reveal F-actin localization. Cells treated with PMA, a PKC activator, form lamellipodial extensions at the cell periphery (B-D) while untreated cells do not (A). F-actin is localized within the lamellipodial extensions.

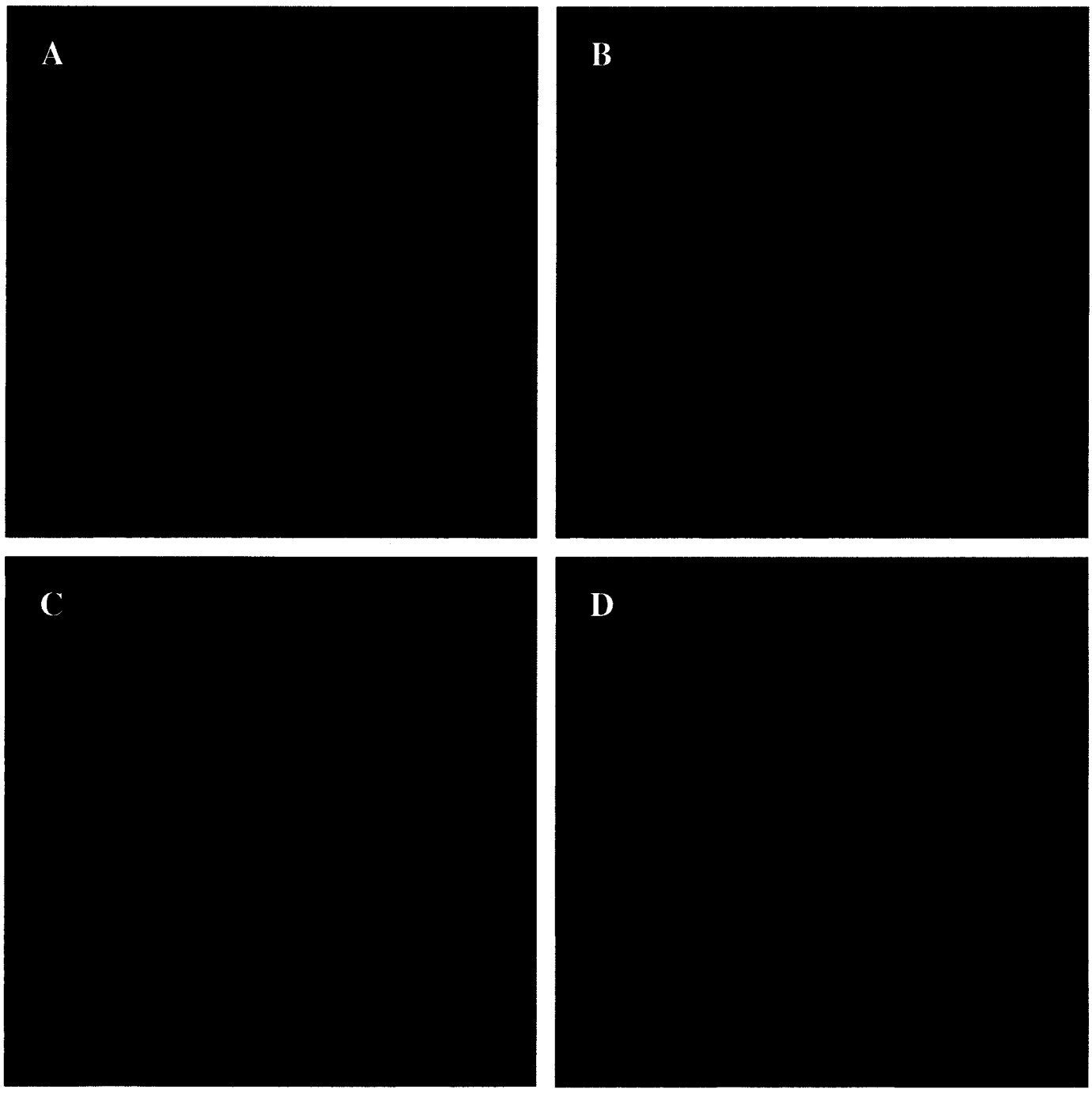


Figure 13.

Discussion

Eukaryotic CAPs are highly conserved actin binding proteins that have been shown to play regulatory roles in cell elongation, cytokinesis, cell migration and polarity, and axon pathfinding in non-vertebrate species (Bahn and Sundstrom, 2001; Barrero et al., 2002; Benlali et al., 2000; Kawai et al., 1998; Noegel et al., 1999; Wills et al., 2002). Two CAP homologs have been identified in all vertebrates studied including mouse, rat and human (Matviw et al., 1992; Swiston et al., 1995; Vojtek and Cooper, 1993). Even though hCAP1 and hCAP2 share 64% amino acid homology most studies have focused on CAP1 and little information exists about the function of CAP2. Although there are differences in the mRNA expression of these two genes in rat, it is not clear if there are functional differences (Swiston et al., 1995).

This chapter describes the localization of CAP2 in HEK293 cells and REF52 fibroblasts. It is essential to have data including endogenously expressed proteins to ensure that the observed effects are valid and not the result of hyperactivity of an overexpression system. Subcellular fractionation of transfected HEK293 cells found CAP2 exists primarily as a cytosolic protein with some membrane affinity, not unlike CAP1. Endogenous CAP2 expression in REF52s is cytoplasmic with some perinuclear localization. Interestingly, CAP2 appears to specifically alter its localization in response to environmental cues. Upon cell wounding, CAP2 localizes to the leading edge of the migrating cell within lamellipodial extensions. Mouse Abp1 also displays a similar pattern of localization (Kessels et al., 2000). The leading edge of a cell is a site of dynamic actin rearrangement. These results suggest a role for CAP2 in cytoskeletal growth through its ability to bind actin monomers and perhaps release them at sites of actin rearrangement.

Treatment of REF52s with PMA also caused relocalization of CAP2. After 1 hour of treatment with PMA, lamellipodial extensions were generated and CAP2 was intensely expressed within them. It was also demonstrated that F-actin is present within the lamellipodia. Growth factors have been shown to be able to induce lamellipodia formation and in COS cells mAbp1 localizes to these extensions (Kessels et al., 2000). Similar lamellipodial extensions have been observed in gastric fibroblasts in response to PMA treatment (Chew et al., 2002). The data presented in this chapter places CAP2 and F-actin together at the cell periphery where actin remodeling is occurring in response to PKC activator by PMA. These results suggest CAP2 is involved in a signal transduction pathway downstream of PKC and may be involved in sequestering and releasing actin monmers during PMA activated signal transduction at sites of high actin turnover. The presence of CAP2 in dynamic regions at the cell periphery is consistent with its possible involvement in actin treadmilling or G-actin sequestering (Freeman et al., 1995; Gieselmann and Mann, 1992; Gottwald et al., 1996; Moriyama and Yahara, 2002).

CHAPTER 4

Conclusions and Future Prospects

General Conclusions

The goal of this thesis was to gain a better understanding of the regulation of mammalian CAP proteins. Studies in yeast have revealed that yeast CAP is involved in an activated Ras2 pathway that leads to the induction of adenylyl cyclase. In mammalian cells, CAP homologues contain a conserved adenylyl cyclase binding domain but have not been demonstrated to interact with adenylyl cyclase. Since yeast CAP interacts with adenylyl cyclase and vertebrate CAPs do not, it is thought that a different mechanism and / or an alternate signaling pathway might regulate vertebrate CAPs.

In this study, human CAP2 was determined to be a phosphoprotein through the use of *in vivo* phosphorylation assays. Two likely phosphorylation sites were mapped to conserved serines 433 and 434. Two-dimensional electrophoresis demonstrated that additional sites of phosphorylation exist within the carboxyl terminus of CAP2. Chemical activators of various signaling pathways were tested for their ability to alter the phosphorylation of CAP2. Activation of PKC, by PMA, was able to increase phosphorylation of CAP2 significantly, in a calcium independent manner. This result indicates that CAP2 is a downstream target of PKC.

The effect of phosphorylation on the ability of CAP2 to bind actin was studied. A mutant with two conserved serine residues (433, 434) altered to alanine (CAP2AA) displayed reduced levels of phosphorylation and also had reduced actin binding. This result suggests phosphorylation of CAP2 on serines 433 and 434 positively regulates actin binding. Another mutant of CAP2 missing both conserved serine residues as well

as the carboxyl terminal actin-binding domain (CAP2 N433) had increased levels of phosphorylation. This result was quite unexpected but can be explained through the concept of autoinhibition. Some proteins, like WASp, have a folded or inhibited conformation as well as an unfolded conformation. Perhaps the truncation of the carboxyl terminus in the CAP2N433 mutant prevents the formation of a folded conformation. This permanently unfolded protein may have more sites that are now accessible for phosphorylation. CAP2N433 is also missing the second dimerization domain. Perhaps CAP2 exists in a dimeric form, concealing phosphorylation sites and without this domain CAP2N433 cannot dimerize to prevent phosphorylation.

Further studies were performed to examine the localization of endogenous and overexpressed CAP2 in a cell culture system. Endogenous CAP2 in REF52 cells is a cytosolic protein with some perinuclear localization. Subcellular fractionation of transfected HEK293 cells also indicated CAP2 is a cytosolic protein with some membrane localization, like its homolog CAP1. In order to further understand the role of CAP2 within the cell, wounding assays were performed. CAP2 was intensely localized to areas of actin remodeling within 30 minutes after wounding. This indicates that CAP2, a monomeric actin binding protein, is playing a role in the reorganization of the actin cytoskeleton. This also indicates that CAP2 is able to rapidly alter its localization perhaps in response to activation by a PKC predicted pathway. CAP2 localization was also able to change in response to PKC activation by PMA. Treatment of REF52 with PMA caused the formation of lamellipodial extensions of the cytoskeleton and CAP2 was localized within these structures. PKC activation is known to induce cytoskeletal effects but since CAP2 expression also changed in response, this further

reinforces the concept of CAP2 being activated in a signaling pathway downstream of PKC.

Future Experiments

Phosphorylation Experiments

The results presented in this thesis demonstrate that CAP2 is a phosphoprotein. Further experiments need to be performed to map all phosphorylation sites of CAP2 and CAP1. There are several methods that can be used to perform this task including phosphoamino acid analysis (PAA) and mass spectroscopy (MS). PAA should be attempted first as it is the more inexpensive of the two methods. This method relies on the fact that phosphorylated serine, threonine, and tyrosine residues are stable in acidic conditions that can cleave peptide bonds. After acid hydrolysis of the protein of interest the peptides can be separated two dimensionally by thin-layer chromatography (Zhou et al., 2001). Phosphorylated residues within the peptides are detected by autoradiography (if ^{32}P-labelled) and with ninhydrin (visible dye binds alpha amino acids). Phosphoamino standards are run concurrently with the target sample for comparison.

Matrix Assisted Laser Desorption Ionization- Time of Flight (MALDI-TOF) MS is designed to examine proteins from smaller than 15 kD. Initially, the protein of interest will be split into two aliquots and subjected to proteolytic digest with acid, to generate small peptide fragments. One group of fragments is treated with calf intestinal phosphatase to remove phosphate groups from phosphorylated residues while the other sample is untreated. The peptides are then mixed with a matrix material and deposited onto a grid or chip. Pulses of laser light ionize the peptides allowing them to enter into the gaseous phase. These ionized peptides are accelerated in an electrostatic field. All

particles have the same kinetic energy but lighter ions travel faster and therefore will reach the detector faster. Ions are detected in a time dependant manner and are analyzed by measuring their mass to charge ratio. A computer computes the mass spectra and the peptide fragments are assigned weights. There are online databases containing actual and expected peptide fragment weights of many proteins. Detection of phosphorylation sites is done by comparison of the fragments from the phosphatase treated sample with the untreated sample. If a fragment within the untreated group weighs 80 Da (or a multiple of 80) more than the same peptide that was exposed to phosphatase, a phosphorylation site has been identified (for an example see (Vacratsis et al., 2002).

Since PKC activation increases the phosphorylation of CAP2, PMA treatment would aid in these methods of detecting phosphorylation sites. Only a small percentage of the protein sample will be endogenously phosphorylated without activation. It would be interesting to determine if these sites are conserved in CAPs of other species. Site-directed mutagenesis could be performed to generate mutant forms of CAP2 that cannot be phosphorylated (change S, T, or Y to A) or are phosphomimicking mutants (change S, T or Y to D or E). These mutants could be used in an *in vivo* phosphorylation assay to further understand the role of phosphorylation in the regulation of CAP function.

Signaling

Research presented in this thesis supports the theory that CAP2 may act as a link between signaling molecules and cytoskeletal dynamics. CAP2 phosphorylation is increased by PKC through activation by PMA, a phorbol ester. While it is known that phorbol esters increase the incorporation of $^{32}PO_4$ into PKC substrates, it is known that PKC activation can lead to the activation of other kinases (Chapline et al., 1998). In order

to determine if CAP2 is directly phosphorylated by PKC, *in vitro* kinase assays can be performed to verify CAP2 is a PKC substrate. All other phosphorylation sites must be mapped and mutants must be generated with alanine substitutions, as was performed for the CAP2AA mutant, so that each individual site can be tested for phosphorylation by PKC.

There are numerous isoforms of PKC, so it should be deduced which one is involved in the pathway leading to CAP2 phosphorylation. REF52 cells express isoforms, α, δ, and ε while HEK293 cells express α, δ, ε, ι, and λ (Farshori et al., 2003; Liao and Jaken, 1993). Only three isoforms are common to both cell types used in this thesis and only two of these are calcium independent, δ and ε. Using antibodies against these forms of PKC their presence in REF52 and HEK293 cells should be verified. An immunoprecipitation of CAP2 could be probed for PKC δ and ε using isoform specific antibodies. If the kinase acts upstream of CAP2 or the interaction of PKC with CAP2 is transient, this experiment is unlikely to produce a positive results. Alternatively an *in vitro* kinase assay may be performed to see if either PKC δ or ε can cause incorporation of radiolabelled $^{32}PO_4$ from ATP into CAP2.

The three small GTPases, Rac, Rho, and Cdc42, of the Rho family are often involved in cytoskeletal signaling. Constitutively active and dominant negative forms of these GTPases can be used in a phosphorylation assay to determine if they are involved in CAP2 phosphorylation. These constructs may also be transfected into REF52s to see if they alter endogenous CAP2 localization. Rho is the most likely of the three GTPases to be involved in CAP signaling because of its role with cofilin, another cytoskeletal protein. Rho activates the Rho associated kinases PAK and ROCK, which in turn activate LIM kinase (LIMK) (Ohashi et al., 2000a; Ohashi et al., 2000b). LIMK

inactivates cofilin by phosphorylation. Cofilin prevents the formation of F-actin by causing fragmentation of F-actin filaments (Ohashi et al., 2000a). It is thought that CAP2 and cofilin may work together to disassemble F-actin into G-actin monomers at sites of actin remodeling. To determine the affect of Rho on CAP2, REF52 cells could be transfected with Rho sense and antisense oligonucleotides (RNAi). Since Rho is an important cytoskeletal signaling molecule, both experiments should cause cytoskeletal changes, as Rho expression will be either upregulated (sense), or knocked down (antisense). CAP2 localization should not be affected unless CAP2 is a downstream effector of Rho.

CAP2 has several conserved domains that could allow it to serve as an adaptor molecule between signaling cascades and actin rearrangement. CAP2 is able to sequester monomeric G-actin and can interact with other proteins via its amino terminal coiled-coil domain, its central poly-proline region or perhaps through dimerization with other proteins using its two dimerization domains. A recent report has found rat CAP2 in circumvallate papillae where it is thought to be involved in taste transduction (Ishimaru et al., 2001).

CAP function

Perhaps the most interesting discovery made in this thesis was that decreased phosphorylation of CAP2 was correlated with reduced actin binding. It would be interesting to see if over-expression of CAP2AA in REF52 cells causes any cytoskeletal abnormalities like impaired migratory abilities or lack of lamellipodial extensions in response to PMA treatment. These results would reinforce the theory that

phosphorylation of conserved serine residues 433 and 434 of CAP2 is essential to cell migration and that PKC is responsible for the phosphorylation of these residues.

In order to learn more about the role of CAPs with the actin cytoskeleton, CAP2 expression can be repressed through RNA inhibition technology (RNAi). Reducing levels of profilin, another actin binding protein, was achieved by perfusing smooth muscle tissue with antisense oligonucleotides (Tang and Tan, 2003). This experiment demonstrated that the absence of profilin caused a reduction in force development. Megakaryocytes treated with WASp antisense causes dysfunction in vesicle formation and F-actin bundling (Miki et al., 1997). If CAP2 RNAi can prevent CAP2 relocalization and formation of lamellipodial extensions than it is clear that CAP2 is regulating the cell's migratory response. It may be necessary to use RNAi against both CAP1 and CAP2 simultaneously to see a cellular response as these two homologs may play a redundant role. REF52 could be further fractionated to isolate a lamellipodial fraction to verify CAP2 localization to the leading edge during cell migration (Kessels et al., 2000).

To investigate how CAP2 interacts with other proteins to perform cytoskeletal or signaling functions, interacting partners must be identified. There are several ways to perform this search. Previously our lab performed a yeast two-hybrid screen of a brain cDNA library and found a neuronal binding partner for CAP1 and CAP2 named neuronal calcium binding protein (NECAB) (Motillo et. al. submitted 2003). MS can be used to search for interacting proteins after a sensitive immunoprecipitation (IP) has been performed. The IP should be performed using endogenous CAP2 either from rat brain or REF52 cells. The immunoprecipitated proteins can be directly acid-hydrolyzed and sent for mass finger printing or two-dimensional (2D) electrophoresis can be performed. Two-dimensional electrophoresis will separate proteins by charge and mass into protein

spots visualized via silver staining. Protein spots can then be cut out and sent for mass fingerprinting. A similar experiment was performed on a CAP2 IP but the MS was unsuccessful as the samples were contaminated. Another experiment, albeit expensive, is to electroblot the 2D gel before silver staining and send it to BD Transduction Labs for power blotting. This company will probe your blot with hundreds of antibodies to detect the proteins creating the spots on the 2D gel.

Autoinhibition

Two-dimensional gels (2D gels) can be used to distinguish between multiple phosphorylated forms of a single protein. More than one phosphorylated form of CAP2 was expected since alteration of two potential phosphorylation sites (Ser 433,434) to alanine did not completely abolish phosphorylation of CAP2. On a 2D gel CAP2 presented three spots while CAP2AA displayed only two. This is readily explained by the loss of two potential phosphorylation sites in the CAP2AA mutant. However, CAP2N433, a mutant missing Ser 433 and 434 presented seven spots on a 2D gel. This result correlated with the increased level of phosphorylation displayed by CAP2N433 in the *in vivo* phosphorylation assays. CAP2N433 is missing the C terminal forty-one amino acids. This deleted fragment contains the actin binding domain, a dimerization domain, and as mentioned, two highly conserved serine residues. It has been demonstrated that CAP2N433 is unable to bind actin, but it has not been determined if CAP2N433 can bind itself or wild type CAP2. Perhaps CAP2 phosphorylation is regulated by autoinhibition. CAP2 could generate an inhibited or folded conformation via an intramolecular interaction using its two dimerization domains. If this theory is assumed, CAP2N433 could not form the folded conformation and it is likely that more

residues are available for phosphorylation in this conformation (**Figure 14**). This suggests CAP2 is mostly present in a folded conformation, as the level of phosphorylation of wild type CAP2 was lower than that of CAP2N433. Other cytoskeletal proteins like WASp and PAK are maintained in an inhibited form.

This theory of autoinhibition needs to be examined further. NMR spectroscopy and X-ray crystallography are the best way to visualize conformational changes that could inhibit activity like actin binding. Unfortunately a full length crystal of CAP2 has not been made and it an unlikely prospect as only pieces of the N and C terminal domains have been successfully isolated (Hofmann et al., 2002; Ksiazek et al., 2003; Rehm et al., 2002). Another way to determine which regions of the protein are participating in autoinhibition is to map the minimal region required for actin binding or a particular protein interaction. This can be accomplished via cloning or partial proteolysis followed by functional pull-down assays (Pufall and Graves, 2002). The intramolecular bonds that hold the inhibited or bent conformation in place need to be mapped and disrupted. In the case of CAPs disruption of the dimerization domains may prevent dimerization. The results of these experiments should provide a clearer picture of how CAP regulates its ability to bind actin. Furthermore, an analysis of whether phosphorylation affects protein folding can be performed.

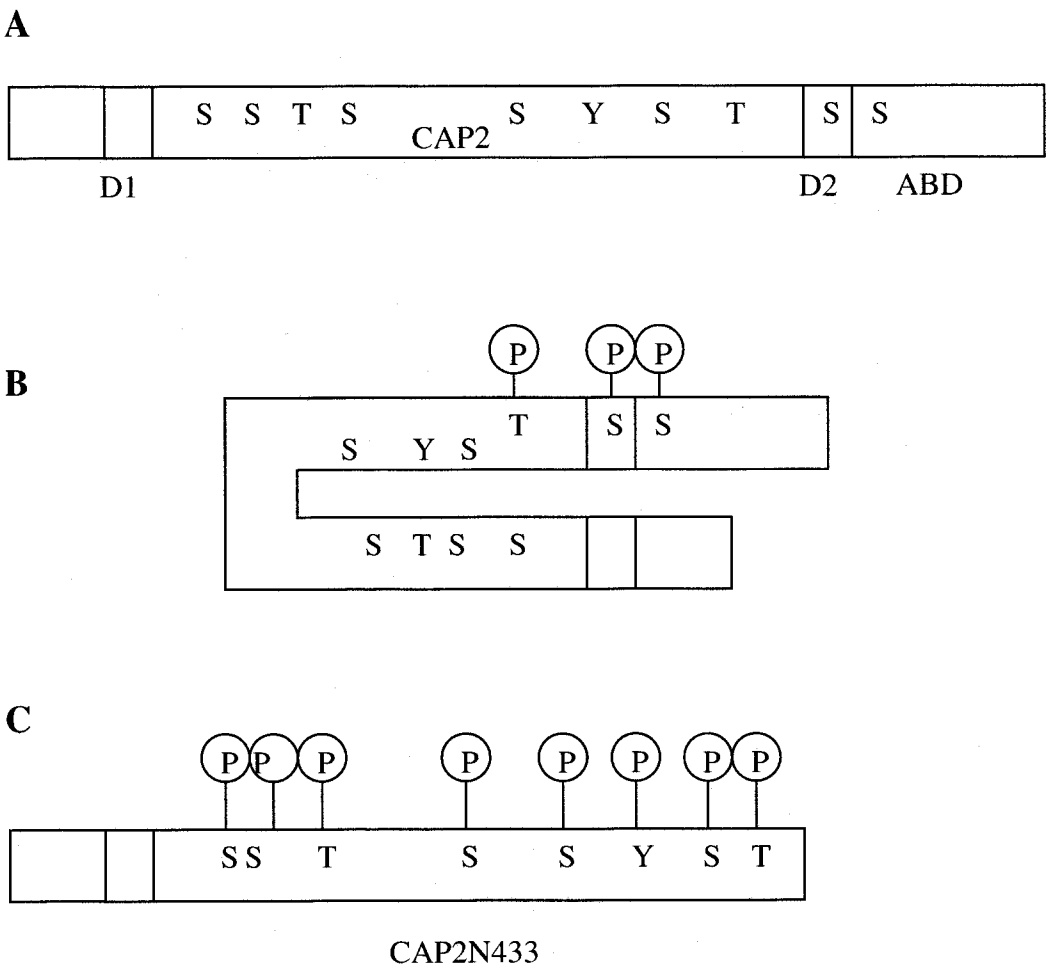


Figure 14: A model of CAP2 autoinhibtion. CAP2 contains two dimerization domains, D1 and D2 depicted in A. Perhaps CAP2 can form an inhibited conformation by interacting with itself via dimerization domains one and two, as shown in figure B. If CAP2 is truncated losing the ABD (actin binding domain) and part of D2 then it cannot form this intramolecular interaction and more residues are available for phosphorylation.

Endocytic Function

A recent publication displayed a computational strategy for analyzing protein-protein interactions. Within a complex chart of over 233 interactions Srv2 (yeast CAP) was associated with Abp1, actin regulating kinase 1 (Ark1), and Protein Kinase C Related Kinase 1 (Prk1) (Tong et al., 2002). These associations are suggestive of a role for CAPs in endocytosis. This specific role was not examined in this thesis but perhaps a future project may explore. The SH3 region of Abp1 is able to interact with the poly proline region of Srv2, suggesting a role for CAP in signaling (Fazi et al., 2002). Abp1 is also able to interact with two serine/threonine kinases, Prk1 and Ark1, via its SH3 domain (Fazi et al., 2002). Prk1 has been shown to phosphorylate Pan1 and prevent the formation of a protein complex that is required for endocytosis, therefore Prk1 is a negative regulator of endocytosis (Tang et al., 2000; Zeng et al., 2001). This suggests that Abp1 regulates endocytic function through protein-protein interactions via its SH3 domain. Since Abp1 interacts with Srv2, perhaps Srv2 is also regulating endocytosis. It would be interesting to see is Prk1 and Ark1 could phosphorylate Srv2. If this event is possible, does it negatively effect endocytosis, prevent a complex formation of Srv2 with other endocytic proteins, or even have an effect on cytoskeletal dynamics? The role of Srv2 in endocytosis is further reinforced by the synthetic lethality of Srv2 with Sla2 (Wesp et al., 1997). Sla2 and Prk1 are synthetic lethal with Abp1 and therefore these proteins share a redundant role (Fazi et al., 2002). It is intuitive that CAP is involved in endocytosis as membrane vesicle attachment and release requires actin rearrangement and CAP is known to localize to such areas (Vojtek and Cooper, 1993).

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APPENDIX A: Oligonucleotides Used

Number	Name	Sequence (5' → 3')
31	lexcap2-f	ttgaattcatggccaacatgcagggactgg
32	lexcap2-r	ttctcgagttaggccataatttctgcagg
108	hacap2c232-f	ttgaattccttttcgagaatgaagcaaaaaaag
109	hacap2n304-r	ttctcgagttaggtgggagattgagtttgccc
161	hancap2433-r	ttctcgagttacacgatctcacagtctaatgc
157	cap2437438aa-f	gagatcgtgagcgccaaggcagctgaaa-tgaacatacttatc
158	cap2437438aa-r	gataagtatgttcatttcagctgccttggcgct-cacgatctc

Vita Auctoris

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December 2003	Completed Master's of Science degree from the University of Windsor
September 2003	Received a Graduate Tuition Scholarship from the University of Windsor
May 2003	Received a two-year Natural Science and Engineering Research Council of Canada (NSERC) Post Graduate Scholarship Award (PGS-A)
May 2003	Received a two-year Ontario Graduate Scholarship (OGS)
September 2002	Received a Graduate Tuition Scholarship from the University of Windsor upon acceptance into the Graduate Biological Sciences Program
May 2002	Received the Holder/Franklin Award for Excellence in Undergraduate Research for an Honor's Thesis from the University of Windsor
April 2002	Completed a four-year Honor's Bachelor of Science degree with Thesis, with a major in Biology and a minor in Chemistry
January 1999	Received a Community Scholar Award from the University of Windsor
June 1998	Competed in Odyssey of the Mind World Finals in Orlando, Florida
September 1997	Received a Community Scholar Award from the University of Windsor
September 1997	Received an Admission Bursary from the University of Windsor

Significant Academic Accomplishments

ASCB Poster Presentation December 2003

Human CAP2 is phosphorylated and localizes to the leading edge of fibroblasts at sites of actin rearrangement. J.M. Sullivan, E.P. Mottillo, A.V. Hubberstey.

ASCB Poster Presentation December 2002

Necab1: a novel human neuronal protein that interacts with Cyclase Associated Proteins (CAPs) E.P. Mottillo, J.M. Sullivan, V. Patel, W. Lepeak, L.A. Elferink, A.V. Hubberstey.

Master's Thesis
Functional Characterization of Human CAP2

Honor's Thesis
Characterization of Human Necab1

Manuscripts in Submission

Sullivan, J., Mottillo, E.P. and A.V. Hubberstey. (2003) Human CAP2 is a cytoplasmic phosphoprotein that concentrates at sites of actin rearrangement in lamellipodia. J. Cell Science

Manuscripts in Preparation

Mottillo, E.P., Sullivan, J., Patel, V., Lepeak, W., Elferink, L.A. and A.V. Hubberstey. (2003) Necab1: a novel human neuronal protein that interacts with Cyclase Associated Proteins. J. Cell. Biochem.