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# Characterization of a novel human CAP2 interacting protein: Neuronal calcium binding protein 1 (Necab1).

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# CHARACTERIZATION OF A NOVEL HUMAN CAP2 INTERACTING PROTEIN: NEURONAL CALCIUM BINDING PROTEIN 1 (NECAB1)

by

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## ABSTRACT

The actin cytoskeleton is implicated in numerous processes such as cellular division, migration, endocytosis/exocytosis, and more recently synaptic plasticity. Important players in controlling cytoskeletal rearrangements are the Cyclase Associated Proteins (CAPs) which are thought to link cell signalling to the dynamics of the actin cytoskeleton, via sequestration of monomeric actin subunits. CAPs are conserved throughout evolution and possess conserved functional domains. Previous studies have suggested a link between CAP proteins and vesicle trafficking and this study focussed on characterizing binding partners of human CAPs. Using a two hybrid screen of a human adult brain cDNA library, a novel human CAP interacting protein named Neuronal calcium binding protein 1 (Necab1) has been isolated. Necab1 is a 351 amino acid protein containing a predicted calcium binding EF-hand and three coiled-coil domains. It is highly expressed in human brain, and similar to CAP2, displays high levels of expression within the hippocampus and cerebral cortex of rat brain. *In vitro* binding and *in vivo* immunoprecipitations demonstrate that CAP2 interacts with Necab1. Two-hybrid interaction assays performed with deletion mutants, in addition to site-directed mutagenesis of the coiled-coils of CAP2 (R10T, L11P) and Necab1 (I218K, L221R), reveal that the central coiled-coil of Necab1 interacts with the amino proximal coiled-coil of CAP2 (RLE motif). It is also demonstrated that Necab1 can interact with the neuronal t-SNARE syntaxin 1a, suggesting a role in synaptic transmission. Furthermore, fractionation studies on rat brain reveal that Necab1 and CAP2 are mainly cytosolic proteins, but also display some membrane localization. Immunolocalization studies in neuronal PC12 cells, demonstrate that CAP2 colocalizes with Necab1 in neurites, but not with F-actin. In order to further understand the functional role for Necab1 and CAP2, immunoprecipitations demonstrate that when CAP2 is bound to Necab1, no actin binding occurs, and that this inhibition is independent of $Ca^{2+}$. Furthermore, actin polymerization assays reveal for the first time that CAP2 is a potent inhibitor of F-actin formation. Necab1 does not demonstrate any concurrent function with CAP2 in these assays, but does inhibit filament formation itself to some degree. To study potential mechanisms of CAP2 regulation, *in vivo* labelling studies were performed. Results demonstrated that CAP2 is phosphorylated in the C-terminus. Analysis of the C-terminus of

CAP2 reveals a domain that is conserved within all CAPs, in which two serine residues are predicted to be potential phosphorylation sites. Thus, results suggest that human CAP2 functions to regulate the formation of actin filaments where it likely responds to cell signalling events. Furthermore, a neuronal role is proposed where CAP2 may function during events of synaptic transmission and plasticity in conjunction with Necab1.

**To Nonna Gina**

**So che saresti fiera di me**

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*I have come a long was since I first began my Masters project. The whole experience has made me a better person in regards to numerous aspects. It has taught me to be more critical, to look in general at the underlying meaning of things, and more importantly that I can accomplish almost anything. These are all things that will help me in the future, be it a at a place of employment or in continued education.*

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## List of Abbreviations

**Abp1** - Actin binding protein 1

**ADF-H** - Actin Depolymerization Factor - Homology

**ATP** - adenosine triphosphate

**AMPA** - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic

**ARF** - ADP Ribosylation Factor

**ARP2/3** - Actin Related Protein 2/3 complex

**AP2** - Alpha-adaptin 2

**BAT** - HLA-B-Associated transcript

**CA** - *cornu ammonis*

**$Ca_{2+}$** - calcium ion

**CaMKII** - α-Calcium-calmodulin– dependent proteinkinase II

**CAP** - Cyclase Associated Protein

**Cdc42** - Cell Division Cycle 42

**CC** - Critical Concentration

**CCP** - clathrin-coated pit

**CCV** - clathrin coated vesicle

**Da** - daltons

**DTT** - dithiothreitol

**End4** - Endocytosis 4

**ER** - endoplasmic reticulum

**F-actin** - Filamentous-actin

**FBS** - fetal bovine serum

**G-actin** - Globular-actin

**GAP** - GTPase Activating Protein

**GAPDH** - Glyceraldehyde 3-Phosphate Dehydrogenase

**GEF** - Guanine nucleotide Exchange Factor

**GFP** - Green Fluorescent Protein

**GST** - Glutathione-S-Transferase

**HA** - hemagglutinin

**HEK293** - Human Endothelial Kidney 293

**HIP1** - Huntington Interacting Protein 1

**HIP1R** - HIP1-Related

**IP** - immunoprecipitation

**IRSp53** - Insulin Receptor Substrate p53

**Lat A** - latrunculin A

**LIMK** - LIM Kinase

**LTP** - Long Term Potentiation

**Munc18** - mammalian unc18

**Necab1** - Neuronal Calcium Binding Protein 1

**NGF** - nerve growth factor

**NMDA** - *N*-methyl-D-aspartate

**NSF** - N-ethylmaleimide sensitive fusion protein

**OD** - optical density

**ONPG** - o-Nitrophenyl β-D- galactopyranoside

**PAK** - p21 Activated Kinase

**pBS** - pBlueSript/SK II$^+$

**PBS** - Phosphate Buffered Saline

**PC12** - pheochromocytoma 12

**PDGF** - Platelet derived growth factor

**PK** - Pyruvate Kinase

**PKA** - Protein Kinase A

**PKC** - Protein Kinase C

**$PIP_2$** - Phosphatidylinositol 4,5-bisphosphate

**RAB** - Ras genes from rat brain

**Ras** - Rat sarcoma

**Rho** - Ras Homology

**ROCK** - Rho associated coiled-coil containing kinase

**RRP** - readily releasable pool

**RT-PCR** - Reverse Transcriptase - Polymerase Chain Reaction

**SCAR** - suppressor pf cAR

**SDS-PAGE** - Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

**SH3** - Src Homology 3

**SLA2** - Synthetically lethal with Abp1 2

**SNAP** - soluble NSF-attachment protein

**SNAP-25** - Synaptosomal Associated Protein of 25 kDa

**SNARE** - soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor

**SNC1** - suppressor of the null allele of *CAP*

**SNIP1** - SNAP-25 Interacting Protein 1

**STIP1** - Synaptotagmin Interacting Protein 1

**Stx** - syntaxin

**t-SNARE** - target SNARE

**VAMP** - Vesicle Associated Membrane Protein

**VASP** - Vasodilator-Stimulated Phosphoprotein

**v-SNARE** - vesicle SNARE

**WASP** - Wiskott Aldrich Syndrome Protein

**WAVE** - WASP family Verprolin-homologous protein

**XB51** - X11L Binding protein 51

**X-gal** - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

# CHAPTER ONE

## Introduction

Actin is the most abundant protein in eukaryotic cells, compromising 5-10% of a cells total protein. In the monomer form, globular actin (G-actin) is a protein of 42 kDa and is conserved from yeast to humans with 90% amino acid homology. Due to actin's intrinsic ATPase activity, at physiological conditions, G-actin can spontaneously self-associate and polymerize into filamentous actin (F-actin), and results in the hydrolysis of the bound ATP molecule. Actin filaments are polar, in that they have two distinct ends; a fast growing barbed (+) end in which actin polymerization is favoured, and a slow growing pointed end (-) where the removal of G-actin is favoured (reviewed in Carlier and Pantaloni, 1997). At steady state, the addition of monomers on the barbed end equals the release of monomers on the pointed end. The critical concentration for actin is 0.1 uM. Thus, if the concentration of G-actin rises above this, actin polymerizes until the critical concentration is reached. However, the concentration of actin in the unpolymerized form is found well above this level in a wide array of organisms (Pollard et al., 2000). This seeming contradiction and the mechanism of equilibrium between G and F-actin is regulated, can be explained by the presence and activity of a plethora of actin binding proteins found in all eukaryotic cells.

Actin binding proteins can be organized into different groups based on their function: filament severing proteins, such as gelsolin and brevin bind to the side of an F-actin filament, breaking it apart and causing a decrease in the viscosity of the cytoplasm; filament depolymerizing proteins, such as the Actin Depolymerization Factor (ADF) family of proteins and cofilin, can bind to side of filaments and break them into G-monomers; capping proteins, such as Cap Z, bind to the end of actin filaments thus, preventing filament elongation and promoting depolymerization; anchoring proteins, such as dystrophin and vinculin, tether the actin cytoskeleton to the plasma membrane; filament bundling proteins, such as actinin, which are capable of crosslinking actin filaments; and finally monomer sequestering proteins, such as the Cyclase Associated Proteins (CAPs) and profilin, which bind G-actin, thus preventing it from being polymerized. Furthermore, these proteins promote F-actin polymerization by delivering monomers to sites of actin turnover. The nature of certain actin binding

proteins is oversimplified as some proteins have other actin binding properties beyond those which have been described. For example, cofilin is able to depolymerize F-actin and bind G-actin depending on pH (Bernstein et al., 2000; Maciver and Hussey, 2002). The CAPs have recently been shown to have other actin binding properties beyond G-actin sequestration, such as depolymerization on the pointed end, and the ability to facilitate the addition of actin monomers on the barbed end (Moriyama and Yahara, 2002). Numerous other actin binding proteins exist, some with specific tissue/organ expression suggesting a functional correlation with their expression pattern.

Actin filaments, monomers and their associated proteins are organized into a highly dynamic structure called the actin cytoskeleton, which is involved in a wide array of cellular functions. Some well studied roles for the cytoskeleton include maintaining cell structural integrity, cellular movements and locomotion, shape changes and cell division/cytokinesis (see references within Schmidt and Hall, 1998). Locomotion and cellular movements are driven by the spread of pseudopods (also known as lamellipods), which are due to the extension of actin filaments on the barbed end (front of the lamellipod), and disassembly at the pointed end (rear of the lamellipod) by an array of actin binding proteins (reviewed in Carlier and Pantaloni, 1997). A role for the actin cytoskeleton is also realized in the processes of endocytosis/exocytosis (vesicle trafficking), and during synaptic plasticity, though the exact function during these processes is still unclear.

**Cyclase Associated Proteins (CAPs)**

The Cyclase Associated Proteins are actin monomer binding proteins that are thought to link cell signalling to morphological changes in the actin cytoskeleton (reviewed in Hubberstey and Mottillo, 2002). CAP was first identified in yeast over ten years ago in two independent studies. Yeast CAP was identified through its ability to alleviate phenotypes associated with an activated *RAS2* allele (Fedor-Chaiken et al., 1990). The second study identified CAP as a complex with adenylyl cyclase (Cyr1p), hence the name CAP (Field et al., 1990). Both studies, identified yeast CAP as a signalling molecule between RAS and its effector Cyr1p. Deletion of yeast *cap* not only revealed that Ras signalling was compromised, but cells also demonstrated four additional phenotypes: inability to grow

on rich medium; perturbations in temperature responses; sensitivity to nitrogen starvation; and an altered cell morphology resulting in larger and rounder cells, thus possessing a perturbed actin cytoskeleton (Vojtek et al., 1991). Since these phenotypes are not associated with Ras signalling, yeast CAP was thought to link temperature and nutritional signalling responses to changes in the actin cytoskeleton. CAP's ability to act through Ras signalling and bind adenylyl cyclase has not been demonstrated in higher eukaryotes, as only yeast *S. cerevisiae* and *S. pombe* have shown to be the case. The N- and C-termini of yeast CAP have different functions, thus mediating the different responses seen in *cap-* cells. The N-terminus was shown to be important for signalling through activated Ras (Gerst et al., 1991; Vojtek et al., 1991) and was further mapped to specific residues of N12, L13, and E28 (Shima et al., 1997). The C-terminus was demonstrated to be necessary for correct cell morphology and nutritional responses, and an actin binding domain was later identified in the terminal 27 amino acids (Gerst et al., 1991; Vojtek et al., 1991; Zelicof et al., 1996).

CAPs are conserved throughout evolution **(Figure 1)** as homologues exist in a wide-array of organisms such as pig, rat, mouse, human, *D. discoideum*, *Candida albicans*, *Arabidopsis*, cotton, *Xenopus*, and *Drosophila,* suggesting a fundamental function for CAPs in regulating actin dynamics (Bahn and Sundstrom, 2001; Barrero et al., 2002; Baum et al., 2000; Gieselmann and Mann, 1992; Gottwald et al., 1996; Kawai et al., 1998; KhosrowShahian et al., 2002; Vojtek and Cooper, 1993; Yu et al., 1994; Zelicof et al., 1993). Furthermore, mammals contain two homologues of yeast CAP, CAP1 and CAP2, but this is likely to extend to other vertebrates, as a *Xenopus* CAP2 homologue has been recently cloned and characterized (unpublished results, KhosrowShahian, F., Mottillo, E., Hubberstey, A. V. and Crawford, M. J.).

All CAPs have conserved functional domains in addition to the previously described actin binding domain and the adenylyl cyclase binding domain (Hubberstey and Mottillo, 2002) **(Figure 2)**. Though adenylyl cyclase binding does not exist beyond yeast, this domain is structurally conserved and reveals a heptad repeat region, otherwise known as a coiled-coil domain. Coiled-coils consist of two to five amphipathic alpha-helices that wind around one another, forming a supercoil, which acts

**Figure 1. CAP phylogenetic tree demonstrating the relationships between all CAPs.** Amino acid sequences of all 14 CAPs were aligned to construct a phylogenetic tree using TreeView software. The values next to the branches indicate the % amino acid identities between different CAPs.

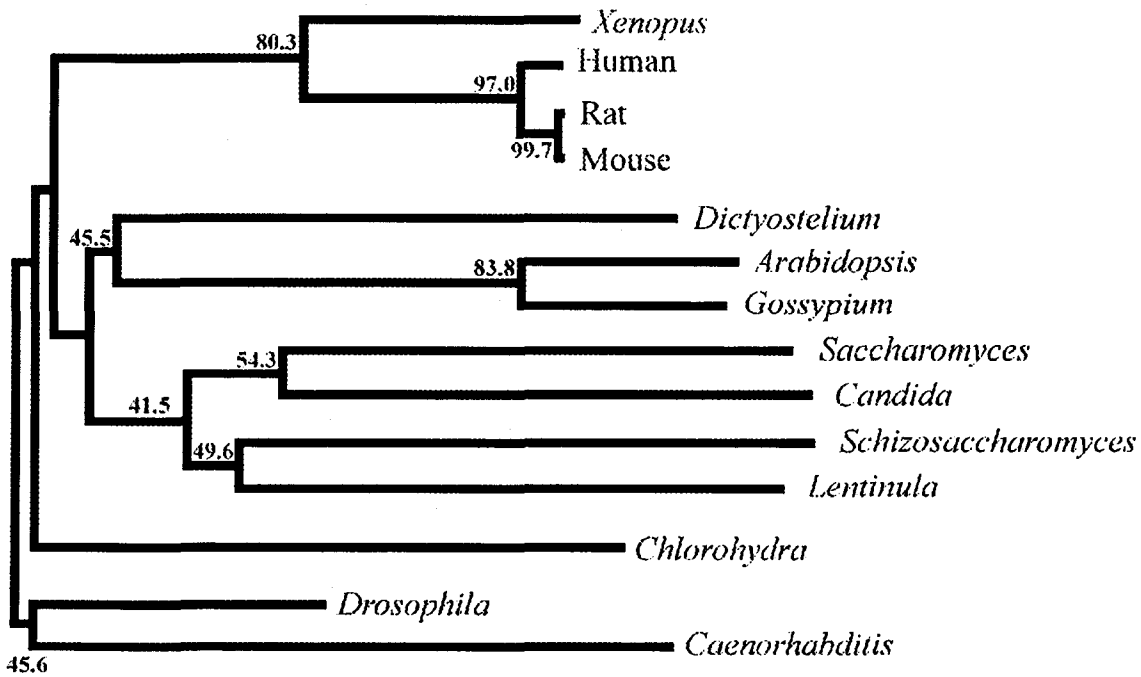



**Figure 1.**

**Figure 2. General conserved structural domains of the Cyclase Associated Proteins (CAPs).** The N-terminus of all CAPs contain a highly conserved sequence of amino acids; alanine, arginine and glutamic acid in repeat, and has been denoted the RLE motif. This RLE motif makes up the larger coiled-coil of CAPs. In yeast adenylyl cyclase binding occurs here, and is also the site of the N-terminal dimerization domain of all CAPs. CAPs also contain a central poly-proline region (PRO), a second dimerization domain localized to the C-terminus (Di) and a C-terminal proximal G-actin binding domain (Act).

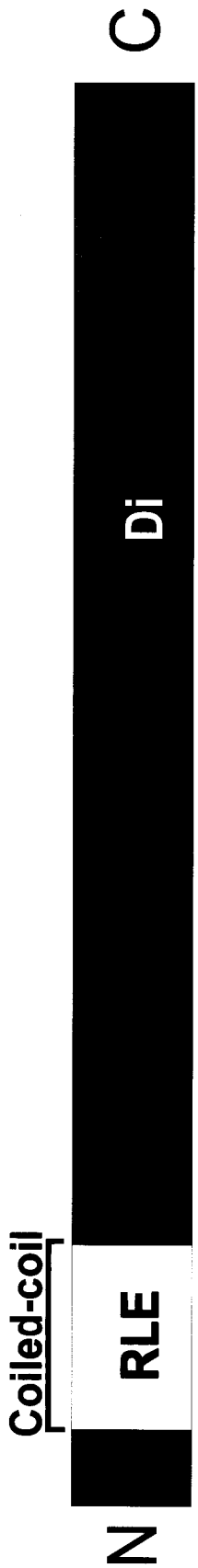



Figure 2.

as a protein folding motif important for mediating protein-protein interactions, such as oligomerization (Burkhard et al., 2001). Not surprisingly, one of the dimerization domains has been mapped in this vicinity, and a second dimerization domain exists within the C-terminus (Hubberstey et al., 1996). Therefore, CAPs are capable of forming multimeric complexes and have also been shown to form oligomers in the size range of 440 - 669 kDa (Moriyama and Yahara, 2002). Another functional domain is the central poly-proline region of CAP, which has been shown to bind SH3 domains of other proteins. This domain binds yeast actin binding protein 1 (Abp1p) (Freeman et al., 1996).

CAPs have been observed to have a multitude of cellular functions in different systems. Firstly, CAPs are thought to play a role in cell elongation, as a cotton homologue of CAP was isolated and shown to be highly expressed during fibre elongation (Kawai et al., 1998). CAPs have also been tied to a role in cell elongation as a CAP homologue in *Arabadopsis*, AtCAP1, was recently cloned and characterized. Overexpression of AtCAP1 reduced F-actin filament formation and generated phenotypes of reduced leaf and petiole size which was attributed to a decrease in cell size and number (Barrero et al., 2002). CAPs have also been shown to have a fundamental purpose in development. A *Drosophila* homologue of CAP (acu/capulet) was first isolated through its ability to induce changes in the morphogenetic furrow of the eye disc. Acu was shown to be required for cell shape changes during this differentiation process, via its ability to prevent actin polymerization. Acu was also demonstrated to have a role in controlling the steps that lead to neuronal differentiation within the eye disc (Benlali et al., 2000). *Capulet* was simultaneously isolated through a screen for genes required in oocyte polarity. A similar actin function for capulet was identified as its accumulation within the oocyte inhibited actin polymerization. Mutants for *capulet* had a disrupted oocyte polarity, as an improper distribution of mRNA was evident, and this polarity function of CAP was also evident within yeast (Baum et al., 2000). A further study on *capulet* identified it as a major player in the spatial control of the actin cytoskeleton. Capulet prevented the formation of actin filaments on the apical side of epithelial cells, and its effects were modulated through the Abl tyrosine kinase, though biochemical proof of this signalling pathway awaits (Baum and Perrimon, 2001). Further proof for the function of CAPs during development is evident in the *Xenopus* homologue, xCAP1. xCAP1 was shown to be

developmentally regulated as it is expressed as a maternal transcript and was subsequently upregulated prior to gastrulation and through to the neurula stages (KhosrowShahian et al., 2002).

An role for CAPs during vesicle trafficking and endocytosis/exocytosis has been apparent for some time. The first indication that CAPs may have a role in endocytosis was demonstrated in yeast by the ability of the homologue of the neuronal v-SNARE synaptobrevin, SNC1, to complement phenotypes associated with deletion of the C-terminus of yeast CAP. Specifically these phenotypes were nutritional and temperature responses, and abnormal cell size, while Ras responsiveness was not restored (Gerst et al., 1992). Yeast CAP (Srv2) was genetically implicated in the endocytic process through a screen for mutants deficient for endocytosis. A recessive negative form of yeast CAP that was unable to internalize pheromone was identified, as the *cap-* strain was not deficient for endocytosis. Also, End4/Sla2 was shown to have a function in endocytosis that is redundant with that of Srv2 and Abp1. An End4 mutant missing its coiled coil domain in both an Srv2 and Abp1 deletion background, was unable to internalize labeled pheromone (Wesp et al., 1997). This study suggests that a complex of proteins consisting of End4, Rvs167, Abp1 and CAP work in concert to mediate the endocytic function of actin. Yeast Abp1 and CAP have been biochemically linked (Freeman et al., 1996), and a characterized role for Abp1 in endocytosis is apparent in both yeast and mammals (Kessels et al., 2001; Wesp et al., 1997). These results imply a possible role for mammalian CAPs in endocytosis, but whether or not this interaction is conserved in mammals, remains to be resolved. In characterizing the *Dictyostelium* homologue of CAP, Noegel *et al.* also discovered a role for CAP in endocytosis. Mutants deficient for CAP demonstrated no disruptions during phagocytosis, but had a substantially reduced fluid phase uptake (Noegel et al., 1999). More recently, a proteomic screen of proteins involved in cell polarity in *S. cerevisiae,* revealed that CAP can interact with other proteins players of endocytosis. Two-hybrid interaction screens revealed that yeast CAP can interact with Rvs167 (Lombardi and Riezman, 2001), a regulator of endocytosis and the actin cytoskeleton; and Sla1p, implicated in coupling the endocytic machinery to the actin cytoskeleton (Warren et al., 2002). Therefore, it is apparent that CAPs in general have an implied function in vesicle trafficking. The studies that identified a role for the CAPs in vesicle trafficking are model systems for vertebrate cellular

function, in addition, the highly conserved function of all CAP merits investigation of the role that mammalian CAPs may play.

## Role of the Actin Cytoskeleton During Vesicle Trafficking

### Endocytosis

Endocytosis is the active process by which cells internalize material such as proteins and lipids from their extracellular surroundings (**Figure 3**). One of the more well studied endocytosis pathways is clathrin-mediated endocytosis, which is initiated by the recruitment of the protein clathrin from the cytoplasm to defined sites on the plasma membrane. Clathrin monomers (consisting of a light and heavy chain) then assemble into a lattice of hexagons and pentagons in the shape of cage-like structure termed a clathrin-coated pit (CCP), followed by cargo recruitment. Detachment of the developed clathrin coated vesicle occurs, followed by transport of the vesicle into the cytoplasm (McPherson, 2002; Robinson et al., 1996). An important player in the late stages of clathrin-coated vesicle (CCV) formation is the large GTPase dynamin which is actively recruited to coated pits. It is thought that in the unbound state dynamin is found in the clathrin lattice, and upon GTP binding, the dynamins form a ring around the invaginated plasma membrane. Upon GTP hydrolysis, dynamins create a force that mediates membrane fission and pinching off of the new vesicle (Altschuler et al., 1998; Urrutia et al., 1997). Another important aspect of vesicle formation is the inclusion of targeting proteins within vesicles, termed SNARE (soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor) proteins, named so on based their ability to bind SNAP, a major component of the membrane fusion apparatus (Sollner et al., 1993). SNAREs are thought to be necessary for membrane targeting and will be further addressed in the section dealing with exocytosis.

In yeast, endocytosis and the actin cytoskeleton have been linked through genetic studies. Mutations in genes required for receptor-mediated endocytosis also result in perturbations of the actin cytoskeleton (Geli and Riezman, 1998; Munn et al., 1995). Additionally, use of actin depolymerizing drugs in yeast disrupts the internalization steps of endocytosis (Munn, 2001). The role for the

**Figure 3. Endocytic and exocytic events within the synapse.** A synaptic bouton is illustrated, showing the endocytic (green) and exocytic (yellow) processes. Actin filaments are illustrated in red, where they function as a scaffold for vesicles at the reserve pool (1), and are though to function in tracking vesicles to the active zone (2) (Doussau and Augustine, 2000) where they dock with the plasma membrane. Prior to exocytosis, actin acts to inhibit the fusion of vesicles (3), where its removal is thought to be required in order for fusion to occur (Doussau and Augustine, 2000). The vesicle fusion reaction is regulated by the SNARE proteins, but $Ca^{2+}$ is the actual trigger for fusion and the subsequent release of neurotransmitter (4) (Brunger, 2000). Fused vesicles are then endocytosed, in which the actin cytoskeleton is thought to play a facilitative role (5) in addition with receptor-mediated endocytosis (6) (Qualmann et al., 2000; Schafer, 2002). Vesicles are then pinched off, in which dynamin plays a major part (7) (Altschuler et al., 1998; Urrutia et al., 1997), and are targeted to endosomes for recycling (light blue; 8) or back to the golgi ( grey; 9). In addition, vesicles that have been endocytosed following exocytosis, can be targeted back to the reserve pool (10) (Shupliakov et al., 2002). Intracellular traffic also occurs between endosomes and golgi (11). Figure adapted from Lin and Scheller (2000).

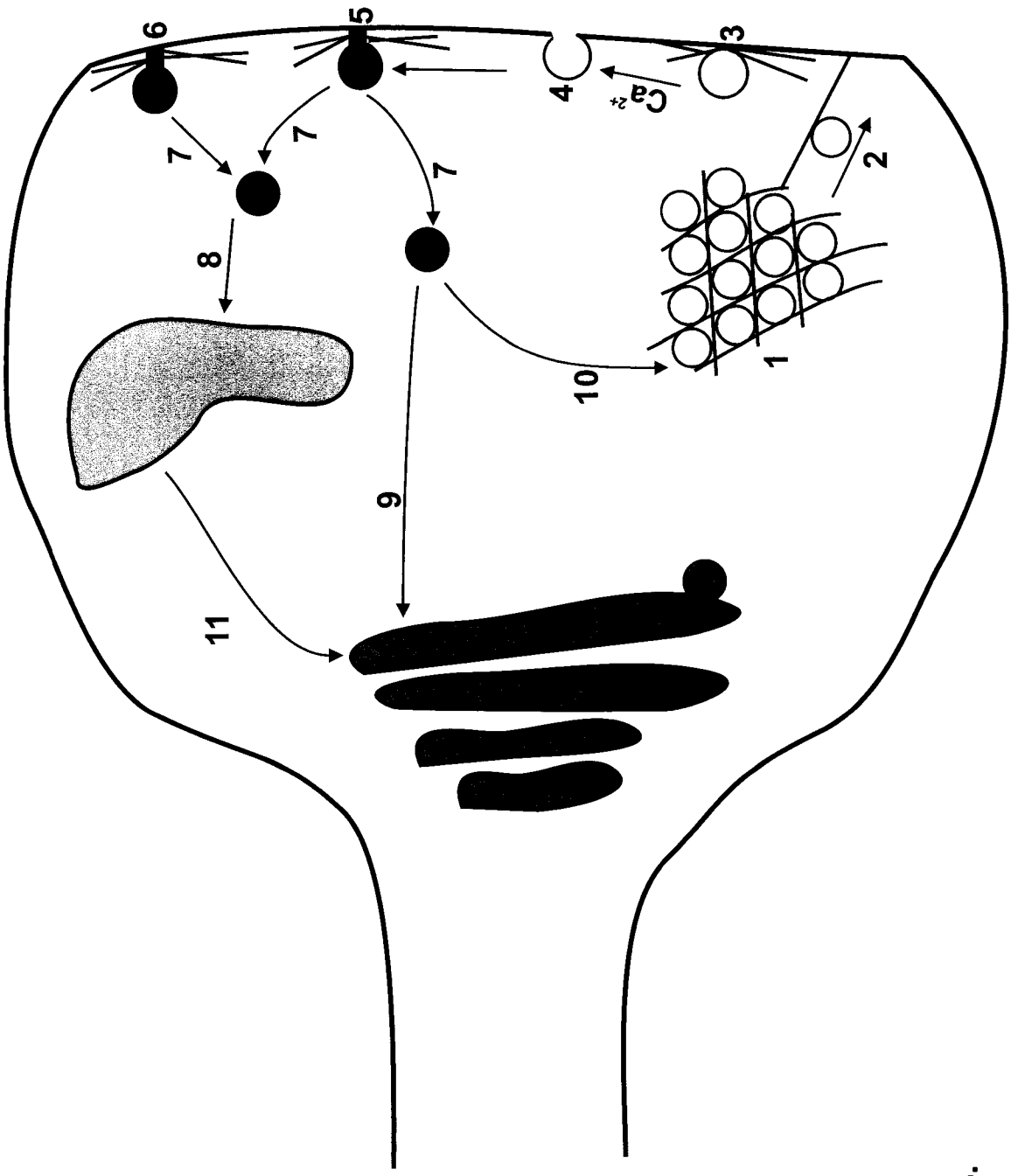



Figure 3.

actin cytoskeleton during endocytosis in mammalian cells is not so clear; few genetic links exist, and the use of actin depolymerizing drugs disrupts endocytosis depending on the assay and cell type used (Geli and Riezman, 1998). More recently, the use of actin perturbing drugs has revealed that the actin cytoskeleton plays more of a facilitative role in receptor-mediated endocytosis (**Figure 3**), but does not inhibit the formation of coated vesicles in all cases tested (Schafer, 2002).

One molecular link in mammals between the actin cytoskeleton and endocytosis involves the protein mammalian Abp1 (mAbp1). As mentioned earlier, in yeast Abp1 and CAP interact with each other in concert with other proteins to mediate the endocytic function of actin. mAbp1 binds filamentous actin via its two actin binding domains consisting of an actin depolymerization factor - homology (ADF-H) domain and also a novel actin binding motif (Kessels et al., 2000). Immunocytochemical studies support mAbp1's F-actin binding properties, as it localizes to the cortical actin cytoskeleton, and furthermore can relocalize to areas of high actin turnover at the leading edges of migrating cells (Kessels et al., 2000). mAbp1 has been linked to endocytosis via dynamin, as *in vivo* studies demonstrate a relevance for this interaction, and is dependent upon mAbp1's SH3 domain (Kessels et al., 2001). The function of mAbp1 in receptor-mediated endocytosis was demonstrated by a decrease in the uptake of labeled transferrin upon overexpression of its SH3 domain. This decrease in uptake was abolished upon overexpression of dynamin, thus strengthening the relevance of the interaction, and mAbp1 as a link between the actin cytoskeleton and endocytosis (Kessels et al., 2001). Another molecular link in mammalian systems involves the Huntington Interacting Protein 1 (HIP1) and HIP-Related (HIP1R), mammalian orthologues of yeast Sla2p (McPherson, 2002). As mentioned, Sla2p and yeast CAP have been functionally associated, thus is another example of a protein that provides an intimate link between CAPs and endocytosis. Both HIP proteins are associated with clathrin coated vesicles and clathrin coated pits, and function to facilitate the assembly of clathrin structures (McPherson, 2002). The HIPs functionally link the cytoskeleton to clathrin coats by their ability to bind F-actin and coats simultaneously. The formation of this actin-clathrin complex through the HIPs, is thought to allow the recruitment and assembly of clathrin at the plasma membrane. Currently no evidence exists for an association between mammalian CAPs and the HIPs

or mAbp1; thus, studies that search for novel mammalian CAP binding partners will be essential in further understanding their function.

The actin cytoskeleton could have numerous different functions during endocytosis, each one occurring at different stages of the endocytic process (Qualmann et al., 2000; Schafer, 2002). Firstly, the actin cytoskeleton may act as a scaffold for the numerous protein players involved in endocytosis and its rearrangement would elicit their function. A possible second role for the actin cytoskeleton is in the invagination of plasma membrane, wherein the actin cytoskeleton would facilitate or support this process. Thirdly, the cortical actin cytoskeleton may act as a barrier at the plasma membrane, thus in this case it would be inhibitory to the process and its removal would be a prerequisite for the invagination of vesicles. A fourth role for the cytoskeleton, is during the membrane fission which results in the liberation of vesicles from the plasma membrane. In this step, the polymerization of actin at the neck of the vesicle would provide the force needed to detach it. A final role for the actin cytoskeleton exists in the movement of endocytic vesicles through the cytoplasm, wherein the polymerization of actin results in the formation of a comet tail, thus providing the force necessary for this movement. The propulsion properties of actin polymerization is clearly evident through the study of the pathogen *Listeria monocytogenes* which uses the cytoskeleton to propel itself through the cytoplasm (Portnoy et al., 2002). Though these numerous associations for the actin cytoskeleton during the different steps of endocytosis may seem convoluted, this can be attributed to the specialized roles that actin may play in different cell areas or types.

**Exocytosis**

Exocytosis is the term used to describe the fusion of a secretory vesicle with the plasma membrane and the subsequent release of its contents into the extracellular area (**Figure 3**). This process is either constitutive, or is highly regulated, were the secretion of cellular messengers such as neurotransmitters, cytokines, and hormones occurs only in response to extracellular signals. The most widely studied form of regulated exocytosis is calcium mediated neurotransmitter release, which occurs in synapses, and is the manner in which the nervous systems conducts cell to cell

communications. The synaptic vesicle cycle first occurs with the synthesis of lipids and membrane proteins on the endoplasmic reticulum (ER) and their subsequent modification at the golgi apparatus. Mature synaptic vesicles are then filled with neurotransmitter at the nerve terminal and are targeted to the presynaptic area where regulated exocytosis occurs, otherwise called the active zone. Once near the active zone, vesicles can become either part of the reserve pool which is bound to cytoskeletal elements, or can become docked to the plasma membrane where they become part of the readily releasable pool (**Figure 3**) (Lin and Scheller, 2000). The subsequent steps all occur within the presynaptic terminal (Sudhof, 1995). Firstly, docking involves contact between the vesicle and plasma membrane, and is followed by a rate-limiting priming step that makes the vesicles competent to $Ca^{2+}$ mediated exocytosis. Once vesicles are primed, they can be stimulated to fuse by the $Ca^{2+}$ spike that occurs during an action potential. Once the synaptic vesicles have emptied their contents, they are internalized through a clathrin-mediated endocytic process. The CCV then shed their coat and are recycled by endosome fusion (**Figure 3**). New synaptic vesicles can now form from the budding of endosomes, and the process repeats itself.

The essential components for vesicle fusion, which are conserved from yeast to humans, can be grouped into three classes of protein (Jahn and Sudhof, 1999). The first class consists of the membrane bound SNAREs which can be further divided into two classes consisting of v-SNAREs (within transport vesicles) and t-SNAREs (localized to the target membrane for the vesicle) (Sollner et al., 1993). The second class of proteins consist of Munc18 homologues which bind to t-SNAREs and regulate their ability to bind v-SNAREs. The third class is made up of the Rab proteins which are small GTPases that regulate vesicular traffic through their ability to bind the SNAREs and Munc18 homologues.

The SNARE hypothesis is an idea formulated to describe the fusion of vesicles with the plasma membrane through the cycling of assembly/disassembly of SNARE complexes. Though the initial hypothesis included the idea that the specificity of v-SNARE/t-SNARE interactions would modulate the specificity of membrane trafficking and targeting (Sollner et al., 1993), to date this has not been proven. The most frequently studied SNAREs are those involved in synaptic vesicle

exocytosis and they represent the minimal machinery needed for fusion to occur. They consist of the t-SNAREs syntaxin 1a and SNAP-25 (membrane bound due to its palmitoylation), and the v-SNARE synaptobrevin (VAMP), wherein syntaxin 1a and synaptobrevin contain C-terminal transmembrane domains. The neuronal SNAREs share homology within a region termed the SNARE motif which consists of a heptad repeat or coiled-coil domain. Through these SNARE motifs, the proteins can form a highly stable tetramer termed the SNARE complex, wherein syntaxin 1a and synaptobrevin contribute one SNARE motif and SNAP-25 contributes two (Jahn and Sudhof, 1999). The current SNARE hypothesis (Brunger, 2000) initiates prior to docking, with syntaxin bound to its negative regulator nSec1, and synaptobrevin likely bound to synaptophysin. Interestingly, as touched upon in the CAP discussion, the yeast homologue of synaptobrevin, SNC1, is able to complement certain *cap*-phenotypes. During docking, the syntaxin-nSec1 and synaptobrevin-synaptophysin interactions are broken, and synaptobrevin is then able to bind syntaxin and SNAP-25. In the priming step, the SNARE complex becomes competent to respond to an increase in intracellular $Ca^{2+}$, likely through a calcium sensor protein such as synaptotagmin. During vesicle recycling SNAP (soluble NSF-attachment protein - not to be confused with SNAP-25) and the ATPase NSF, bind the SNARE complex and dissociate it upon ATP hydrolysis.

The role of the actin cytoskeleton in exocytosis/neurotransmitter release is not as well studied as that of endocytosis and fewer molecular links exist. As is the case with endocytosis, different roles for the actin cytoskeleton have been postulated depending on the cell and assay type used. In bovine chromaffin cells (of endocrine type) the role of the actin cytoskeleton has been clearly established during the secretory process (Trifaro et al., 2000). In these endocrine cells, a ring of filamentous actin exists beneath the plasma membrane acting as a barrier to release, thus trapping secretory granules and preventing their fusion with the plasma membrane. Upon stimulation the cortical cytoskeleton becomes depolymerized, and this facilitates the docking and fusion of vesicles (Doussau and Augustine, 2000; Trifaro et al., 2000). Additionally, actin filaments are highly concentrated in presynaptic terminals (Morales et al., 2000) and different roles have been postulated for the cytoskeleton during presynaptic function. Two pools of neurotransmitter vesicles exist within the

synaptic terminal; a readily releasable pool (RRP) at the active zone which is primed for fusion, and a reserve pool, situated above the RRP (Lin and Scheller, 2000). A network of actin filaments along with cytoskeletal proteins exists at the reserve pool and its role is to act as a structure that maintains these vesicles (**Figure 3**) (Doussau and Augustine, 2000; Lin and Scheller, 2000). During steady neurotransmitter release a role for the actin cytoskeleton has been proposed in trafficking vesicles from the reserve pool to the RRP. Likewise, actin may serve as a track for vesicles to move between the two pools, or another idea is that depolymerization of actin filaments results in the release of reserve vesicles (reviewed in Doussau and Augustine, 2000).

Interestingly, recent studies suggest an inhibitory role for the actin cytoskeleton in neurotransmitter release (**Figure 3**). Ohnishi *et al.* (2001) demonstrated that the use of actin depolymerizing drugs facilitated the release of $Ca^{2+}$ dependent neurotransmitter in the rat neuronal cell line PC12 and cerebrellar granule cells. Morales *et al.* used biochemical and electrophysiological studies to elucidate the step at which the inhibitory role occurred. Miniatures excitatory postsynaptic currents (mEPSCs), which represent the fusion of a single synaptic vesicle, were used to measure neurotransmitter release in primary hippocampal neurons. The use of the F-actin depolymerizing drug latrunculin A (Lat A), led to an enhancement of neurotransmitter release independent of $Ca^{2+}$, while cytochalasin D did not (Morales et al., 2000). The authors propose that the actin cytoskeleton within the active zone acts as a structural component, or possibly a scaffold prior to the last stage of vesicle priming, and thus would need to by reorganized prior to the transition to the final stage.

One molecular link between the reserve pool of vesicles and the actin cytoskeleton is the synapsin family of proteins. These proteins demonstrate different actin binding properties, such as the ability to bind actin monomers and nucleate them, thus facilitating F-actin formation, and the ability to bind F-actin filaments and bundle them (reviewed in Doussau and Augustine, 2000). Functional studies such as the microinjection of synapsin antibodies or knockout mice of synapsin, disrupts the reserve pool, thereby inhibiting the release of subsequent neurotransmitter and demonstrate synapsins as regulators of the reserve pool (Doussau and Augustine, 2000).

**Role of the Actin Cytoskeleton During Neuronal Plasticity**

During brain development synaptic wiring occurs, wherein neurons extend axons and dendrites, thus making new synaptic connections. This active process is called neurite outgrowth and involves the actin cytoskeleton (Luo, 2002). The leading edge of a neurite contains an actin rich structure called a growth cone, which is composed of filopodia, and meshworks of actin termed lamellipodia situated between filopodia. The finger shaped filopodia are composed of F-actin bundles and are constantly extending due to the addition of actin monomers at the leading edge. At the trailing edge F-actin filaments are broken down and allows the growth cone to move forward. Simultaneously, at the veil shaped lamellipods, a net F-actin flow away from the leading edge causes filopodia and lamellipodia to retract. The lamelipodia are then built forward where they meets the tip of filopodia. New filopodia then extend from the leading edge and the process repeats itself. Thus, the net rate of growth is controlled by independently adjusting the rates of actin polymerization and F-actin flow (Luo, 2002). The regulation of growth cone formation is also an important feature of axon guidance. Well after development, morphological changes in neurons continue, such as growth and pruning of neuronal connections, the addition or removal of synapses, and changes in synaptic size and shape, all of which are thought to be associated with learning and memory formation. More importantly, these changes are dependent on the dynamics of the actin cytoskeleton (Luo, 2002).

One of the fundamental questions in neuroscience is how the nervous system modifies itself to create memories and facilitate learning. Synaptic plasticity is the term used to describe this apparent ability of the nervous system to change the strength of synaptic connections (Yuste and Bonhoeffer, 2001). One of the more widely studied forms of plasticity is Long Term Potentiation (LTP) which is a prolonged enhancement of synaptic strength, and is argued to be the mechanism behind learning and memory formation (Malenka and Nicoll, 1999). Specifically, LTP occurs within the hippocampus during strong periods of stimulation, when a synaptic input is activated at the same time that depolarization is occurring in the postsynaptic cell (Malenka and Nicoll, 1999). The site of LTP has been pinpointed to areas where the majority of excitatory synaptic inputs occur: between presynaptic terminal of axons and the postsynaptic terminals of dendrites called dendritic spines

(Yuste et al., 2000). Dendritic spines function to integrate the multiple input signals that are received from presynaptic terminals of innervating axons. Spines are thought to act as separate structures that compartmentalize and restrict calcium and proteins involved in synaptic function. Consequently, signals received on one spine do not spread to another on the same dendrite allowing for each synaptic contact within a neuron to be adjusted separately (Yuste et al., 2000). This idea is thought to be the basis for the relationship between LTP function and learning and memory formation.

Research in the last ten years has demonstrated that the chemical and structural modifications that occur within dendritic spines are fundamental mechanisms behind LTP, thus learning and memory formation (reviewed in Yuste and Bonhoeffer, 2001). Interestingly, actin is highly enriched within dendritic spines (Smart and Halpain, 2000) and recent research has focussed on studying if the structural modifications within them are due to the actin cytoskeleton. A role for the actin cytoskeleton during LTP was deduced through the use of actin depolymerizing drugs on hippocampal mouse slices, where maintenance of LTP within slices was impaired. The effects of Cytochalasin B were reversible, as washing returned the slices to a state where LTP could occur. Actin depolymerizing drugs act on dynamic filaments, and the fact that removal of Cytochalasin B returned actin to a dynamic state, suggests that actin dynamics are essential to synaptic plasticity (Krucker et al., 2000). In another study the use of the depolymerization drug Lat A in cultured hippocampal neurons was used to study the role of actin in synapse stability and formation. The authors demonstrated that F-actin is pertinent to the development and maintenance of synapses, as young hippocampal neurons treated with the drug demonstrated a complete loss of synapses. The use of Lat A had no effect on mature neurons, suggesting that a stable F-actin cytoskeleton is critical to the maintenance of mature synapses (Zhang and Benson, 2001). An elegant study by Colicos *et al.* allowed a real-time view of the pre- and postsynaptic changes that occur during LTP. They utilized the property that the electrical conductivity of silicon is enhanced when exposed to light. By layering hippocampal neurons on a silicon wafer and illuminating a single neuron, this resulted in the activation of that single neuron. The advantages of this technique is that it is noninvasive, and the effects on a single neuron can be studied. By using the fluorescent reporter gene *GFP* and its derivatives, the authors revealed that the elicitation of LTP

resulted in the reorganization of actin, as presynaptic actin advanced toward the synapse, and postsynaptic actin moved away from the stimulated synaptic terminal. Furthermore, these actin dependent changes during LTP result in what seemed to be the formation of new active pre- and postsynaptic connections (Colicos et al., 2001). Further studies are needed to discern if these actin-mediated synaptic changes result in synaptic plasticity.

A study by Meng *et al.* used a LIM kinase 1 (LIMK-1) knockout mouse to study its regulatory role in neuronal function. Abnormal expression of LIMK-1 along with other proteins results in the human developmental disorder Williams syndrome, which is characterized by mental retardation and deficits in visuospatial cognition. LIMK is an important regulator of actin dynamics via its ability to inactivate the actin binding protein cofilin. Cofilin is an important player in binding G-actin and severing F-actin, thereby resulting in the treadmilling of filaments (reviewed in Bamburg, 1999; Bamburg et al., 1999). Levels of activated cofilin were substantially higher in the brain of LIMK-1 knockout mice, and the distribution of actin filaments in dendritic spines and branches was significantly altered, likely due to increased activity of cofilin. The knockout mice also manifested abnormalities in dendritic spine morphology and in synaptic function, and more interestingly demonstrated an enhancement in LTP. The mice also showed alterations in fear responses and spatial learning suggesting a role for LIMK-1 in behaviour. These results suggest that LIMK-1 is involved in spine morphogenesis and synaptic plasticity through its regulation of the actin cytoskeleton (Meng et al., 2002). Recently, the human CAPs have been biochemically linked to cofilin, likely aiding in the turnover of actin filaments (Moriyama and Yahara, 2002), and suggestive in that they too may play a role in neuronal plasticity.

**Signalling to the Actin Cytoskeleton**

An important aspect of actin is the signalling cascades that occur following the binding of a morphogen onto the cell surface, resulting in morphological changes of the cytoskeleton. Signalling to the actin cytoskeleton in mammalian cells is controlled by the Rho family of GTPases which include Rho, Rac and Cdc42. GTPases are small proteins that act as molecular switches and are involved in a wide array of cellular functions (reviewed in Matozaki et al., 2000). When in the GTP-bound state

GTPases are active, while when bound to GDP they are inactive. When extracellular signals are received, a GEF (Guanine nucleotide Exchange Factor) facilitates the exchange of GDP for GTP, activating the GTPase, and causing a conformational change. The active GTPase can now bind to downstream effectors and activate them, hence eliciting a signal transduction cascade. Conversely, GAPs (GTPase Activating Proteins) are negative regulators as they hydrolyse bound GTP to GDP, therefore inactivating the GTPase and the signalling cascade. Studies from fibroblasts have revealed that each member of the Rho family of GTPases act in response to different stimuli and control distinct morphological changes, but also act upon each other through a hierarchy of Cdc42 → Ras → Rho (Hall, 1998; Schmidt and Hall, 1998). Rho can illicit the formation of actin stress fibres and focal adhesions, acting in response to fetal calf serum and lysophosphatidic acid. Rac induces lamellipodia formation and membrane ruffles, and acts in response to different growth factors. Cdc42 is responsive to bradykinin and controls the formation of filopodia.

Though downstream effectors of the Rho family have been characterized, the full sequence of each cascade have yet to be determined. One fairly well characterized cascade is that of the Rho GTPase (Matozaki et al., 2000). ROCK is a serine/threonine kinase that is a downstream effector of Rho since it is stimulated by active Rho-GTP. Active ROCK can phosphorylate numerous targets such as the myosin light chain phosphatase, thereby inhibiting it, and leading to an induction of smooth muscle contraction. ROCK also acts on another pathway consisting of LIMK, wherein LIMK is phosphorylated/activated, and subsequently acts to phosphorylate and inactivate cofilin. Another effector of Rho is mDia which acts to bind profilin and control actin polymerization. Cdc42 controls the *de novo* polymerization of actin filaments through its downstream effector N-WASP (Luo, 2002). Active Cdc42 along with the lipid messenger $PIP_2$, can bind to N-WASP and abolish its auto-inhibitory conformation. Active N-WASP can bind to the ARP2/3 complex and stimulate the formation of new barbed ends via the nucleation of actin monomers (reviewed in Welch and Mullins, 2002). Additionally, Cdc42 can activate the serine/threonine kinase effector PAK, which phosphorylates LIMK, leading to its activation. Interestingly, the Rac GTPases also act through PAK. Rac can also activate the ARP2/3 complex, but through a different pathway. Active Rac turns on the effector IRSp53

activating SCAR/WAVE, SCAR/WAVE then stimulates ARP2/3 to perform its actin nucleation tasks. Thus, it is apparent that there is significant crosstalk between the Rho family of GTPases.

Members of the Rho family have also been shown to be activators/regulators of numerous actin-dependent cellular processes. The Arf and Rab family of GTPases control vesicle trafficking, specifically the budding and targeting of vesicles, respectively (Chavrier and Goud, 1999). Intriguingly, Rho family members are also implicated in endocytic traffic (Ellis and Mellor, 2000). Firstly, Rac and Rho function in clathrin-mediated endocytosis, as activated forms block the internalization of transferrin. This idea agrees with the recent implication in endocytosis for the F-actin binding protein HIP. Cdc42 and Rac mediate the formation of membrane protrusions that occur during phagocytosis such as the engulfment of bacteria. Rho is also implicated in phagocytosis, specifically during type II, wherein the particle to be phagocytosed enters into actin-lined invaginations in the plasma membrane (Ellis and Mellor, 2000). Additionally, the Rho GTPases RhoD and B are involved in endosomal sorting, while Cdc42 regulates endocytic and secretory pathways at the basolateral membrane in polarized cells. The Rho family has also been implicated in $Ca^{2+}$ mediated neurotransmitter release. Doussau *et al.* (2000) revealed that Rac1 fractionates to synaptic vesicles and used activators/inhibitors to elucidate that Rac1 regulates a late stage in neurotransmitter release (Doussau et al., 2000). The step of Rac1 function was further demonstrated to occur after vesicle docking and during fusion competence (Humeau et al., 2002). Rho, Rac and Cdc42 also function in neurite outgrowth, mostly acting through different effectors. Rac and Cdc42 act to promote neurite outgrowth (mainly through PAK), while Rho acts through its effector ROCK to promote growth cone collapse (Nikolic, 2002). A neuronal function closely related to growth cone formation that the Rho family is implicated in, is axon guidance. Similarly, axon attraction is mediated through Cdc42 and Rac, while Rho acts in an antagonistic manner (Patel and Van Vactor, 2002). Rac and Rho are also involved in the maintenance of dendritic spines and branches, wherein Rac controls the maintenance of spine density, and Rho through its effector ROCK is involved in inhibiting the formation of dendritic branches (Nakayama et al., 2000). This study suggests that the Rho GTPases may also have a role in signalling during synaptic plasticity.

**Thesis Objectives**

The major objective of the thesis was to further characterize the human Cyclase Associated Proteins CAP1 and CAP2. In particular, the objective was to identify new binding partners for hCAPs that may play a role in vesicle trafficking/neuronal function. In order to fulfill this aim, major experiments were performed that addressed four specific questions/objectives:

1. Identify new binding partners for hCAPs.
2. Characterize the interaction between hNecab1 and CAP2.
3. Determine the subcellular localization of CAP2 and Necab1.
4. What is the functional purpose of the Necab1-CAP2 interaction and how is CAP2 regulated?

A yeast two-hybrid screen of a human adult brain cDNA library was performed using hCAP2 as the bait protein. A novel protein with unknown function named Synaptotagmin Interacting Protein 1 (STIP1) was isolated that interacted with both hCAPs. A function in synaptic vesicle trafficking was suggested for STIP1 since it contains a predicted calcium binding motif, and since synaptotagmins are thought to be calcium sensors for calcium mediated exocytosis (Augustine, 2001). However, the putative interaction with Synaptotagmin 1 was shown to be physiologically insignificant, and so the protein has been subsequently re-named Neuronal Calcium Binding Protein 1 (Necab1) (Sugita et al., 2002; Sugita and Sudhof, 2000). Thus, Necab1 and the interaction was further characterized, as it implied a role for the hCAPs in vesicle trafficking (Chapter Two). Hereafter, this novel hCAP interacting protein will be referred to as Necab1 in this thesis. Necab1 is a 351 amino acid protein, containing a predicted single EF-hand calcium binding domain, and three repetitive coiled-coil domains. It is highly and preferentially expressed in human brain, with lower levels in kidney, trachea and lung. Further examination of expression within rat brain reveal that rat Necab1 and CAP2 have a similar distribution. *In vitro* binding studies demonstrate that both CAP1 and CAP2 interact with Necab1. Surprisingly, immunoprecipitation analysis reveals that only CAP2 can bind Necab1 *in vivo*. Yeast two-hybrid assays performed with deletion mutants and site directed mutagenesis reveal that the coiled-coil domains of both proteins are critical for CAP-Necab1 binding. The central coiled-coil

of Necab1 interacts with the amino proximal coiled-coil of CAP2 (termed the RLE motif). In mammalian cells, site-directed mutagenesis of the amino terminal coiled-coil of CAP2 (R10T, L11P) along with immunoprecipitation analysis has revealed that CAP2 (R10T, L11P) does not interact with Necab1 *in vivo*. Furthermore, it is shown that Necab1 is a protein capable of dimerization. A closer inspection of Necab1 reveals that it has a potential SNARE motif. It is also demonstrated that the neuronal t-SNARE syntaxin 1a interacts with Necab1 through its SNARE motif.

Fractionation studies were performed in order to identify the subcellular localization of CAP2 and Necab1 (Chapter Three). These studies reveal that endogenous Necab1 and CAP2 are mostly soluble cytosolic proteins, that show some localization to other compartments. The potential functional significance of the Necab1 - CAP2 interaction was also studied (Chapter Four). This was accomplished through functional immunoprecipitations involving Necab1 - CAP2 - actin complexes which demonstrated that CAP2 cannot bind actin when bound to Necab1. Actin polymerization assays demonstrate that CAP2 is a potent inhibitor of F-actin formation; but, Necab1 has no effect on this function. Necab1 itself may have an effect on polymerization, although a direct interaction between Necab1 and actin was not demonstrated. In order to determine if CAP2 was involved in a cell signalling cascade, and to understand how it is regulated, we performed *in vivo* phosphorylation assays which revealed that CAP2 is highly phosphorylated in the C-terminus. The significance of these functional assays, with relation to the interactions described, and future experiments, will be discussed in Chapter Five.

# CHAPTER TWO

## Characterization of Necab1: a Novel CAP Binding Partner

### Introduction

Protein interactions are the basis for all cellular processes that occur within a cell, and can be illustrated by the multitude of actin binding proteins which act hand in hand to rearrange the actin cytoskeleton. For example, yeast CAP is thought to function in concert with other proteins to mediate the function of the actin cytoskeleton during endocytosis (Wesp et al., 1997), and also during cell polarity development (Drees et al., 2001). Thus, teasing apart these protein complexes will be essential to assembling the puzzle of how actin functions during cellular processes. An additional example of the importance that protein interactions play in cellular processes is exemplified in the SNARE proteins, which mediate the release of neurotransmitter (Sudhof, 1995).

Another important aspect of neuronal function is $Ca^{2+}$ signalling, since it plays a principal regulatory role. An increase in intracellular $Ca^{2+}$ is the signal for the release of neurotransmitter between neurons of the brain. This signal is regulated by the $C_2$ class of calcium binding proteins such as synaptotagmins; likely the calcium sensors for vesicle fusion (Augustine, 2001; Sudhof, 2002). Usually the binding of $Ca^{2+}$ elicits a conformational change in the protein, allowing other proteins to interact with it. The second class of calcium binding proteins is the EF-hand motif family which consist of helix-loop-helix motifs, wherein the loop can co-ordinate a calcium ion. Usually calcium binding proteins of the EF-hand family contain an even pair of motifs that allow the proper co-ordination of the calcium ion. EF-hand proteins that contain only one motif demonstrate the ability to form homodimers in order to bind the calcium ion (Lewit-Bentley and Rety, 2000).

An aspect of neuronal function that has been ignored up until recently, is the role that actin may play in not only neurotransmitter release, but also synaptic plasticity. Further understanding the protein players involved in the reorganization of the actin cytoskeleton, and their putative binding partners within the brain, will allow a role for actin dynamics in neuronal function to be elucidated.

In this chapter, the isolation and characterization of a novel neuronal binding partner of the human CAPs named Neuronal Calcium Binding Protein 1 (Necab1) is described. We demonstrate

that Necab1 is highly and preferentially expressed in the brain. Further examination within the brain reveals that Necab1 has an expression pattern similar to that of CAP2. Through the use of *in vitro* and *in vivo* binding assays, Necab1 is shown to be a legitimate binding partner of CAP2. Further two-hybrid mapping using deletion mutants and immunoprecipitation analysis, along with site-directed mutagenesis on the CAP2 coiled-coil, demonstrate that the Necab1 - CAP2 interaction occurs through coiled-coil domains. It is also shown that Necab1 is a protein that can self-associate, likely to form dimers in a calcium-independent manner. Analysis and alignment of Necab1's central coiled-coil, reveals that it has homology with neuronal SNAREs. Binding studies demonstrate that Necab1 can also directly interact with the t-SNARE syntaxin 1a, and this interaction is mediated through syntaxin's Q-SNARE motif. The justification for Necab1's interactions and its similar brain expression pattern with CAP2 suggests an important neuronal role for Necab1. Furthermore, it suggests that Necab1 may be a modulator of CAP2 function within the brain.

## Materials and Methods

*Yeast Two-Hybrid Library Screen*

Full-length human CAP2 was cloned into the yeast two-hybrid expression vector pBTM116 that contains the LexA coding sequence under the control of the yeast *ADH1* promoter, and also the *TRP1* gene for selection, to create pLEX-CAP2. This in frame fusion with the LexA DNA binding domain was used as a bait to screen a human adult brain cDNA library (cloned into the activation domain plasmid pGAD10, Clontech). Briefly, the yeast L40 strain was first transformed with pLEX-hCAP2 and subsequently transformed with the brain two-hybrid cDNA library using lithium acetate method. Approximately $1\times10^6$ yeast transformants were screened for their ability to grow in the absence of histidine. Of these histidine prototroph transformants, ten yeast colonies were obtained and analyzed for their ability to turn blue in the presence of the colourimetric substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Of ten positive interacting clones, clone #5 (GAD10#5) demonstrated a positive interaction with both hCAP1 and hCAP2, and no ability to activate transcription by itself

(transactivation). In order to isolate library plasmid, yeast transformants were plated onto media which contained tryptophan that cured the bait plasmid (pLEX-CAP2). The loss of bait plasmid was confirmed by performing a β-gal assay on the cured yeast colonies. Library plasmids from cured transformants were subsequently isolated through membrane disruption of yeast with glass beads. Purified DNA plasmid was then subsequently electroporated into the *E. coli* strain DH10B. Clones were sequenced using dye-deoxy terminator reactions using either an ABI sequencing system (York University) or a Visible Genetic Long Read tower system using a Thermo Sequenase Cy 5.5 Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). Subsequent BLASTX search of clone GAD10#5 showed it had high homology to a partial human and full-length rat GenBank sequences named Neuronal Calcium Binding Protein 1 (Necab1) (GenBank AF 193756, AF 193755, Sugita and Sudhof, 2001).

*cDNA Cloning of Full-length Necab1*

GAD10#5 from the two-hybrid library screen encoded a clone of Necab1 missing the 5' end. In order to clone full length Necab1, we performed a BLASTN search of the Human Genome Project database. Upon analysis of the chromosomal sequence it was determined that 12 base pairs were missing from the 5' end. This analysis, along with the full length sequence of rat Necab1 (GenBank AF 193755), allowed us to PCR full length Necab1 from the human brain cDNA library using the synthesized forward (*#97*) and reverse (*#99*) primers containing Xho1 sites (see **Appendix A**). The PCR product was gel purified to remove any contaminating library plasmid, and was subsequently subcloned into the Xho1 site of pBlueSript/SK II$^+$ (Stratagene) to create pBS-Necab1. This clone was subsequently used to clone into other vectors.

*Molecular Cloning*

A Full-length Necab1 fragment with Xho1 sites (from pBS-Necab1) was used to clone into the Sal1 site of the His-Tag (pET-33b(+)), HA-tag (pCI-HA), and MYC-tag (pCI-MYC) vectors and the Xho1 site of GST vector (pGEX-KG) to create in frame fusion proteins. Polymerase chain reaction (PCR) was used

to generate VP16 fusions of full length hNecab1 and the appropriate deletion mutants using the primers listed in **Appendix A**. Primers were designed in such a way that both carboxyl and amino terminal deletion mutants had three variations of coiled-coil organization (See **Figure 9** for schematic diagrams), and were used as follows: full-length VP16-Necab1 primers *#125* and *130*; Necab1-N284 primers *#125* and *127*; Necab1-N250 primers *#125* and *128*; Necab1-N200 primers *#125* and *129;* Necab1-C156 primers *#136* and *130;* and Necab1-C100 primers *#138* and *130*. The LEX-Necab1 fusion was made by PCR of Necab1 using primers *#137* and *99*, and cloned into the Xho1 site of pBTM116. PCR was used to amplify *CAP2* from pCI-HA-CAP2 in a 100 µl reaction using Vent DNA polymerase (New England BioLabs) and forward (*#31*) and reverse (*#32*) primers designated in **Appendix A**. The PCR product was digested with EcoR1 and Xho1, and subsequently cloned into yeast two-hybrid expression vector pBTM116 (digested with EcoR1/Sal1) to create an in frame fusion between CAP2 and LEX. Cloning of GST-CAP2 and GST-CAP2-N304 fusion proteins was performed by PCR of fragments of full length CAP2 (primer *#123* and #32) and the N-terminal 912 nucleotides (primer *#123* and *#109*), respectively. This created 3' EcoR1 sites and 5' Xho1 sites that were used to clone into the pGEX-KG that was double digested with EcoR1/Xho1.

For cloning of the CAP2 coiled-coil mutant ($CAP2_{TPE}$) site directed mutagenesis was performed in a one step PCR using the forward primer 5'-TTGAATTCATGGCCAACATGCAGGGAC TGGTGGAAACACCGGAACGA GCTCTC-3' that contained nucleotide substitutions (underlined) to convert the first RLE motif to TPE (R10T, L11P) and the reverse primer LEX-CAP2-R (*#32* - **Appendix A**). This PCR product was subsequently used to clone into pCI-MYC and pBTM116. The Necab1 mutant containing the abolished central coiled-coil domain ($Necab1_{2CC}$) was created via an overlap extension mutagenic strategy, involving a two-step PCR reaction, with HA-Necab1 as a template. Forward 5'-AACCAGTGGATGACCCAGAAAAATAGACGCCAGAAATTAATTGAT-3' (*#153*) and reverse primers 5'-ATCAATTAATTTCTGGCGTCTATTTTTCTGGGTCATCCACTG GTT-3' (*#154*) were constructed in which both contained nucleotide substitutions (underlined) that changed two nonpolar residues in the second coiled-coil to polar residues (I218K, L221R) and also created an Hsp92 restriction site. Briefly, the first PCR reaction involved amplifying fragments that contained the

mutated primers, that is using the forward primer *#125* and reverse primer *#154* in one reaction, and forward primer *#153* and reverse *#130* in another. These PCR fragments are complimentary to each other in respect to the mutated sequence. Thus, a subsequent second PCR using these purified fragments as templates, in addition to the forward *#125* and reverse primer *#130* resulted in a full-length Necab1 containing the desired mutation. This final product was cloned into the Not1 site of pBTM116, and positive clones containing Necab1$_{2CC}$ were subsequently screened for the additional Hsp92 restriction site. All PCR products were amplified using High Fidelity PCR Supermix (Invitrogen Inc.) unless otherwise stated. Clones were subsequently sequenced as described previously.

*Yeast Two-Hybrid Interaction Assays*

For two-hybrid interaction assays, full length Necab1 or carboxyl and amino terminal deletion mutants were fused to the VP16 activation domain. CAP1, CAP2 and Necab1 were cloned into the LEX fusion pBTM116 along with the CAP2 coiled-coil mutant (CAP2$_{TPE}$). To quantify two-hybrid interactions, liquid β-galactosidase assays were performed. Yeast double transformants were grown in 4ml of Yc-ULTK +Amp liquid media to an optical density (OD) at 600 nm of greater than 0.500. 1.5 -3.0 mL of cells were pelleted and resuspended in Z buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1mM $MgSO_4$, 1 mM DTT), a drop of chloroform and 0.1% SDS was added to each tube and vortexed to rupture yeast cells. ONPG (o-Nitrophenyl β-D- galactopyranoside, Sigma) was added to a final concentration of 0.8 mg/ml, the time of addition was recorded and tubes were incubated at 30°C until a prominent yellow colour developed (24 hour maximum). Once the intensity of the yellow colour reached a maximum, the reaction was stopped by adding 500 µl of 1M sodium bicarbonate and the elapsed time was recorded. Tubes were then centrifuged for 1 minute to pellet cell debris, and 1.2 mL of supernatant was transferred to a cuvette and absorbance at 420 nm was measured and recorded. The amount of β-galactosidase activity was calculated using the equation below and the values of three to four trials per clone were averaged.

Lac Z activity = $1000 * OD_{420} / t * v * OD_{600}$

$OD_{420}$ = absorbance of 1 mL reaction at 420nm
t = time elapsed (minutes)
v = concentration factor
$OD_{600}$ = absorbance of 1 mL culture at 600nm

*RT-PCR*

Total RNA from brain, kidney, liver, heart, trachea, and lung (Clontech) were reverse transcribed into first strand cDNA using an 18-mer oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen Inc.) as per manufacturer's instructions. Briefly, 25 µl reactions containing 0.5 µg of oligo(dT), 1 µg RNA, 1 µl 10 mM dNTP mix, and sterile RNase free $ddH_2O$ were heated at 65°C for 5 min and chilled on ice. 4 µl of 5x first strand buffer, 2 µl 0.1 M DTT, and 1 µl RnaseOUT Recombinant Ribonuclease Inhibitor (40 units/µl l) (Life Technologies Inc.) were added and incubated for 2 min at 42°C prior to addition of 1 µl (200 units) of Superscript II and another incubation at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min. PCR products were amplified by using 1 µl of cDNA templates, 1 µl of each forward and reverse primer (100 pmol/µl), and 30 µl of PCR Supermix (Invitrogen Inc.). PCR reactions first involved a 5 min hot start at 94°C, then 35 cycles of 94°C for 45 s, 55-58°C for 45 s, 72°C for 1.5 min, and a final extension step of 72°C for 5-7 min. If Necab1 mRNA was present in tissues a 600 bp fragment would be amplified using the forward primer #93 and the reverse primer #99. GAPDH is a constitutively expressed housekeeping gene that was used as a control for quality of cDNA, generating a fragment of 750 bp using the forward #52 and reverse primer #53. **(Appendix A)**.

*Cell culture and Transfections*

HEK293 cells were maintained in Dulbecco's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 100 units/ml of penicillin/streptomycin (Invitrogen/Life Technologies). HEK293 cells were transiently transfected in 35 mm dishes with 4 µg of denoted plasmid constructs via Lipofectamine 2000 reagent (Invitrogen) as per manufacturer's instructions.

*GST Fusion Protein Preparations*

For GST fusion protein expression, clones in pGEX-KG were transformed into the *E. coli* expression strain BL21-DE3. Protein preparations were prepared from a 5 ml starter overnight at 37°C. After a 16 hr incubation, starter cultures were diluted 1:100 in 500 ml LB media (in 2L flasks) for approximately 2 hr until the O.D. reached ~0.5. For GST-CAP1, GST-CAP2, and GST-CAP2-N304 the temperature was them reduced to 30°C and grown for 15 -20 more min. Cultures were then induced with freshly made IPTG to a final concentration of 0.2 mM and grown for 4-6 more hours. Each 500 ml culture was then spun down and resuspended in 25 ml of phosphate-buffered saline (1×PBS) (14 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.75 mM KH2PO4, pH 7.4) containing a protease inhibitor cocktail (Complete, Roche) and left at 4°C until the next morning. Bacterial cells were then sonicated on ice 7-8× on setting three, and cells were allowed to cool on ice in between sonications. Triton X-100 was added to a final concentration of 0.8% and mixed gently on ice for a half hour to aid in solubilization of recombinant proteins. Lysed cells were then centrifuged at 10,000 × g for 15 min, and the supernatants from each 25 ml sonicate were incubated with 500 µl of a 50% glutathione-agarose slurry for 1 hr at 4°C. Glutathione beads containing the GST-fusion proteins were then washed four times for 20 min each with 1×PBS containing 0.8% Triton X-100. Finally, fusion proteins were eluted in Glutathione Elution Buffer (20 mM reduced glutathione, 50 mM Tris, pH 8.0). Eluted proteins were then buffer exchanged and concentrated into 1×PBS using a centricon tube (Fisher). For regular GST all growth incubations were done at 37°C, and for GST-Necab1, instead of a 30°C incubation, the temperature was 25°C. When necessary, recombinant proteins were cleaved free of GST prior to the elution step by incubating beads in Thrombin Cleavage Buffer (20 mM Tris, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) containing thrombin (Amersham Pharmacia Biotech) (~3 units/1000ug of recombinant protein), overnight at room temperature. Thrombin was inactivated by the addition of 1 mM PMSF.

*Antibodies*

An anti-Necab1 rabbit polyclonal antibody was raised against full length bacterially expressed Necab1 cleaved free of GST, and was a kind gift from Dr. Lisa Elferink (University of Texas Medical Branch).

A rabbit polyclonal anti-CAP2 antibody was raised against the N-terminal 304 amino acids of CAP2 (CAP2-N304) which was first expressed bacterially as a GST fusion protein and cleaved free of the GST. The purified protein was sent off to Covance Inc. where rabbit anti-CAP2-N304 antibodies were generated. CAP2 antibodies from whole sera were then affinity purified using CAP2-N304 as an antigen on a CNBr-activated Sepharose 4B column (Amersham Biosciences). Other antibodies used in this study are as follows: anti-syntaxin1 (HPC-1, Sigma), anti-GST (Sigma), and anti-actin (Chemicon). The anti-HA (12CA5), anti-Myc (9E10) antibodies were a kind gift from Dr. Dallan Young (University of Calgary).

*Western Blot Analysis*

For immunoprecipitations 10-20 µg of proteins were loaded in total extract lanes. Proteins were resolved on a 12% SDS–PAGE gel and transferred to nitrocellulose for Western blot analysis. HA and MYC tagged proteins were detected using a monoclonal anti-HA antibody 12CA5 (1:10,000 dilution) and a monoclonal anti-MYC antibody 9E10 (1:800) respectively. A secondary horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody was then used (1:3300) (Roche). For rat tissue samples and brain sections, freshly dissected organs or brain sections were flash frozen in liquid nitrogen, ground with a mortar and pestle, and extracted with 2% SDS. Protein (25 µg) samples were resolved on 12% SDS-PAGE gels. For detection of endogenous Necab1 and CAP2, rabbit polyclonal antibodies were used at a dilution of 1:5000 and 10,000 respectively, and a secondary anti-rabbit IgG HRP conjugate (Promega) was used at a dilution of 1:10,000. The anti-syntaxin1 HPC-1 antibody was used at a dilution of 1:10,000 anti-actin at a dilution of 1:2000, anti-GST at a dilution of 1:5000, and all were detected via a secondary horseradish peroxidase (HRP)-conjugated sheep anti-mouse antibody. Protein concentrations were determined using the Protein Bio-Assay Reagent (Bio-Rad). For western blot analysis, proteins were visualized using a Lumi Light chemiluminescence detection kit (Roche) as per manufacturer's instructions. If required, blots were stripped using Restore (Pierce) as per manufacturer's instructions.

*In Vitro Binding Assay*

GST-fusion proteins or GST (10 µg) alone immobilized on Glutathione agarose or beads alone were incubated with 250 ng of bacterially recombinant Necab1 in 300 µl of Binding Buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.2 % Triton X-100, 0.5 mM EGTA) for 1.5 hrs at 4°C. Glutathione beads were then washed 4× at 4°C with 500 µl of Binding Buffer containing 1% Triton X-100, boiled off in sample buffer and subjected to SDS-PAGE. Western blot analysis was performed using a polyclonal antibody against hNecab1 and GST fusion protein binding was verified by Coomassie blue staining or via the use of an anti-GST antibody.

*Immunoprecipitation analysis*

HEK293 cells were transiently transfected via Lipofectamine 2000 (Invitrogen) in 35 mm dishes as described. After leaving DNA/Lipofectamine complex on cells for 6 hrs, cells were trypsinized, and replated into 35 mm and 60 mm plates and left for approximately 36 hrs. Cells were then rinsed quickly with ice-cold PBS, and scraped off with a cell scraper in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM KCl, 1% IGEPAL CA-630 (NP-40), 10% glycerol) containing a protease inhibitor cocktail (Complete Mini, Roche). When binding studies involved calcium, immunoprecipitations were done in either the presence of 3 mM $CaCl_2$ or in 3 mM of the chelator EGTA. Cell were then briefly sonicated on ice and centrifuged for 10 min at 12,000 g at 4°C. Protein concentrations were determined using the Protein Bio-Assay Reagent (Bio-Rad). Total protein extracts(200-300 µg) were precleared with Protein A agarose beads (Sigma) for 30min in a total volume of 500 µl. Extracts were then incubated with Protein A beads that were chemically crosslinked with monoclonal anti-HA antibody (12CA5) for 1.5 hrs. Immune complexes were then washed 4×10 min each with 500 µl of RIPA minus glycerol and protease inhibitors (or with 3 mM $CaCl_2$ or 3 mM EGTA). The proteins were then boiled in 1× sample buffer and analyzed via Western blot analysis using a monoclonal anti-HA antibody and a monoclonal anti-MYC antibody (9E10). All immunoprecipitation analysis was carried out at 4°C.

*Rat Brain Synaptosomal Pull-down Assays*

Rat brain synaptosomes were prepared as previously described and all procedures were done at 4°C (Chapman et al., 1996; Thomas et al., 1999). Briefly, rat brains were homogenized in 320 mM sucrose with a Teflon-glass dounce homogenizer and subsequently centrifuged at 5000 rpm for 2 min in an SS34 rotor. The supernatants were collected and spun at 11,000 rpm for 12 min to generate the crude synaptosomal pellet. Synaptosomes were then solubilized in buffer B (50 mM HEPES-HCl, pH 7.6, 100 mM NaCl, and 1% Triton X-100) containing protease inhibitors at a detergent-to-protein ratio of 10:1 with 10 strokes in a Teflon-glass homogenizer. Homogenized synaptosomes were then mixed for 2 hr at 4°C and subsequently clarified of insoluble material by centrifugation at 50,000 rpm for 15 min. Binding studies were performed at 4°C by incubating 800 µg of the cleared solubilized synaptosomes in the presence of 3 mM $CaCl_2$ or 3 mM EGTA with 15 µg of the immobilized GST, GST fusion proteins, or glutathione-agarose beads alone in buffer C (10 mM HEPES-NaOH, pH 7.4, 0.15 M NaCl, 2 mM $MgCl_2$, and 0.2% Triton X-100). Protein complexes were washed 4× for 10 min each in Buffer C (with 3 mM $CaCl_2$ or 3 mM EGTA), boiled in 1× sample buffer, resolved via SDS-PAGE, and analyzed for Western blot using a monoclonal anti-syntaxin 1 (HPC-1) antibody.

## Results

### Necab1: a Novel Human CAP Interacting Protein

A two hybrid screen of a human adult brain cDNA library, using hCAP2 as a bait revealed ten histidine prototroph colonies. Only one of these ten colonies, GAD#5, demonstrated the ability to interact with both hCAP1 and hCAP2, and showed no transactivation properties by itself (**Table 1**). Retransformations, along with further two-hybrid analysis of Necab1, revealed that it interacted with LEX fusions of hCAP1 and hCAP2, but not with negative control baits such as LEX-Lamin, LEX-BAT, LEX-BAT313, and LEX-BAT380N (**Figure 4**). Upon sequencing and performing a BLASTX search, GAD#5 was shown to encode a protein that shared homology with a rat protein named Neuronal Calcium Binding Protein 1 (Necab1) (GenBank AF 193756, Sugita and Sudhof, 2001). Further

**Table 1: Analysis of clones isolated from yeast two-hybrid screen using LEXCAP2 as bait**

| GAD10 Clone # | Transactivation | Interaction after LEXCAP2 retransformation | Interaction with LEXCAP1 |
|---|---|---|---|
| 1 | NO | NO | NO |
| 2 | NO | NO | NO |
| 3 | NO | YES | NO |
| 4 | NO | NO | NO |
| 5 | NO | YES | YES |
| 6 | YES | YES | NO |
| 7 | NO | YES | NO |
| 8 | NO | NO | NO |
| 9 | YES | YES | NO |
| 10 | NO | NO | NO |

**Figure 4. Two-hybrid analysis of Necab1 following retransformations.** The original clone GAD10#5, that encoded a partial clone of human Necab1 (GAD10Necab1) was tested against numerous bait plasmids in the β-galactosidase colony filter assay using LacZ as a substrate. Positive interactions are denoted by a blue colour and the intensity of the interaction can be corelated with colour intensity.

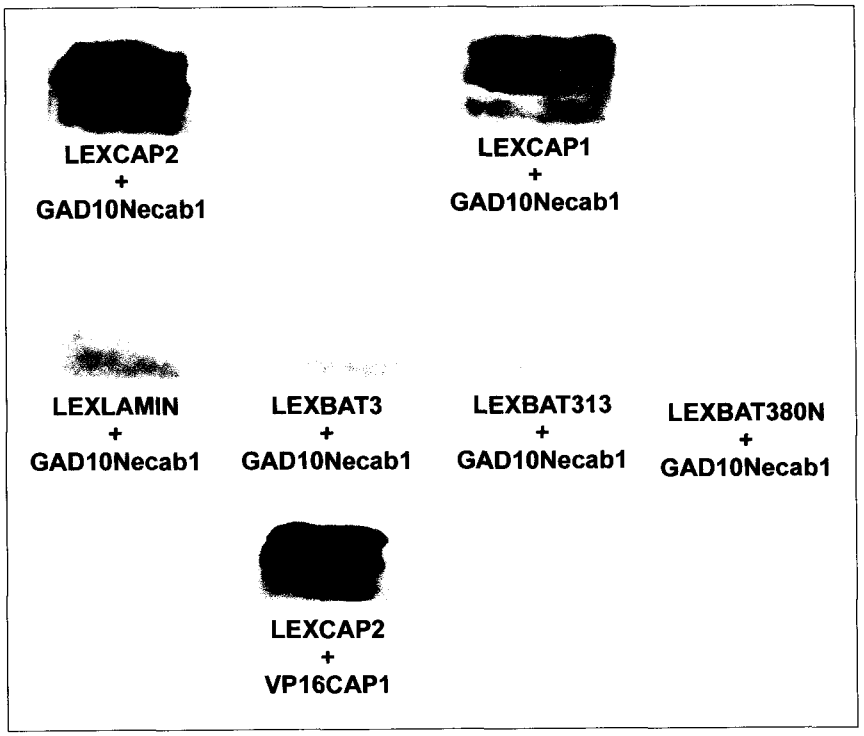



**Figure 4.**

analysis revealed that GAD#5 did not contain the full length sequence of Necab1, but was missing the 5' end. A subsequent BLASTN search of the Human Genome Project database was performed to obtain the chromosomal sequence. Analysis of this sequence along with the full length sequence of rat Necab1 (GenBank AF 193755), lead to the determination that 12 base pairs were missing from GAD#5. Primers were designed to PCR full length Necab1 from the brain cDNA library. This PCR product was subsequently cloned into pBS (Stratagene) and sequenced.

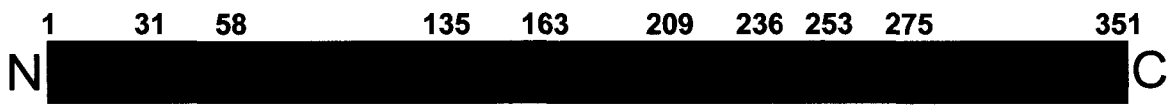
Analysis of the full length protein sequence of Necab1 (**Figure 5A**), reveals an N-terminal calcium binding domain (EF-hand), and three coiled-coil domain in tandem (**Figure 5B**). Coiled-coil domains are thought to mediate protein-protein interactions with coiled-coils of other proteins, and even act as homo-dimerization domains (Burkhard et al., 2001). Further analysis of the central coiled-coil motif reveals that it is a potential SNARE motif, as Necab1 has homology to the neuronal SNAREs in this region (**Figure 5C**). The t- and r-SNAREs have been reclassified into Q- and R-SNAREs based on conserved structural motifs (Fasshauer et al., 1998). This was based on amino acid sequence alignment of the conserved residues from the crystal structure of the SNARE complex. Specifically, each SNARE contributes an amphipathic $\alpha$-helix to the complex, and alignment within reveals a layer of highly conserved hydrophobic residues. Upon closer inspection, a zero layer of ionic residues occupying the centre of the $\alpha$-helices of all SNAREs is evident. This ionic residue can be either arginine (R) or glutamine (Q); thus, the designation of the names. The SNARE complex has been shown to constitute three Q-SNAREs and one R-SNARE. Alignment of the central Necab1 coiled-coil (residues 207-260) with the hydrophobic layer of the neuronal SNAREs reveals that Necab1 shares homology, specifically with synaptobrevin (a v-SNARE). Within the zero layer of Necab1, the residue is a lysine instead of an arginine, but this change is tolerated as a synaptobrevin family member also contains a lysine at the zero layer (Fasshauer et al., 1998).

**Figure 5. Analysis of the amino acid sequence of human Necab1 and SNARE alignment.** ***A***, Complete 351 amino acid sequence of Necab1. The EF-hand motif is highlighted in red, and coiled-coil domains are highlighted in green. The amino acid sequence that was used to perform the SNARE alignment is highlighted in bold and underlined. ***B***, domain structure of Necab1 drawn to scale. The N-terminal EF-hand (*EF*) is shown in red, with the three repetitive coiled-coils (*CC*) are highlighted in green. Coiled-coils were predicted using the COILS Server at http://www.ch.embnet.org/software/COILS_form.html. Numbers indicate the amino acid residues that border each of the respective domains. ***C***, Alignment of the four neuronal SNAREs motifs based on their respective hydrophobic layers. *hNECAB1* (hNECAB1; 207-260) was aligned against these segments in like manner. *Sx1a* (syntaxin 1a; 202-255); *SN1* (SNAP-25; 28-82); *SN2* (SNAP-25; 148-203), *Sb2* (synaptobrevin; 31-85). The core layers that comprise the 15-layered hydrophobic environment of the amphipathic bundle are boxed in green and the ionic, zero (0) layer residue indicated in red.

A

[1]MEDSQETSPSSNNSSEELSSALHLSKGMSIFLDILRRADKNDDG
KLSFEEFKAYFADGVLSGEELHELFHTIDTHNTNNLDTEELCEYFS
QHLGEYENVLAALEDLNLSILKAMGKTKKDYQEASNLEQFVTRFL
LKETLNQLQSLQNSLECAMETTEEQTRQERQGPAKPEVLSIQWP
GKRSSRRVQRHNSFSPNSPQFNVSGP**GLLEEDNQWMTQINRLQ**
**KLIDRLEKKDLKLEPPEEEIIEGNTKSHIMLVQRQMS**VIEEDLEEF
QLALKHYVESASSQSGCLRISIQKLSNESRYMIYEFWENSSVWNS
HLQTNYSKTFQRSNVDFLETPELTSTMLVPASWWILNN[351]

B




C

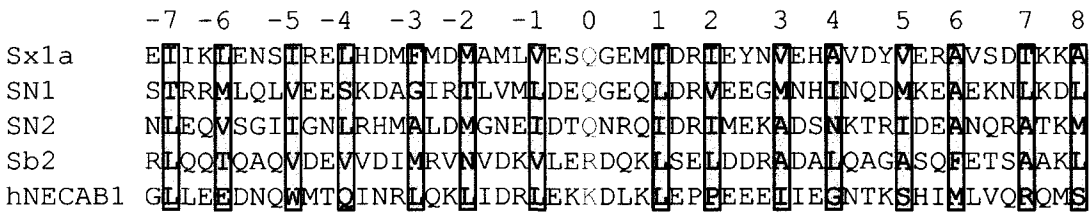



**Figure 5.**

**Necab1 is Preferentially Expressed in Brain Along with CAP2**

To further aid in deducing the function of Necab1 and CAP2, rabbit polyclonal antibodies against both proteins were developed. The antibody against full-length Necab1 reacts specifically with recombinant Necab1 and an endogenous protein of approximately 42 kDa, since Anti-Necab1 antibodies preabsorbed with GST-Necab1 do not (**Figure 6A**). No specific immunoreactivity was seen with preimmune serum (results not shown). A rabbit polyclonal antibody against CAP2 was generated using the first 304 amino acids as an antigen. Preimmune serum does not show any specific reactivity (results not shown). The CAP2 antibody reacts specifically with HA-tagged CAP2 and CAP2-N304 (antigen used for the antibody), and an endogenous protein of approximately 58 kDa, but does not demonstrate any cross-reactivity with HA-CAP1 (**Figure 6B**). The band above 79 kDa in **Figure 6B** is a non-specific band. Anti-CAP2 antibodies react specifically with the 58 kDa band in rat brain, while antibodies that have been immunodepleted with GST-CAP2-N304 do not (**Figure 6C**).

In order to deduce Necab1's tissue expression, RT-PCR was performed on total human RNA using specific primers. RT-PCR results revealed that Necab1 mRNA is preferentially and highly expressed within the human brain, but with lower levels in kidney, trachea and lung (**Figure 7A**). GAPDH was used as a control for cDNA quality and quantification. Further analysis of Necab1 expression within rat organs and tissues was performed using the anti-Necab1 rabbit polyclonal antibody. Western blot analysis demonstrated similar RT-PCR results, except that no Necab1 expression was seen in rat kidney, and Necab1 was also detected within spinal cord (not tested in RT-PCR) (**Figure 7B**). Actin was used as a control for protein sample quality and as a loading control. Further analysis of rat brain regions reveals that Necab1 and CAP2 have a similar brain expression pattern (**Figure 7C**). Both are highly expressed within the frontal and posterior cortexes and the hippocampus, and both are expressed at low levels in the medulla, pons, and spinal cord. Necab1 and CAP2 expression only differs within the cerebellum, as CAP2 is present, whereas Necab1 is absent, agreeing with published reports on Necab1 (Sugita et al., 2002). Actin was used as a protein loading control, and syntaxin 1 as a positive control, agreeing with previously published results (Chen et al., 1999).

**Figure 6. Specificity of the anti-Necab1 and anti-CAP2 polyclonal antibodies.** ***A***, anti-Necab1 antibody shows specific immunoreactivity in brain extract. Anti-Necab1 antibodies (*Anti-Necab1)* or those preabsorbed with the GST-Necab1 antigen (*Cleared*) were used for Western blot to detect recombinant and endogenous Necab1. Note that immunoreactivity is almost abolished in the Cleared blot and the band in *Brain* extract is only present in *Anti-Necab1*. ***B***, specificity of the anti-CAP2 antibodies. Rat Brain and mammalian cell extracts expressing empty vector (*HA*), HA-tagged *CAP1*, *CAP2*, or the antigen used for the antibody - CAP2-N304 (*N304*) - were loaded on SDS-PAGE. Western blot analysis was performed using anti-HA or the anti-CAP2 antibodies. Note that *anti-CAP2* antibody only reacts with *CAP2* and *N304*, but not *CAP1*, and specifically reacts with a band in *Brain* extract not present in the *HA* blot. ***C***, immunodepleted anti-CAP2 antibodies (*Cleared*) do not react with CAP2. Blots were performed as in ***A***, except that mammalian extracts expressing empty vector (*HA*) or *HA-CAP2* were used. Note that the Cleared antibodies react with other bands, but not with those specific for *Anti-CAP2*. Approximate protein standard sizes are indicated in kDa.

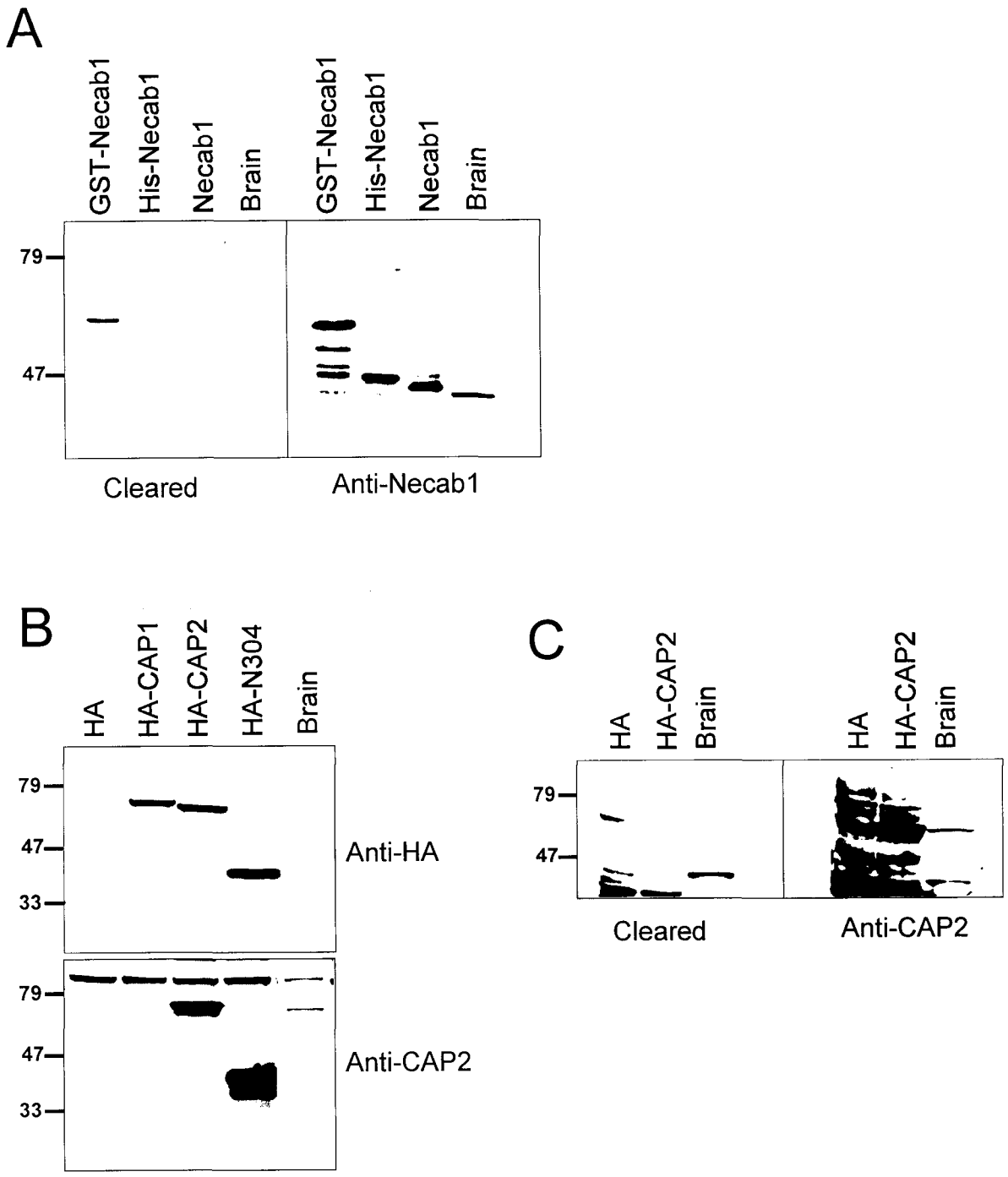



**Figure 6.**

**Figure 7. Necab1 tissue expression and similar brain distributions of Necab1 and CAP2.** ***A***, RT-PCR of Necab1 expression. Total RNA of different human tissues was used to make cDNA and PCR products were amplified using specific primers. *GAPDH* was used a positive control for quality of cDNA. Base pair sizes are indicated at side of gel. ***B***, western blot of Necab1 expression in rat tissues. Tissues were extracted in SDS buffer and 25 ug of protein was loaded on SDS-PAGE and immunoblotted using *anti-Necab1*, and *anti-actin* as control for both quality and amount of protein loaded. ***C***, Brain distribution of Necab1 and CAP2. Brain regions were dissected, extracted with SDS buffer and 25 ug loaded on 12% SDS-PAGE. Blot were probed with *anti-Necab1*, *anti-CAP2*, *anti-Syntaxin 1* as a control for proper distribution, and *anti-actin* as a control for amount of protein loaded.

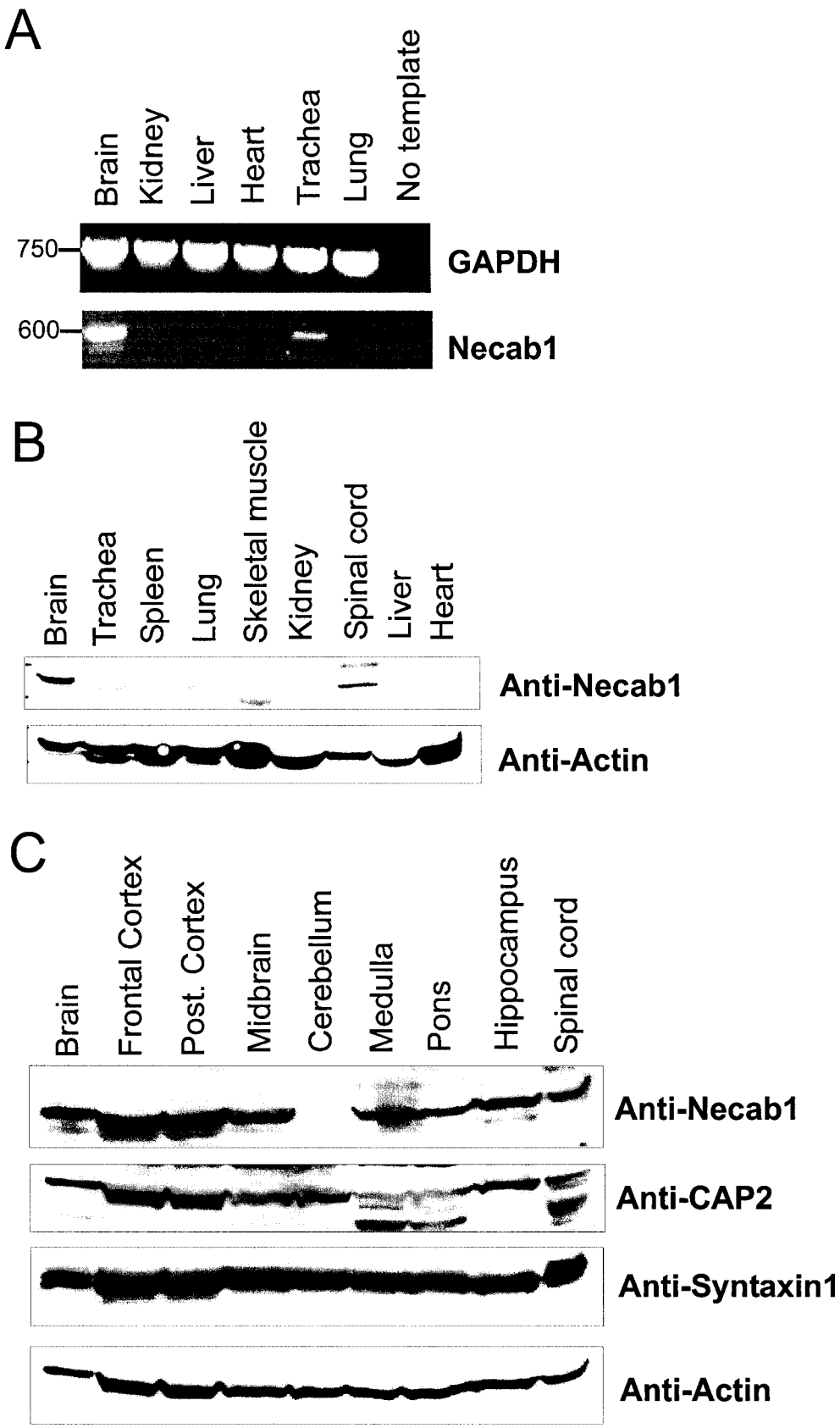



**Figure 7.**

**Necab1 - CAP2 Interaction is Specific and Dependent on Coiled-coil Domains**

In order to demonstrate that Necab1 is indeed a genuine binding partner of the human CAPs, we performed *in vitro* binding studies using recombinant bacterially expressed proteins. As demonstrated in **Figure 8A**, Necab1 directly interacts with both CAP1 and CAP2, but not GST or beads alone, in which proteins have been immobilized onto glutathione beads as shown by the coomassie blue stain to the left. The * denotes GST-CAP1 that have been degraded within the C-terminus as it is still pulled down by the glutathione beads and was also verified by performing anti-GST western blot (results not shown). The *in vitro* interaction fortified Necab1 as a CAP binding partner and lead us to further characterize the interaction *in vivo* using mammalian cells lysates expressing HA and MYC-tagged proteins. Interestingly, immunoprecipitation of HA-Necab1 using the anti-HA antibody (12CA5) demonstrates that the interaction may be specific for CAP2 since CAP1 does not show any interaction with Necab1 *in vivo* (**Figure 8B**). Cell lysates from HEK293 cells expressing HA- and MYC-tagged proteins reveals that all constructs are expressed and immunoprecipitations reveal that MYC-CAP2 does not bind without the presence of HA-Necab1.

To further characterize the interaction between human CAPs and Necab1, we mapped the interaction domains necessary for binding using the yeast two-hybrid system. Different deletion mutants of Necab1 were constructed and expressed as fusions with the VP16 activation domain, such that both carboxyl and amino terminal deletion mutants had three variations of coiled coil organization (see schematic in **Figure 9**). These deletion mutants were then tested for their ability to interact with the LEXCAP1 and CAP2 fusions containing the DNA binding domain in the yeast two hybrid system (**Figure 9**). Results demonstrated that the N-terminal 250 amino acids (N250) of Necab1 containing the first two coiled-coils interacted with both CAPs, while N284 containing a small C-terminal deletion showed a reduced interaction which may be explained by protein instability. N200 that contained only the first coiled-coil did not exhibit any interaction. Furthermore, C156 containing only the last two coiled-coils, demonstrated a substantial interaction, while C100 does not. Since results suggested that the central coiled-coil of Necab1 is where CAP binding occurs, a mutant of Necab1 was constructed via site-directed mutagenesis that had this domain abolished ($Necab1_{2CC}$). $Necab1_{2CC}$ does not

**Figure 8. *In vitro* and *in vivo* interaction between CAPs and Necab1.** ***A***, in vitro binding of bacterially expressed His-Necab1 to immobilized GST-hCAPs. 250 ng of His-Necab1 was incubated with immobilized GST-fusions on glutathione-agarose beads or beads alone (*Beads*). Beads were washed extensively with binding buffer, boiled off in sample buffer and subjected to SDS-PAGE and Western blot analysis using a polyclonal antibody against Necab1. Coomassie blue stains were performed to verify binding of GST-fusion proteins to glutathione-agarose and is depicted to the right (* denotes a C-terminally degrade product of GST-CAP1 since the fusion protein is pulled down by glutathione agarose beads). ***B***, in vivo interaction demonstrating Necab1 co-immunoprecipitates hCAP2 but not hCAP1. HEK293 cells were transiently transfected with hemaglutammin (HA) or MYC tagged constructs. Protein extracts were incubated with pre-conjugated 12CA5 (anti-HA) Protein-A beads. Immune complexes were washed extensively with RIPA buffer and boiled off in sample buffer. Protein extracts samples (*Extracts*) along with immunoprecipitations (*IP*) were subjected to SDS-PAGE, and western blot analysis was performed using *anti-HA* (12CA5) or *anti-MYC* (9E10) antibodies. Presence (+) and abscence (-) of constructs used is denoted. Approximate protein standard sizes are indicated in kDa.

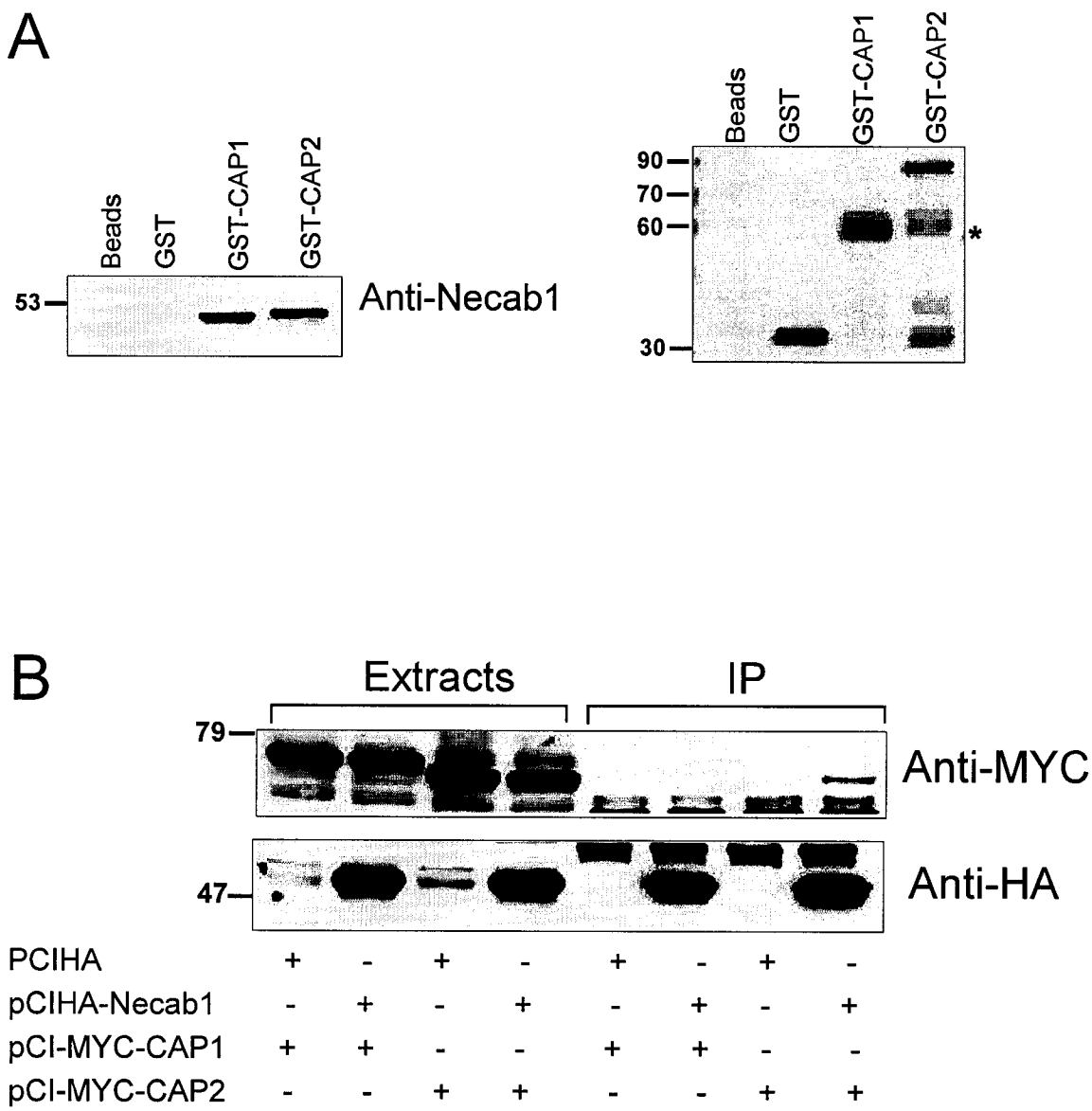



**Figure 8.**

**Figure 9. Two-hybrid mapping of Necab1-CAPs interaction domains.** A schematic of full length Necab1, deletion mutants, and a mutant in which the second coiled has been mutated (I218K, L221R; *Necab1*$_{2CC}$) are illustrated on the left. The EF-hand calcium binding domain (*EF*), and the three predicted coiled-coil domains (*CC*) in tandem repeat are denoted. The interaction of these VP16-Necab1 constructs were tested in a yeast two-hyrid assay with LEX-CAP1 and LEX-CAP2. The ß-galactosidase activity of each interaction was quantified using ONPG (o-Nitrophenyl ß-D-galactopyranoside) as a substrate, normalized, and expressed as a percentage of the activity of full-length Necab1. The amino acid residues are shown as numbers next to each construct. Results are representative of three to four trials, and the mean standard error (±) of each interaction is shown as a percentage.

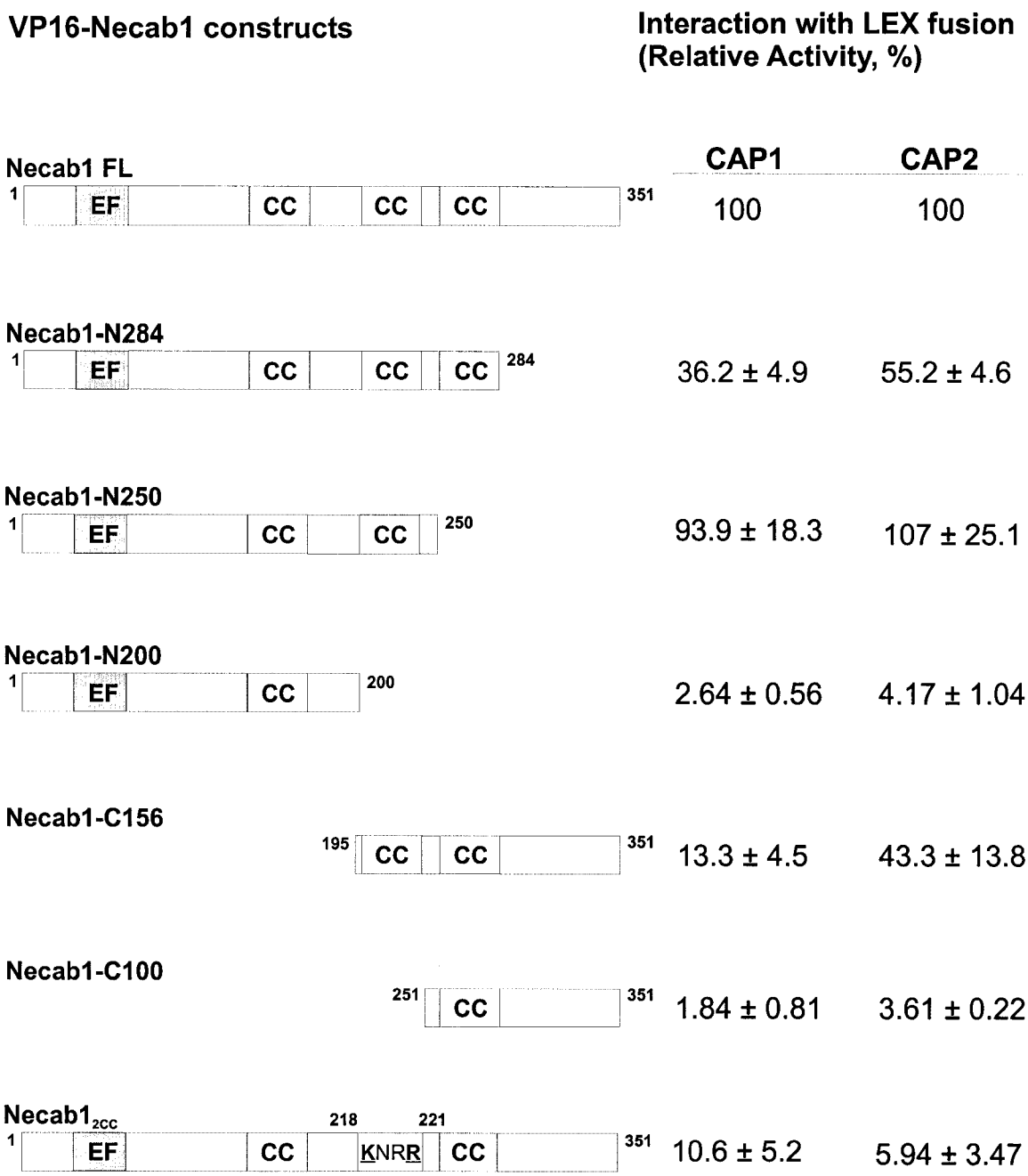



**Figure 9.**

demonstrate any substantial interaction with either of the hCAPs (**Figure 9**), indicating that the central coiled-coil domain of Necab1 is essential for hCAP binding.

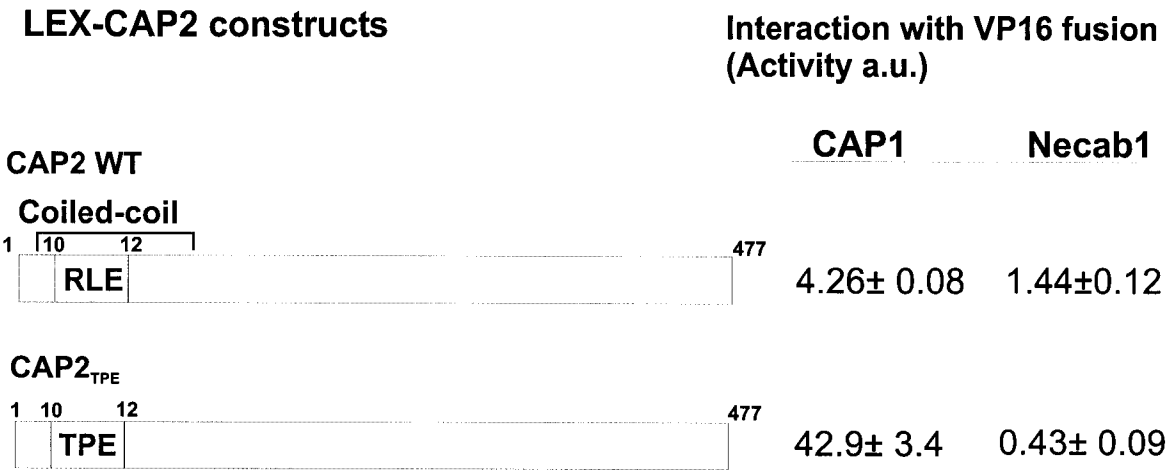
Since Necab1 likely bound to the human CAPs through its central coiled-coil domain and that CAPs also contain an N-terminal coiled-coil, suggested the interaction may be coiled-coil mediated. Thus, a mutant of CAP2 was created via one step site directed mutagenesis PCR strategy with the coiled-coil structure abolished ($CAP2_{TPE}$). All CAPs have a conserved N-terminal domain termed the RLE motif, due to three highly conserved residues that are thought to constitute the coiled-coil structure (Hubberstey and Mottillo, 2002). Changing the charged arginine to an uncharged polar threonine, and the leucine to proline changes the secondary structure, and results in the abolishment of the coiled-coil structure (Nishida et al., 1998). The LEX fusion of the CAP2 coiled-coil mutant (R10T, L11P) along with wild-type CAP2, were tested for their ability to interact with VP16Necab1 in the two-hybrid system (**Figure 10A**). Results demonstrated that wild-type CAP2 can interact with Necab1, while $CAP2_{TPE}$ does not. As a positive control, we employed VP16CAP1 to demonstrate that the coiled-coil mutant is expressed properly and is still capable of interacting with proteins. We further tested the ability of $CAP2_{TPE}$ to interact with Necab1 *in vivo*. Immunoprecipitation analysis reveals that CAP2 interacts with Necab1 through its N-terminal coiled-coil (**Figure 10B**). Immunoprecipitation of HA-Necab1 using the anti-HA antibody (12CA5) demonstrates that wild-type CAP2 interacts with Necab1 but the coiled-coil mutant does not. Western analysis reveals that all constructs are expressed properly (**Figure 10B**). Therefore, Necab1 and CAP2 interact through a coiled-coil mechanism.

**Necab1 Dimerizes Independent of Calcium**

Analysis of Necab1 revealed only a single EF-hand within the N-terminus. Since most calcium binding proteins contain at least two EF-hands, it was postulated that Necab1 could dimerize in order to bind calcium. Single EF-hand domain proteins can form homodimers in order to bind $Ca^{2+}$ (Lewit-Bentley and Rety, 2000). Therefore, *in vivo* binding studies were performed in order to test whether Necab1 could interact with itself. HEK293 cells were cotransfected with HA- and MYC-tagged Necab1 and immunoprecipitations using anti-HA antibodies were performed in the presence of 3 mM $CaCl_2$

**Figure 10. The Necab1-CAP2 interaction is mediated through coiled-coil domains.** ***A***, mutation of the CAP2 coiled-coil domain necessary for Necab1 binding. A schematic on the left illustrates the highly conserved residues (*RLE*) of wild-type (*WT*) CAP2 necessary for the coiled-coil structure, and those same residues (*TPE*) in the mutant (R10T, L11P; $CAP2_{TPE}$) in which the coiled-coil structure is abolished. The interaction of these LEX-CAP2 constructs were tested in a two-hybrid assay with VP16-Necab1, and VP16-CAP2 which was used as a positive control for the interaction with $CAP2_{TPE}$. The interactions were quantified as stated in **Figure 9** except that the activity is stated in arbitrary units (*a.u.*) and the mean standard error is shown. The amino acid residues are shown as numbers next to each construct. Results are representative of three to four trials, and the mean standard error (±) of each interaction is shown as a percentage. ***B***, immunoprecipitation analysis reveals that CAP2's coiled-coil is necessary for binding Necab1 *in vivo*. Immunoprecipitations were performed as described in **Figure 8B**. Presence (+) and absence (-) of constructs used is denoted. Approximate protein standard sizes are indicated in kDa.

A




B

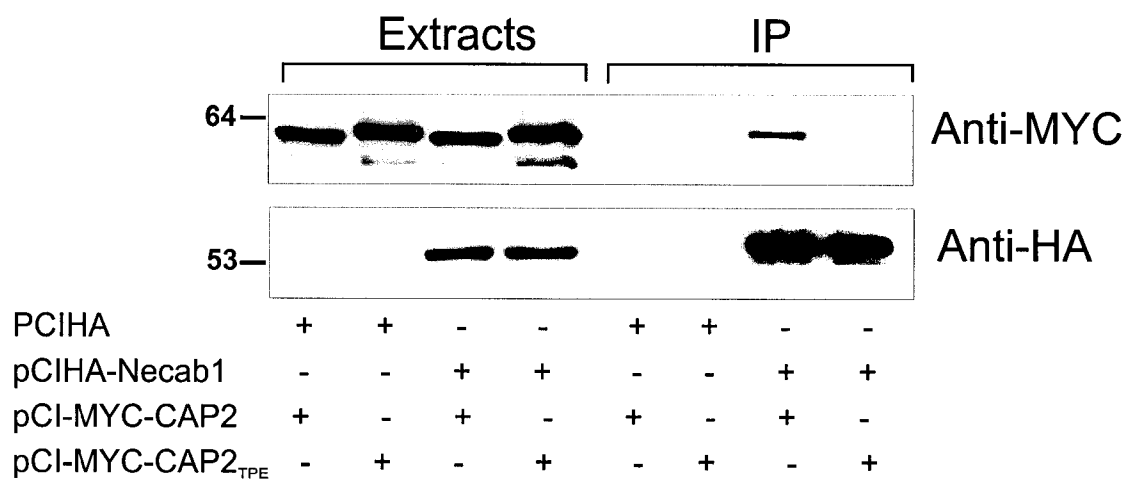


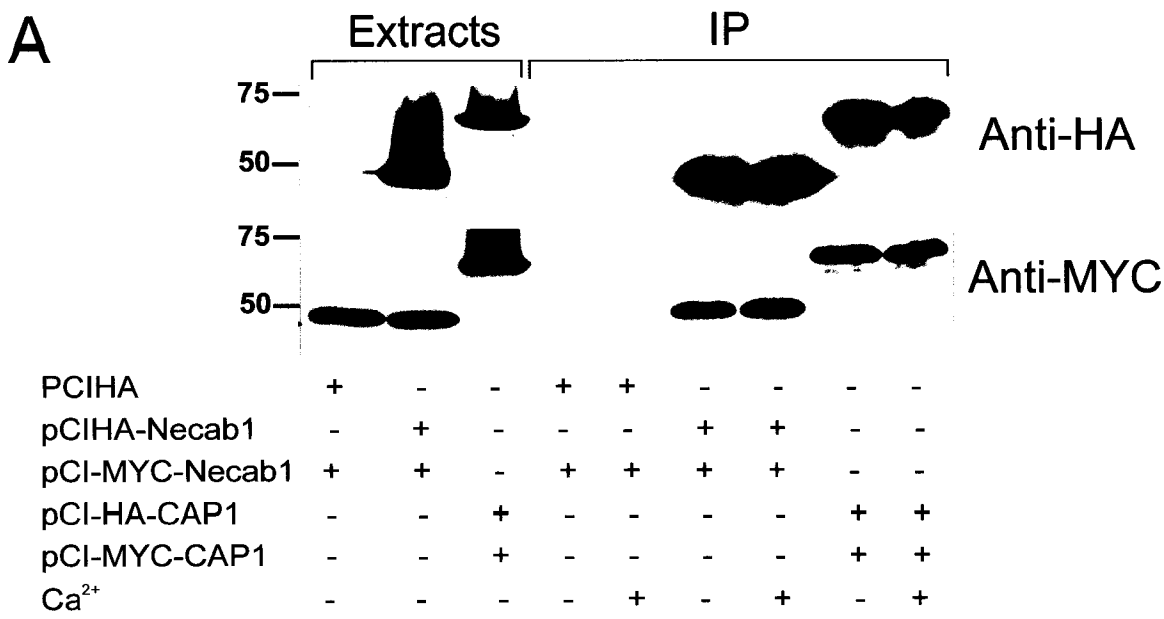

**Figure 10.**

or in the presence of the chelator EGTA (3 mM) (**Figure 11A**). Surprisingly, results demonstrated that Necab1 can indeed dimerize, however, the interaction is calcium independent. The dimerization between HA-CAP1 and MYC-CAP1 was used as a positive control, and no MYC-Necab1 binds without the presence of HA-Necab1. Western analysis reveals that all constructs are expressed properly. The domain necessary for Necab1 dimerization was mapped using similar VP16 deletion mutants of Necab1 used to map the CAP binding domain. These activation domains fusions were tested for their ability to interact with the DNA binding domain fusion of LEXNecab1 in the β-galactosidase colony filter assay (**Figure 11B**). Positive interactions are denoted by a blue colour. All constructs tested demonstrated a positive interaction, except Necab1-C100 that expresses only the last coiled-coil. This establishes that Necab1 dimerization is likely mediated through its first two N-terminal coiled-coils. Though, the involvement of the EF-hand in mediating the dimerization cannot be excluded.

**Necab1 Associates with the Neuronal SNARE Syntaxin 1a**

Necab1 was first isolated through its ability to associate with neuronal synaptotagmin 1 through affinity chromatography (Sugita et al., 2002; Sugita and Sudhof, 2000). However, this interaction was not shown to be physiologically significant, as an *in vivo* interaction was not demonstrated (Sugita et al., 2002). Prior to these results being published, we also wanted to further characterize the Synaptotagmin1 - Necab1 interaction, and also found that it does not occur *in vivo* (results not shown). We hypothesized that Necab1 could possibly bind to neuronal SNAREs due to its SNARE homology. Necab1 is a putative R-SNARE, and R-SNAREs bind specifically to Q-SNAREs, suggestive to the idea that that Necab1 could interact with a Q-SNARE such as syntaxin 1a. In order to test this, *in vivo* rat brain synaptosomal pull-down assays were performed. This assay involves immobilizing GST-Necab1 onto glutathione-agarose beads and incubating solubilized synaptic vesicle enriched brain extracts (synaptosomes) to pull down endogenous protein complexes. GST pull-down assays demonstrated

**Figure 11. Necab1 dimerization and domain mapping.** ***A***, Immunoprecipitational analysis demonstrating that Necab1 can dimerize independent of calcium ($Ca^{2+}$). Bindings were performed as described in **Figure 8B**, except that IPs were done in the presence (+) of 3 mM $CaCl_2$ or containing 3 mM EGTA (-). Presence (+) and absence (-) of constructs used is denoted. Approximate protein standard sizes are indicated in kDa. ***B,*** two-hybrid mapping of Necab1 dimerization domains to the first two coiled-coils. Mapping was performed as described in **Figure 9**, except that the Necab1-LEX fusion was used and the β-galactosidase colony filter assay was performed as described in **Figure 4**. Positive interactions are denoted by a blue colour.



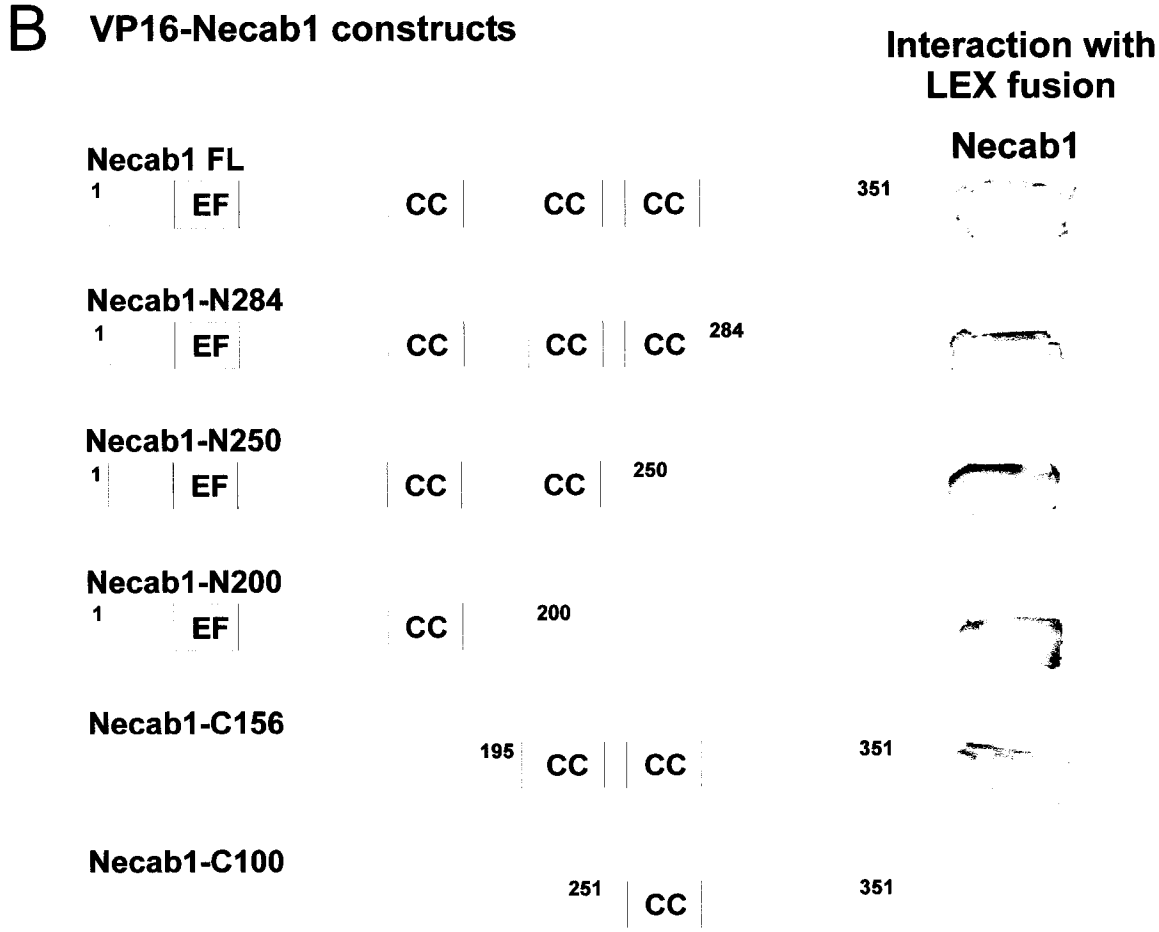




**Figure 11.**

that Necab1 bound Syntaxin 1a *in vivo* in a calcium independent manner (**Figure 12A**). Results also suggest that Necab1 may associate with syntaxin 1a at the membrane, as syntaxin 1a is an integral membrane protein and synaptosomes are a membrane enriched fraction. GST itself did not demonstrate the ability to pull-down substantial syntaxin 1a and neither did beads alone. To further characterize the calcium dependance of the interaction GST or GST-Necab1 were incubated with increasing μM concentrations of free $Ca^{2+}$and solubilized synaptosomes. Results demonstrated that $Ca^{2+}$does not have any effect on the Syntaxin 1a - Necab1 interaction (**Figure 12B**).

To determine if Necab1 and syntaxin 1a directly interact with each other, *in vitro* binding studies were performed using recombinant proteins. Immobilized GST, GST-syntaxin 1a, or beads alone, were incubated with bacterially produced Necab1, and protein complexes were verified by performing a western against Necab1. Only GST-syntaxin 1a binds Necab1, while GST or beads alone do not (**Figure 12C**). GST and fusion proteins were verified for bead binding by performing a western against GST. We further hypothesized that Necab1 - Syntaxin 1a binding occurred through Syntaxin's Q-SNARE motif since Necab1 was a putative R-SNARE. As shown in **Figure 12D**, Necab1 can specifically bind to the immobilized GST-H3 SNARE motif of Syntaxin 1a, but once again it cannot bind to GST or glutathione beads alone. Therefore, Necab1 can physiologically interact with the SNARE syntaxin 1a which is likely mediated through SNARE binding.

## Discussion

In this study, novel CAP interacting protein named Neuronal Calcium Binding protein 1 (Necab1) was isolated and characterized. The interaction is physiologically specific to CAP2, although CAP1 can also interact in the two-hybrid system. The specific brain expression pattern and calcium binding properties of Necab1 suggests that it may have a function during long term potentiation. More importantly, it may modulate a neuronal function of CAP2. We also demonstrated that Necab1 is a physiological binding partner of the neuronal SNARE syntaxin 1a; suggestive of a function for Necab1 during synaptic transmission. Thus, we suggest Necab1 may serve to link CAP2 to neuronal function.

**Figure 12. Interaction of Syntaxin 1a with Necab1 is independent of calcium and mediated through its SNARE motif.** ***A***, *in vivo* interaction of Necab1 with endogenous Syntaxin 1a using rat synaptosomal GST pull-down assays. The cytoplasmic domain of synaptotagmin (*GST-Syt*) or hNecab1 (*GST-nNecab*) were expressed as GST fusion proteins, immobilized onto glutathione-agarose beads and incubated with detergent solubilized rat brain synaptosomal extracts in the presence 1 mM $Ca^{2+}$ or 1mM *EGTA*. Glutathione beads containing protein complexes were washed with appropriate buffers, boiled off in sample buffer and loaded on SDS-PAGE. Western blot analysis (*Wn. Ab*) using an anti-syntaxin 1 mouse monoclonal antibody (*anti-Stx*) were then performed. *GST* and beads alone (*Con*) were used as negative controls and GST-Syt as a positive control. ***B***, the Necab1 - Syntaxin 1a interaction is not affected by increasing calcium concentrations. Immobilized *GST- hNecab* or *GST* alone were incubated with detergent solubilized rat brain in the absence (*EGTA*) or presence of the indicated amounts of free $Ca^{2+}$. Bindings and western blot analysis were performed as described in ***A***. ***C***, interaction of Necab1 with Syntaxin 1a is independent of other proteins. Immobilized GST or the cytoplasmic domain of syntaxin 1a (*GST-Stx*) or beads alone (*Con*) were incubated with recombinant hNecab1 and binding studies were performed as described in **Figure 8A**. Western blot analysis was performed using *anti-hNecab* and anti-*GST* antibodies (*Wn. Ab*). GST alone (*GST*) or beads only (*Con*) were used as controls. ***D***, the Necab1-Syntaxin 1a interaction is mediated through Syntaxin's SNARE motif. Binding and explanation is as described in **12C**, except that the SNARE motif of syntaxin 1a (*H3*) was used rather than the cytoplasmic domain.

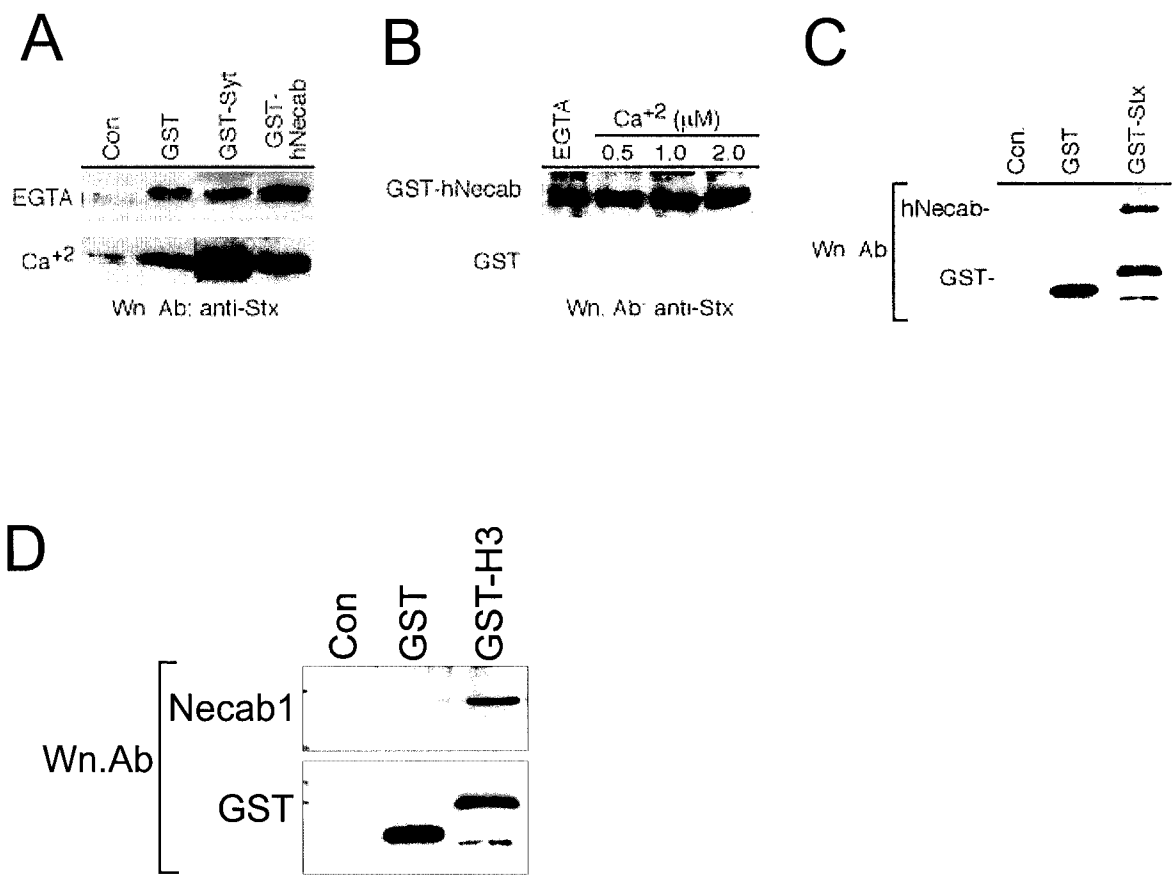



**Figure 12.**

## The Association of CAP2 with Necab1 Implies a Neuronal Function

Necab1 is a recently characterized calcium binding protein, with an unknown function (Sugita et al., 2002). It is a member of a family of calcium binding proteins that are primarily expressed within the brain with the existence of two other orthologues, Necab2 (Bernier et al., 2001) and Necab3 (Lee et al., 2000). Specifically, Necab1 has been shown to have restricted neuronal expression, as it is highly expressed within layer 4 of pyramidal neurons of the cerebral cortex, and within the hippocampus, where only inhibitory neurons and pyramidal neurons of the CA2 region reveal Necab1 expression (Sugita et al., 2002). These results suggest that Necab1 likely has a specialized function within the brain. We have independently isolated and characterized Necab1 through its ability to interact with human CAP2 in a yeast two-hybrid library screen. Necab1 is a protein of approximately 42 kDa that contains an N-terminal EF-hand, and three predicted repetitive coiled-coil domains. Binding studies reveal that Necab1 binds both CAP1 and CAP2 *in vitro* and in the two-hybrid system, but only associates with CAP2 *in vivo*. The *in vivo* interaction with CAP2 suggests that it is physiologically relevant, while the interaction with CAP1 may not be relevant. This does not rule out other possibilities, as CAP1 may be complexed with other proteins and unable to interact with Necab1, or that the interaction with CAP1 is tightly regulated and a regulatory signal is needed for an interaction to occur.

The interaction between CAP2 and Necab1 has been mapped and is mediated through coiled-coils domains. This is suggestive of a specific interaction, as domains necessary for eliciting protein-protein interactions are involved (Burkhard et al., 2001). The specificity of the interaction was also demonstrated in the initial two-hybrid as Necab1 was not able to interact with other proteins. We have determined that Necab1 is highly and preferentially expressed within the brain of both human and rat. RT-PCR from human tissues show that Necab1 exhibits lower levels of expression within trachea, and even lower expression within kidney and lung. Western blot analysis demonstrates a similar expression, except no Necab1 is detected within rat kidney. This could simply be explained by a difference in expression of Necab1 between human and rat, as the orthologue Necab3 (XB51) demonstrates different expression patterns between these two organisms (Lee et al., 2000). In addition, western blot analysis is not as sensitive of a technique as RT-PCR. Another possibility is that

the kidney mRNA is expressed but the protein is not. The contradictory results are not likely due to non-specific primer binding or non-specific antibody interaction, as both primers were designed against a sequence specific for Necab1, and the antibody specifically detects overexpressed HA-Necab1 and an endogenous band of ~42 kDa. Also, antibodies that were pre-absorbed with GST-Necab1 do not demonstrate any specific immunoreactivity. The results that we present differ from those published on Necab1 by Sugita *et al.*, who demonstrated that Necab1 is expressed only within the brain (Sugita et al., 2002). We are confident that our results reveal the true expression pattern of Necab1. Firstly, Sugita *et al.* used Northern and Western analysis to look at the expression of Necab1. RT-PCR is similar in specificity to Northern analysis, but is more sensitive, since in theory, if a single mRNA molecule is expressed it will be detected by PCR. Secondly, the specificity of our antibody is revealed in the fact that our brain expression pattern of Necab1 agrees with that of Sugita *et al.*, whom demonstrated that Necab1 is highly expressed in the cerebral cortex and hippocampus, but not expressed in the cerebellum (Sugita et al., 2002). Thirdly, the expression in trachea was not studied by Sugita *et al.* Thus, the discrepancies in Necab1 expression can probably be explained due to different techniques employed. Different sized bands were observed in the western blot analysis of Necab1 protein expression (**Figure 7B**) and cannot be fully ruled out as alternatively spliced isoforms since northern blot analysis is performed.

The expression of CAP2 mRNA differs from CAP1 which is more ubiquitously expressed, suggestive that CAP2 may have a more specific, refined role in actin reorganization (Swiston et al., 1995). CAP2 mRNA is expressed highly in testes and brain, with moderate levels in heart and skeletal muscle, and lower levels in kidney, small intestine, lung and skin. Interestingly, Necab1 and CAP2 are both expressed in tissues where regulated secretion occurs (*i.e.* brain and kidney). The similar and specific expression pattern for Necab1 and CAP2 in brain is highly suggestive of a specialized function. Both are highly expressed within the cerebral cortex and hippocampus, and at lower levels in the medulla, pons, and spinal cord. Furthermore, this fortifies the idea that the CAPs are more than just mere ubiquitously expressed housekeeping proteins, but instead are spatially regulated; indicative of a specific function. This idea is in agreement with other studies as the *Drosophila* CAP homologue is

spatially regulated (Baum et al., 2000; Baum and Perrimon, 2001); additionally CAP is also developmentally regulated in *Xenopus* (KhosrowShahian et al., 2002).

The expression of Necab1 within the cerebral cortex and hippocampus has been localized to pyramidal excitatory pyramidal neurons, with additional expression in inhibitory interneurons of the hippocampus (Sugita et al., 2002). This is highly intriguing, since excitatory pyramidal neurons act as a convergence point to organize all the inputs that are received from axons of other neurons. Specifically, hippocampal pyramidal neurons of the CA (*cornu ammonis*) region is where the process of long term potentiation (LTP) is localized. LTP is a prolonged enhancement of synaptic strength, and is thought to be the fundamental mechanism behind learning and memory formation. Necab1 is particularly expressed in the CA2 region of the hippocampus (Sugita et al., 2002). The function of the CA2 field is unclear, but it function in slow transmission, directing signals from the CA3 region to CA1 (Sekino et al., 1997). The fact that Necab1 and CAP2 are physiological binding partners, and highly expressed within the cerebral cortex and hippocampus, is suggestive to a neuronal role for CAP2 that is likely moderated through Necab1. The possibility that Necab1 is simply just a $Ca^{2+}$ buffering protein is highly unlikely, as $Ca^{2+}$ buffering proteins are usually of very small molecular weight and are composed of mainly just one EF-hand (Caillard et al., 2000; Hof et al., 1999). EF-hands can also act to modulated a protein binding region in response to $Ca^{2+}$ binding, which is the case with calmodulin (Lewit-Bentley and Rety, 2000). In addition to its EF-hand, Necab1 contains multiple coiled-coil domains and bears R-SNARE homology, thus the possibility to form multiple protein-protein interactions. Hence, it would be intriguing to speculate that Necab1's EF-hand acts to mediate its protein interactions through $Ca^{2+}$ signalling. That Necab1 may be an effector of CAP2 during neuronal function is not unconventional, as other examples exist of proteins having specific neuronal modulators. Calcineurin has been shown to modulate the function of dynamin 1during synaptic vesicle exocytosis (Lai et al., 1999). Dynamin functions during endocytosis with other proteins in order to facilitate pinching of vesicles from the plasma membrane. This calcium-dependent interaction with calcineurin results in a calcium-sensing mechanism for internalization of synaptic vesicles. The possible functional and neuronal role for the Necab1 - CAP2 interaction will be further discussed in Chapter Four and Five.

**Necab1 is a Potential Effector of Synaptic Transmission**

It is evident that many of the protein-protein interactions involved in vesicle docking, fusion and SNARE regulation, are mediated through coiled-coil domains (Brunger, 2000). Necab1 contains an R-SNARE homology motif, and R-SNAREs bind to Q-SNAREs. Interestingly, we have demonstrated that Necab1 is a physiological binding partner of the neuronal Q-SNARE syntaxin 1a and that the interaction occurs through syntaxin's SNARE motif. Thus, it is highly conceivable that Necab1 binds to syntaxin through this domain. Interestingly, this is the domain where CAP binding has been mapped. Whether or not these interactions of Necab1 are independent of each other, remains to be resolved. Nevertheless, if Necab1 does bind syntaxin through its central coiled-coil, this would potentially interfere with CAP2 binding.

Necab1 dimerization was demonstrated to be calcium independent, and may function as a manner of regulating its interaction with other proteins. Necab1's affinity for itself is very high as is evident from the two-hybrid results. Thus, Necab1 homodimerization may be a preferred state, and in this state binding of other proteins may be prevented, as dimerization likely forms through the first two coiled-coils. The alleviation of the dimer would allow Necab1 to bind other proteins. Necab1 dimerization was first postulated to be a manner in which it could bind calcium, since it is a single EF-hand protein. Although this was not demonstrated, as dimerization occurred in the absence of calcium, the possibility still exists that calcium binding occurs when Necab1 homodimerizes. Necab1 may also form higher order multimer structures, however, this would have to be verified by size-exclusion chromatography.

Necab1's connection to the t-SNARE syntaxin 1a is very intriguing. Syntaxin 1a, along with the other neuronal SNAREs SNAP-25, and synaptobrevin, constitute the complex necessary for fusion of synaptic vesicles. Furthermore, Necab1's specific expression within the cortex and hippocampus suggests a role in long term potentiation; a specific form of neuronal communication that results in a postsynaptic enhancement. It is plausible that Necab1 provides a link between SNARE function and LTP since postsynaptic membrane fusion and LTP have been associated. The correlation between membrane fusion and LTP has been demonstrated by the introduction of blockers of synaptic fusion

within hippocampal neurons. These blockers such as peptide inhibitors, and botulinum toxin reduced the induction of LTP (Lledo et al., 1998). Additionally, introduction of SNAP into the postsynapse, an activator of synaptic transmission, resulted in a mechanistic enhancement similar to what occurs during LTP (Lledo et al., 1998). Thus, the molecular machinery that regulates exocytosis is present within the postsynapse of dendrites and acts to provoke LTP. Two scenarios exist where membrane fusion can function during LTP. Firstly, the machinery is thought to be involved in transport of a retrograde messenger that increases the probability of release (Malenka and Nicoll, 1999). In this case, the exocytic machinery would be made more competent to neuronal stimulation and result in an increase of neurotransmitter release. Secondly, the delivery and/or insertion of additional glutamate receptors within the postsynaptic membrane is thought to result in an increase in synaptic strength (Lledo et al., 1998). In agreement, the insertion and movement of new α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors occurs through SNARE-dependent exocytosis during LTP (Borgdorff and Choquet, 2002; Lu et al., 2001). Thus, it is plausible that Necab1 could modify vesicle fusion machinery in either the pre- and/or postsynapse, thereby resulting in an increase in synaptic strength.

The idea that Necab1 is a SNARE effector is not outlandish, as regulators of SNARE function do exist. For example, SNIP1 is a binding partner of SNAP-25 and acts to tether SNAP-25 to the submembranous actin cytoskeleton (Chin et al., 2000). Another SNAP-25 binding partner, spring, acts to regulate neurosecretion by inhibiting the formation of the SNARE complex (Li et al., 2001). In addition, amisyn, a syntaxin 1a binding protein, also regulates the formation of the SNARE complex (Scales et al., 2002). Moreover, the genetic connection between CAP and the yeast synaptobrevin homologue SNC1, suggests that CAP may function during SNARE dependent events.

# CHAPTER THREE

## Subcellular Localization of CAP2 and Necab1

### Introduction

The actin binding proteins, CAPs, contain a central poly-proline region, which is a protein interaction module that binds SH3 domains of other proteins (Mayer, 2001; McPherson, 1999). The yeast CAP poly-proline stretch binds SH3 domains of other proteins, and specifically bind Abp1 through this domain. Additionally, localization studies revealed that the poly-proline region is important for CAP targeting to cortical actin patches (Freeman et al., 1996). This localization was demonstrated to be dependent upon Abp1, as studies in an *abp1-* strain revealed that yeast CAP does not localize to cortical actin patches (Lila and Drubin, 1997). Further proof of the interaction-dependent localization was demonstrated through the inability of a poly-proline mutant of CAP to localize to cortical actin cytoskeleton patches.

The importance of a protein's localization is exemplified by the syntaxin family which display different subcellular localizations which correlate to different cellular functions (Teng et al., 2001). The Syntaxins (Stxs) mediate the fusion of vesicles on different organelles along the exocytic and endocytic pathways. For example, Stx 1a and b are localized to the presynaptic plasma membrane, and mediate the release of neurotransmitter vesicles. The more ubiquitously expressed Stx 5, localized to ER-Golgi boundary, and Stx 7 and 8, localized to endosomes, function during ER-Golgi transport and late endosome fusion; respectively. A second example is actin and its binding proteins through their localization to dendritic spines and neurite outgrowths (Kuhn et al., 2000; Luo, 2002; Smart and Halpain, 2000). Actin, ADF/cofilin, ARP2/3, VASP, and WASP are localized to neuron growth cones, and are also either present, or predicted to be at dendritic spines. The presence and function of these proteins correlates with the role for the actin cytoskeleton in outgrowths, and maintenance of spine stability. Thus, characterizing a protein's subcellular localization provides insight into its function.

In part due to the localization-dependant function of yeast CAP, we wanted to characterize the subcellular localization of human CAP2 and Necab1, in hopes of gaining insight into their functions and

the subcellular compartments of their interactions. We had previously shown that Necab1 interacted with membrane bound Syntaxin 1a (Chapter Two), which suggests that this interaction occurs at the plasma membrane. Previous studies (Vojtek and Cooper, 1993), in addition to our own overexpression studies in mammalian cells, have shown the hCAPs to be mainly cytosolic proteins. We further wanted to identify if CAP2 and Necab1 were localized to the plasma membrane, as this would suggest a function during membrane dependent process of vesicle exocytosis. Thus, subcellular fractionation studies were performed on mammalian cells overexpressing tagged HA-Necab1 and MYC-CAP2, and also on the endogenous proteins, in rat brain. It is demonstrated that Necab1 and CAP2 act mainly as soluble cytoplasmic proteins, but also have a membrane localization which is resistant to extraction. In the rat neuronal cell line PC12, it is demonstrated that endogenous CAP2 is localized to neurite extensions, where it co-localizes with overexpressed Necab1, but not with F-actin.

## Materials and Methods

*Cell Culture and Transfections*

PC12 cells were maintained in DMEM supplemented with 10% FBS, 5% horse serum (Sigma), and 100 units/ml of penicillin/streptomycin (Invitrogen/Life Technologies) in a humidified 5% CO2 atmosphere at 37°C. HEK293 cells were transiently transfected in 35 mm dishes with 4 µg each of pCI-HA-Necab1 and pCI-MYC-CAP2 expression plasmids via Lipofectamine 2000 reagent (Invitrogen/Life Technologies) as per manufacturer's instructions. PC12 cells were transfected at 90% confluency in 35 mm dishes with empty vector (pCI-HA) or HA-Necab1 with 4 µg of pCI-HA-Necab1 and 10 µl of Lipofectamine 2000 for 6 hr, as per manufacturer's instruction.

*Subcellular Fractionations*

Subcellular fractionation of HEK293 cells was carried out as per Kessels *et al.*(Kessels et al., 2000), with slight modifications. HEK293 cells were transiently transfected with HA-Necab1 and MYC-CAP2, as described above in two 35 mm dishes, and after the 6 hr incubation with the Lipofectamine - DNA

complex, were trypsinized off and replated into one 100 mm plate. 36 hrs post-transfection, confluent cells from two 100 mm plates were rinsed in ice-cold 1×PBS, and scraped off in 750 µl homogenization buffer (250 mM sucrose, 10 mM EGTA, 2mM EDTA, 20 mM Tris pH 7.5 plus protease inhibitor cocktail)/plate. Cells were then homogenized with 50 strokes of a glass/Teflon dounce homogenizer, and subsequently passed through a 25 gauge needle 20×. Homogenates were centrifuged at 3000 × g for 20 min to give S1 and P1, which contains unbroken cells, nuclei, mitochondria, and large pieces of plasma membrane. The first wash of P1 was pooled with the S1 fraction, and S1 was subsequently centrifuged for 30 min at 25,000 × g to give S2 and P2, which contains small pieces of plasma membrane, endoplasmic reticulum, and endosomes. S2 was centrifuged at 176,000 × g for 1 hr, to give P3 which contains light microsomes and small vesicles, and S3 which contains soluble, cytosolic proteins. Pellets were washed twice with homogenization buffer. Protein concentrations were determined using the Protein Bio-Assay Reagent (Bio-Rad), and 25 µg of each fractions was resolved on SDS-PAGE. Western blot analysis was performed using antibodies against HA, MYC, and actin as described in Chapter Two. Pyruvate kinase (1:5000) was used as a cytosolic marker, and AP2 (1:2000) was used as a membrane and vesicle marker. Results are representative of three trials. For endogenous localization in brain, a frozen rat brain was diced and homogenized in a similar manner in 6 ml of homogenization buffer, and the same procedure was performed.

*Rat Membrane Extractions*

For rat brain membrane extractions, procedure was followed as per Scales *et al.* and references therein (Scales et al., 2002). Fractionations were performed by homogenizing a rat brain in 7 ml of HB buffer (20 mM Hepes pH 7.2, 10 mM sucrose,10 mM KCl, 2mM EDTA, 2 mM EGTA, 6 mM $MgCl_2$, 1 mM dithiothreitol plus protease inhibitor cocktail) with fifty strokes of a dounce homogenizer, and subsequently passing through a twenty-gauge needle 10×. Homogenates (*H*) were centrifuged for 15 min at 1000 × g to give a post nuclear supernatant (*PNS*) which was then subsequently centrifuged at 20,000 × g for 15 min to give a membrane pellet (*M*), and a 20,000 × g cytosol (*20k-C*). The membrane pellet was washed once with 10 ml of HB buffer and then resuspended in 5 ml of buffer. 20k-C was

then centrifuged for 1 hr at 100,000 × g to generate a supernatant that contained true, soluble proteins (*C*). For the membrane extractions, the resuspended membrane pellet was aliquoted into 1 ml fractions in 2 ml micro-ultracentrifuge tubes, and 1 ml of one of the following was added to the tubes: distilled water as a negative control, 3 M NaCl, 4 M Urea, 0.4 M Sodium Bicarbonate pH 11, or 4% Triton X-100. The solutions were left on ice for 30 min to extract membranes, then subsequently centrifuged for 1 hr at 100,000 × g to generate a supernatant (*S*) and an insoluble pellet (*P*). The pellets were then subsequently resuspended in 2 ml of HB buffer, and equal portions of each *S* and *P* were fractionated via SDS-PAGE. Western blot analysis was then performed using anti-Necab1, anti-CAP2, anti-syntaxin 1a, and anti-Pyruvate Kinase antibodies as described.

*Immunocytochemistry and Confocal Microscopy*

6 hr post-transfection, PC12 cells were plated into Lab-Tek permanox chamber slides (Nalge-Nunc) that were coated with Matrigel basement membrane matrix (Becton Dickinson) (diluted 1:50) at approx. 30% confluency. Cells were differentiated for 4-5 days in DMEM containing 1% FBS, 0.5% HS plus penicillin/streptomycin, and 75-80 ng/ml nerve growth factor (NGF) (Sigma). After differentiation, cells were fixed in freshly prepared formaldehyde diluted to 3.7% in 1×PBS, and subsequently permeabilized in 0.5% Triton X-100 in 1×PBS. For detection of HA-tagged Necab1, the anti-HA monoclonal antibody (12CA5) was diluted 1:200 in 1×PBS, and visualized with an Alexa 568 conjugated goat anti-mouse secondary antibody (1:2000) (Molecular Probes) in 1×PBS. For detection of endogenous CAP2, anti-CAP2 rabbit polyclonal antibodies were diluted 1:40, and detected via a secondary Alexa 488 conjugated goat anti-rabbit secondary antibody (1:500) (Molecular Probes). F-actin filaments were visualized with Alexa 568-phalloidin (Molecular Probes), diluted 1:50 in 1×PBS. After rinsing in 1×PBS 0.05% Tween 20, once in 1×PBS, and briefly in distilled water, coverslips were placed on a drop of Slow Fade Light anti-fade reagent (Molecular Probes) and mounted on a microscope slide and sealed with nail polish. Visualization of subcellular localization was achieved using an MRC 1024 laser scanning confocal microscope (Bio-Rad). Images were processed using Confocal Assistant. Pre-immune sera and CAP2 antibodies clarified with GST-CAP2-N304 were used as controls for

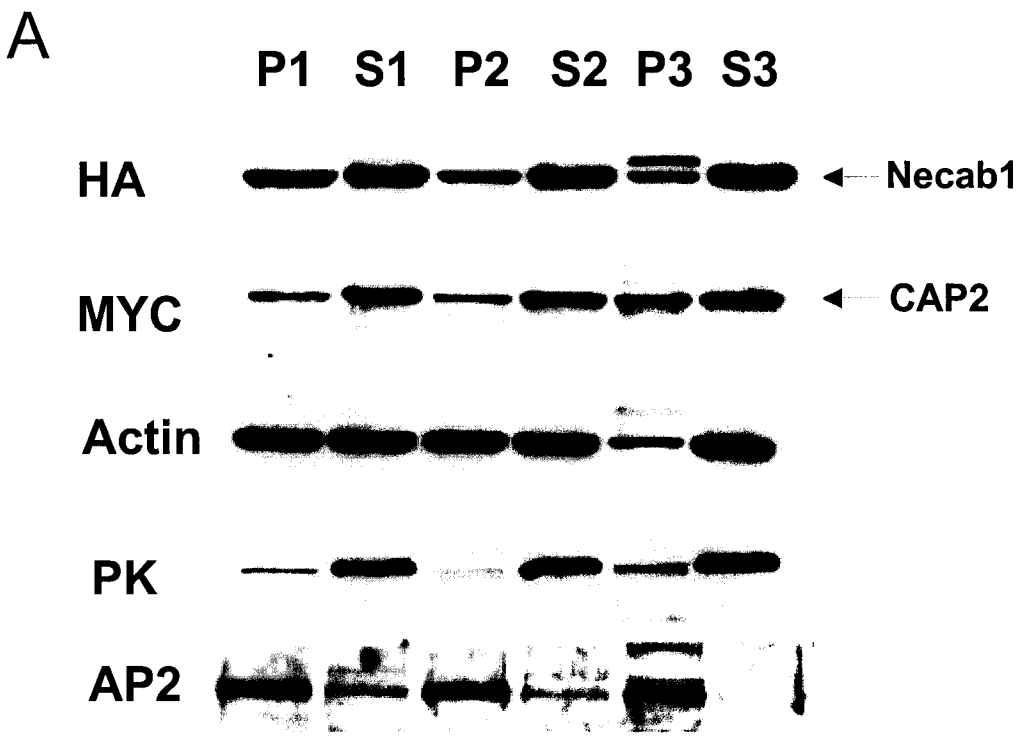
immunofluorescence.

## Results

### Necab1 and CAP2 are Cytosolic Proteins that also show a Membrane Localization

In order to further understand the function of Necab1 and CAP2, subcellular fractionations were performed in HEK293 cells overexpressing both proteins. As **Figure 13A** illustrates, Necab1 and CAP2 act mainly as cytosolic proteins, as they are mostly present in the supernatants, but are also present in comparable amounts in other fractions. Specifically, a substantial amount of Necab1 is found in P1 which contains large pieces of plasma membrane and nuclei. With CAP2, a substantial amount is found associated with P3 which contains microsomes and vesicles. Actin is not concentrated in any particular fraction, but is present in lower amounts in P3. The cytosolic protein Pyruvate Kinase (PK) was used to ensure proper cell disruption had occurred, and is concentrated only in supernatants. Alpha-adaptin 2 (AP2) was used as a membrane and vesicle compartment marker, and is only found within pellets. Since the antibodies against Necab1 and CAP2 were developed, the localization of the endogenous proteins was studied to validate the overexpression results. Fractionations were performed as with HEK293 cells, except that rat brain was used. As shown in **Figure 13B**, results for endogenous Necab1 and CAP2 agree with those of the overexpression studies. The proteins are mainly cytosolic, but also demonstrate some localization to other fractions. Specifically, a substantial amount of CAP2 is found in the microsomal/vesicle pellet, agreeing with the overexpression fractionation.

**Figure 13. Subcellular localization of CAP2 and Necab1.** ***A***, Subcellular fractionation of cells ovexpressing Necab1 and CAP2. HEK293 cells were co-transfected with HA-Necab1 and MYC-CAP2 and cells were subsequently fractionated via differential centrifugation. *P1* contains unbroken cells, nuclei, mitochondria, and large pieces of plasma membrane, *P2* contains small pieces of plasma membrane, endoplasmic reticulum, and endosomes, *P3* contains light microsomes and small vesicles, and, *S3* contains soluble, cytosolic proteins. Equal protein amounts of each fraction were then separated via SDS-PAGE and analysed by western blot for indicated proteins. Pyruvate Kinase (*PK*) was used as a cytoplasmic marker and *AP-2* as a microsomal and membrane compartment marker. *S*, supernatant, *P*, pellet. Results are representative of three trials. ***B***, subcellular fractionation of endogenous Necab1 and CAP2 in rat brain. Fractionations were performed as in ***A***, except that western blot analysis using the *anti-Necab1* and *CAP2* antibodies was performed, demonstrating similar results. *H*, homogenate.



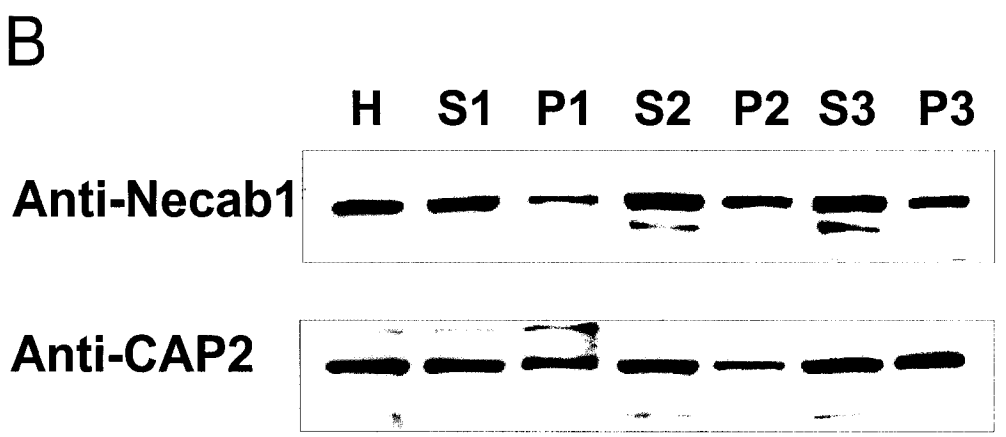




**Figure 13.**

The association of Necab1 with syntaxin 1a, led to the proposition that Necab1 may be localized to the plasma membrane; similarly with CAP2. Rat brain fractionations were performed to study the endogenous proteins and their presence in membrane fractions. Differential fractionation was performed to obtain a post-nuclear supernatant, membrane pellet, and cytosol. Necab1 and CAP2 once again behave as soluble proteins, as they are enriched in the cytosolic (*C*) fraction, but also display some membrane localization (*M*), similar to that of membrane bound syntaxin 1a (**Figure 14A**). Pyruvate Kinase was used as a cytoplasmic control, and demonstrates little membrane localization which can be considered background. To ensure that membrane enriched Necab1 and CAP2 are indeed membrane associated and tightly bound, membrane extractions were performed using different solute conditions. The membrane pellets from **Figure 14A** were extracted with either a control buffer, 1.5 M NaCl, 2M Urea, 0.2 M Sodium Bicarbonate pH 11, or 2% Triton X-100. As **Figure 14B** demonstrates, the membrane bound Necab1 and CAP2 resist extraction in the same manner as that of syntaxin 1a, which agrees with previously published reports (Scales et al., 2002). On the other hand, the cytoplasmic marker PK is washed out with control buffer, pH 11, and is completely extracted with Triton X-100. These results demonstrate that membrane enriched Necab1 and CAP2 are indeed tightly bound, and do not represent background cytosolic proteins.

**CAP2 is Localized to Neurites and Co-localizes with Necab1, but not F-Actin**

In order to gain further insight into a possible neuronal role for CAP2, immunocytochemistry on adrenal pheochromocytoma PC12 cells differentiated with nerve growth factor (NGF)was performed. Use of the anti-CAP2 antibodies demonstrated that CAP2 has a cytosolic localization within the cell body, but is also localized and concentrated to neurite outgrowths (*arrowheads*) (**Figure 15A**, **B** and **C**). The tips of neurite outgrowths contain filopodia and lamellipodia which are regions of high actin turnover. Pre-immune serum does not show any signal (**Figure 15D**), and neither do anti-CAP2 antibodies that were pre-absorbed with GST-CAP2-N304 (**Figure 15E**), demonstrating that the localization in **15A**, **B** and **C**, is specific to CAP2. The localization of CAP2 with F-actin filaments and

**Figure 14. Distribution of endogenous Necab1 and CAP2 in rat brain fractions.** ***A***, Necab1 and CAP2 are mainly cytosolic. A rat brain was homogenized (*H*) and fractionated to obtain the following: *PNS*, post nuclear supernatant; *M*, membrane pellet; *20k-C*, 20,000 × g cytosol; and *C*, 100,000 × g cytosol, containing soluble proteins. ***B***, membrane fractions of Necab1 and CAP2 resist extraction. The membrane pellet from ***A*** were divided in equal parts and extracted with different conditions. Treatments are as follows, *Con*, control buffer; *NaCl*, 1.5 M NaCl; *Urea*, 2 M urea; *pH 11*, 0.2 M sodium bicarbonate, pH 11.0; or *TX100*, 2% Triton X-100. Aliquots representing an equal percentage of each supernatant (*S*) and pellet (*P*) were loaded on 12% SDS-PAGE and immunoblotted with indicated antibodies. *Syntaxin 1* was used as a positive control for the membrane fraction and extractions, and pyruvate kinase (*PK*) was used as a negative control.

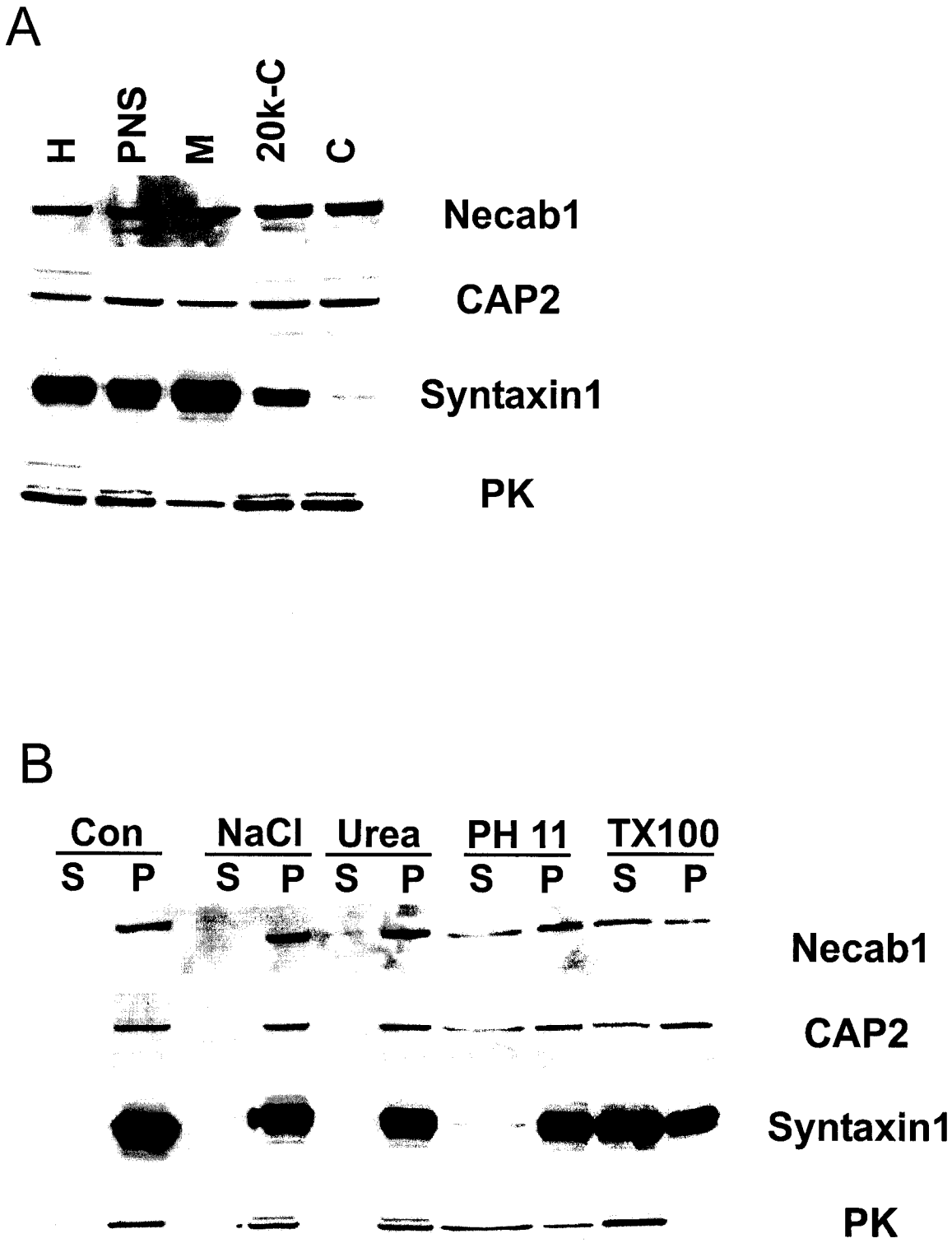



**Figure 14.**

**Figure 15. Localization of CAP2 in NGF-differentiated PC12 cells.** **A**, **B**, and **C** endogenous distribution of CAP2. PC12 cells were differentiated with 75 ng/ml of NGF over a five day period. Immunocytochemistry was performed using the anti-CAP2 polyclonal antibody and visualized via an secondary Alexa-488 anti-rabbit antibody conjugate. Arrowheads point to neurite tips where CAP2 is highly localized. ***D***, preimmune serum was used instead of the anti-CAP2 antibodies and does not result in any significant signal. ***E***, anti-CAP2 antibodies were preabsorbed with GST-CAP2-N304 and used for immunocytochemistry. No signal is observed indicating the immunolocalizations in ***A***, ***B*** and ***C*** are specific to CAP2.

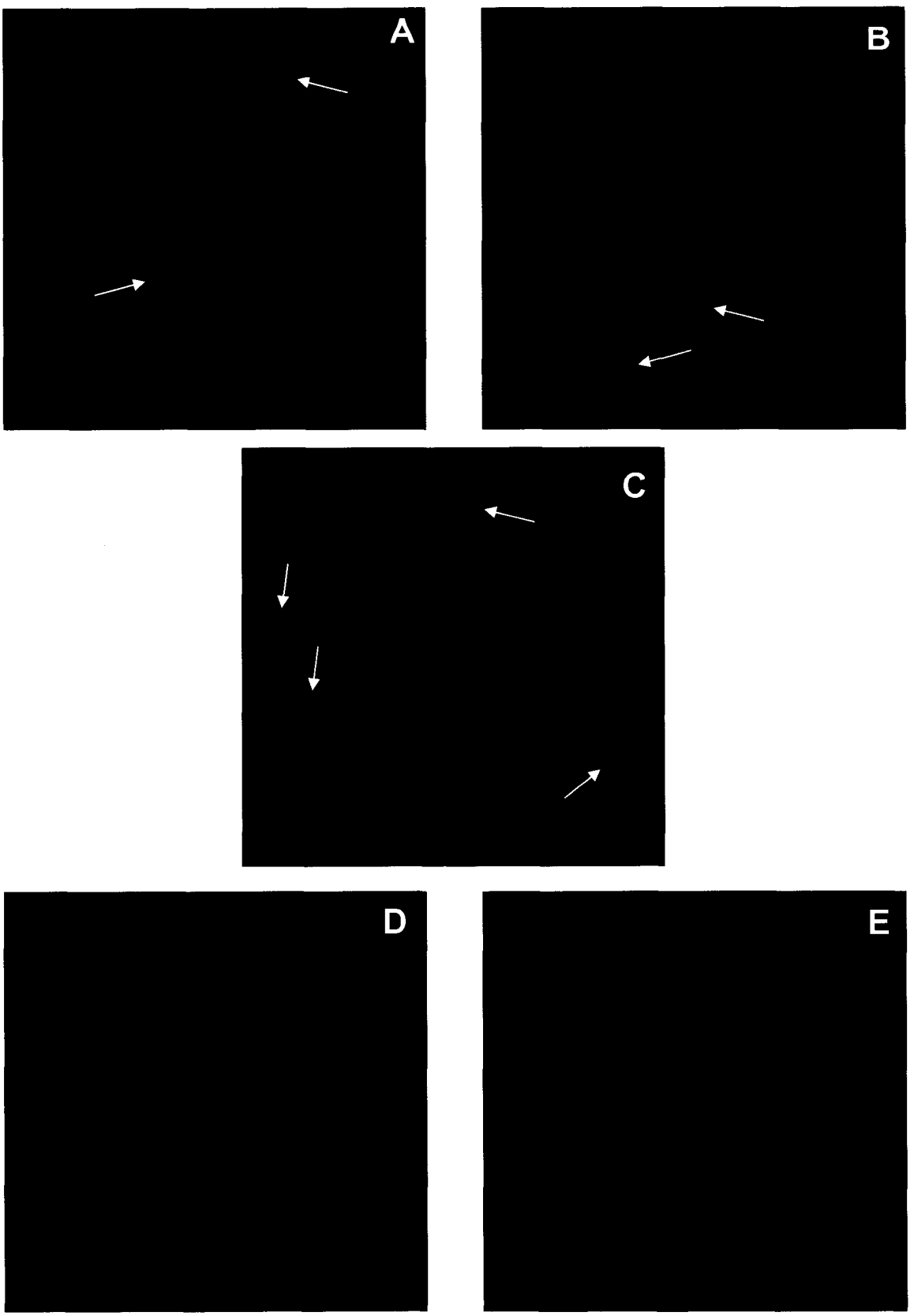



**Figure 15.**

Necab1 was also studied. Cells were stained for endogenous CAP2 and also for filamentous actin using an Alexa 568-phalloidin conjugate. Double labelling of cells shows the network of F-actin filaments (**Figure 16A** and **D**) and endogenous CAP2 (**16B** and **E**). The merged images reveals that CAP2 and F-actin do not co-localize, even though both are highly concentrated within neurites (**16C** and **F**). *Arrowheads* in these images depict examples where F-actin is localized to the very tips of outgrowths, with CAP2 localized to the periphery. Much to our surprise and lament, we were unable to find a cell line that expressed Necab1 (results not shown). Therefore, to study the co-localization of Necab1 and CAP2, PC12s were transfected with HA-Necab1 and double labelling experiments were performed with endogenous CAP2. **Figure 16G** and **J** demonstrate that Necab1 is present throughout the cytoplasm, and within neurites, agreeing with previously published reports (Sugita et al., 2002). In the same cells, CAP2 is once again present within the cell body, and concentrated to neurites (**16H** and **K**). The merged images demonstrate the co-localization (yellow) of Necab1 and CAP2 to neurites, and to a lesser degree within the cell body (**16I** and **L**). It is important to note that Necab1 is found at the very tip of neurite extensions, while CAP2 is not (*arrowheads*).

## Discussion

In this study, we demonstrate that Necab1 and CAP2 are mainly cytosolic proteins, but a fraction is also tightly bound to the plasma membrane. Immunolocalization studies in differentiated PC12s, reveals that CAP2 is concentrated to neurite outgrowths where it co-localizes with Necab1, but not F-actin. Overall, results demonstrate that both proteins are localized to regions where calcium mediated exocytosis and neurite outgrowth occurs, in which the dynamics of the actin cytoskeleton is essential to both processes.

**Figure 16. CAP2 co-localizes with Necab1 but not with F-Actin in NGF-differentiated PC12 cells.** PC12 cells were treated as in Figure 15 except that HA-Necab1 was visualized with the anti-HA antibody and an Alexa-568 secondary conjugate, and F-actin via Alexa 568-Phalloidin. Distribution of F-actin (***A***, ***D***), and overexpressed HA-Necab1 (***G***,***J***), with endogenous CAP2 (***B***, ***E***, ***H***, ***K***) in differentiated PC12s via double immunofluorescence labelling of cells. The superimposed images of F-actin and CAP2 (***C***, ***F***) demonstrates that their distribution does not overlap, while that of Necab1 and CAP2 does (***I***, ***L***). Note, arrowheads point to examples of the very tip of neurites where CAP2 does not co-localize with F-actin (***C***, ***F***) or Necab1 (***I***, ***L***).

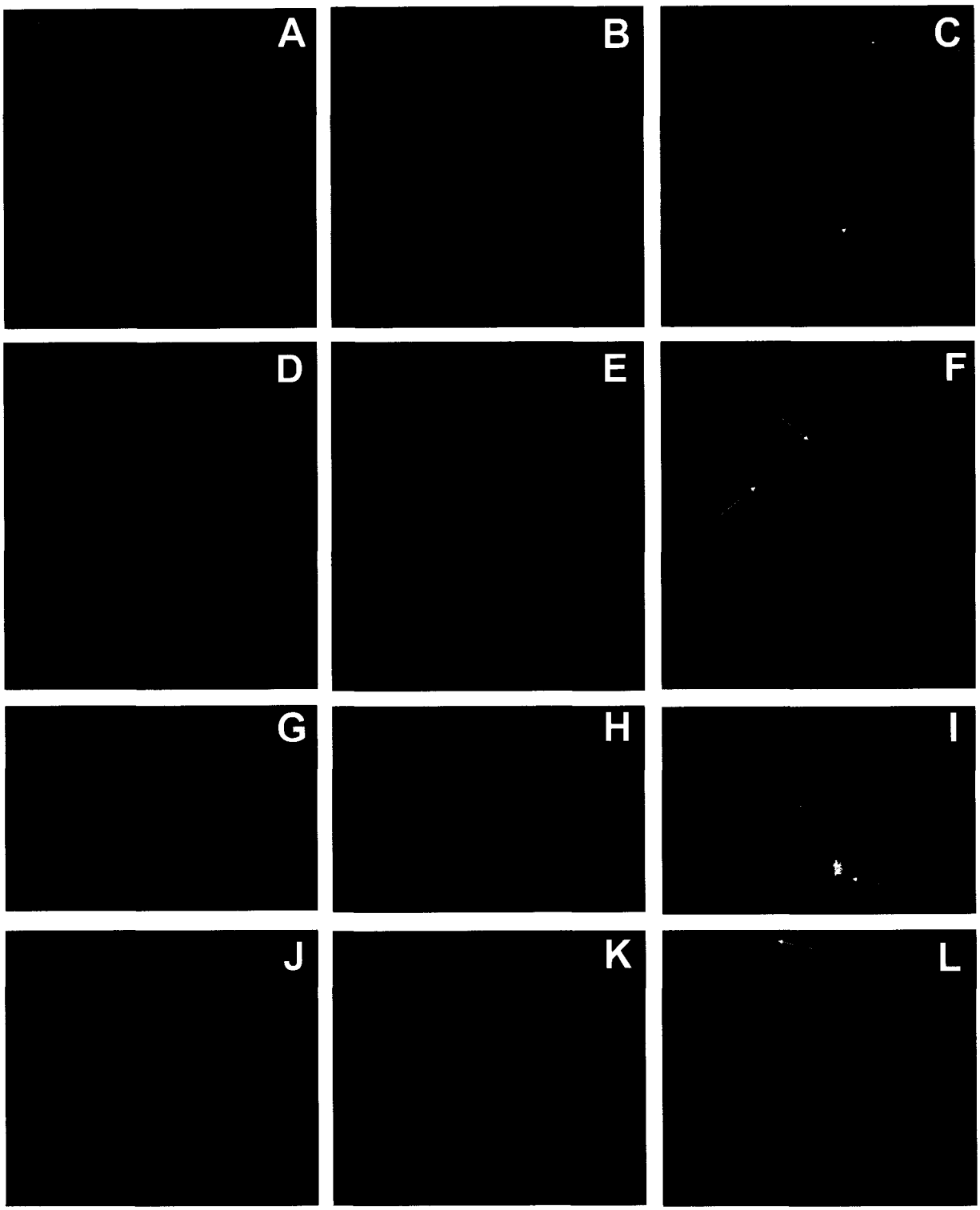



**Figure 16.**

**Co-Localization of Necab1 and CAP2 Suggests a Role in Neuronal Function**

We have demonstrated that Necab1 and CAP2 are cytosolic proteins that are also associated with the lipid membrane. Interestingly, since Necab1 can associate with syntaxin 1a *in vivo* (Chapter Two), this could be one manner in which it is localized to the plasma membrane. It is doubtful that Necab1 is associated with the membrane through weak electrostatic bonds, as it is not extracted with NaCl or pH 11.0 which are known to disrupt such interactions (Scales et al., 2002). This suggests that the localization may occur through covalent bonds. A mechanism for Necab1's membrane localization may occur through two predicted N-myristoylation sites (PROSITE motif search). Whether CAP2 is bound to the plasma membrane through syntaxin 1a is highly unlikely; we are unaware of any SNARE like motif within CAPs, and the mechanism of its localization can only be speculated. One possibility is that it may occur through the lipid messenger $PIP_2$. In *Dictyostelium*, CAP has been shown to have its actin binding properties inhibited by $PIP_2$,and is dependent upon the N-terminus of CAP (Gottwald et al., 1996). Further study of *Dictyostelium* CAP, reveals that its N-terminus is essential in localizing the protein to plasma membrane regions, and this property is independent of the poly-proline region (Noegel et al., 1999). Thus, $PIP_2$ may be important for the membrane localization of CAPs in general, though no proof exists for an interaction in mammalian homologues.

The membrane enrichment of CAP2 and Necab1 within rat brain is an important result, as it localizes these proteins to areas of neurotransmitter release. Also, brain fractionations revealed that a substantial amount of CAP2 is found within the vesicle/microsomal pellet, suggestive of CAP2 being associated with vesicles. Interestingly, the HIPs are actin binding proteins that are also associated with vesicles (McPherson, 2002). During neurotransmitter release the function is strengthened by the co-localization of Necab1 and CAP2 to neurites in NGF-differentiated PC12 cells. PC12 are rat brain cells of a neuroendocrine lineage that have many characteristics of sympathetic neurons, such as the release of neurotransmitter, and response to nerve growth factor (Greene and Tischler, 1976). Thus, they are a good model system for the study of basic neurobiology. Within such cells, the actin cytoskeleton is known to be present at the active zone of the presynaptic terminal of neurons. It is thought to act as a structural component of the terminal, forming a scaffold at the last step prior to

vesicle priming. The belief is that a dynamic change would be needed in the actin cytoskeleton which would then allow the transition to the last stage (Morales et al., 2000). This dynamic change has been demonstrated to occur through the GTPase Rac. Following the docking of vesicles, Rac acts through a yet unknown membrane effector(s), to allow the fusion competence of exocytosis (Humeau et al., 2002). The possibility that CAP2 may be a downstream effector in this signalling pathway will be discussed in Chapter Five. The co-localization of Necab1 and CAP2 also suggests a role during neurite outgrowth. Numerous proteins involved in the turnover of actin filaments also function in outgrowth of neurite extensions (Luo, 2002). These are areas of high actin turnover, and since CAPs are important regulators of actin dynamics; perhaps CAPs also have a function in this process. Interestingly, both Necab1 and CAP2 demonstrate an increase in expression during postnatal brain development. Necab1 is present at the embryonic stage and its expression substantially increases postnatally (Sugita et al., 2002). CAP2 has been reported to have a postnatal increase in expression within the brain (Fukuda et al., 2002). These results bode well with the idea that both proteins are involved in synaptogenesis, as other proteins that function during synapse formation display a similar expression pattern (Sugita et al., 2001). In vertebrates, the overall structure and makeup of the brain is formed prenatally, but synapses in brain are formed postnatally, which correlates with the dramatic postnatal increase in expression of synaptic proteins, such as synapsin, synaptobrevin 2, synaptotagmin 1, and synaptophysin (Daly and Ziff, 1997). Thus, CAP2 and Necab1 may have a similar function in the formation of synapses in immature neurons, and the maintenance of synaptic connections in mature neurons.

Different theories exist for the functional role of CAPs; one is that they act merely as actin monomer sequester proteins, thus acting to negatively regulate F-actin turnover (Gieselmann and Mann, 1992; Gottwald et al., 1996), while another theory is that they play a more active role in actin dynamics; acting as a monomer delivery molecules, thus aiding in the turnover of actin filaments (Moriyama and Yahara, 2002). That CAP2 does not co-localize with actin filaments structures within neurites supports the former theory. On the other hand, the fact that CAP2 is localized to the very periphery of F-actin rich networks lends support to the latter theory. In this case, CAP2 could assume

an active role in the delivery of monomers to these highly dynamic actin-rich structures. Thus, we suggest an amalgamation of these two ideas where the CAPs act to determine the spatial and temporal distribution of actin filaments; capable of preventing the formation of actin filaments, and when required, also promoting their formation. The form of role that CAPs assume would be dependent upon cell type, cellular processes and possibly even cell signalling events. This idea is supported by studies on the *Drosophila* homologue of CAP, capulet. Capulet was identified through a search for genes required for *Drosophila* oocyte polarity (Baum et al., 2000). *Capulet* germline clones had additional actin rich structures especially at polar sites, where the protein would normally localize, thus demonstrating that capulet accumulates in areas of the oocytes to inhibit actin polymerization. Furthermore, capulet was shown to function in limiting the formation of actin filaments in *Drosophila* epithelial cells (Baum and Perrimon, 2001). This role was antagonist to the function of cofilin and profilin, both proteins that promote the turnover of actin filaments. Another study that independently isolated *Drosophila* CAP, demonstrated that it is required to prevent the formation of actin filaments within the eye disc (Benlali et al., 2000). Thus, the distribution and actin binding properties of CAP2 would have to be meticulously regulated in order to maintain a proper functioning actin cytoskeleton. In further support of this theory, we made the observation that PC12 cells containing high levels of CAP2, had few actin filaments. In contrast, cells with large amounts of actin filaments expressed low levels of CAP2 (results not shown). Interestingly, Necab1 co-localizes with CAP2 at neurites, but is also found at the very tips where filopodia and lamellipodia are active. One possibility is that Necab1 may act as a modulator of CAP2's actin binding properties. How actin binding in mammalian CAPs is regulated is still a mystery. Necab1 may be an additional regulatory mechanism of CAP2 within neurons; preventing it from sequestering actin and disrupting dynamic actin structures at the very apex of neurite outgrowths. The functional aspects of the CAP2 - Necab1 interaction will be further addressed within Chapter Four and Five. Finally, our inability to find any cell line that expresses Necab1, suggests that Necab1 has an important physiological role that is not manifested within a cell line, but this role may be present in the grand scheme of neuronal function.

# CHAPTER FOUR

## Potential Functional Role for the CAP2 - Necab1 Interaction in Neuronal Processes

### Introduction

It is becoming clear that the actin cytoskeleton has an important role in the development and formation of neurons, and furthermore, their subsequent maintenance and function (Luo, 2002; Smart and Halpain, 2000). This is apparent, as the actin network involved in the formation of morphological structures such as filopodia, microvilli, and stereocilia, is also involved in the formation of neurons, and more specialized structure such as dendritic spines (Rao and Craig, 2000). For example, the neck of dendritic spines resembles structures of microvilli and sterocilia, actin rich structures of the post-synaptic density resemble that of filopodia, and the filamentous network within mature spine heads resembles that of lamellipodia (Rao and Craig, 2000). Similarly, proteins such as ARP2/3, cofilin, profilin, WASP, and Ena/VASP which function in the formation of basic actin networks, are also proposed to function in the morphological changes that occur in dendritic spines.

The array of actin binding proteins within the brain constitutes a manner in which these proteins need to be controlled, and different measures of control exist. One important regulatory mechanism of actin dynamics in the brain is LIMK1 which acts to phosphorylate and inactivate cofilin (Bamburg, 1999). Phosphorylation is a post-translational modification, and is one manner in which proteins are able to be regulated. Phosphorylation is carried out by a catalytic enzyme termed a protein kinase that can transfer a phosphate group from ATP, and incorporate it into another protein. The result is either a negative (inactivating), or positive (activating) effect on that target protein. Alternatively, a phosphatase can remove a phosphate from the protein, thus acting antagonistically to the kinase. The significance with regards to LIMK1, is that it allows it to act as a negative regulator in the turnover of actin filaments. Additional discovery of the regulatory mechanism in actin dynamics will provide a clearer picture on the function of the actin cytoskeleton.

In yeast, CAP is known to be involved in a cell signalling cascade involving Ras and its effector adenylyl cyclase, but this has not been demonstrated to occur in other eukaryotes. The concept of human CAPs linking cell signalling events to the dynamics of the cytoskeleton is not fully apparent outside of yeast and the manner in which CAPs in general are regulated is unknown. Therefore, the possibility that CAP2 was regulated at the level of phosphorylation, and thus involved in a cell signalling cascade was explored. *In vivo* labelling assays reveal that CAP2 is indeed phosphorylated and occurs within the C-terminal 232 amino acids. In addition, through immunoprecipitation analysis it is revealed that when CAP2 is bound to Necab1, it is unable to bind actin. Furthermore, through the use of actin polymerization assays, it is for the first time demonstrated that CAP2 is a potent inhibitor of actin polymerization. Necab1 does not seem to have any effect on the ability of CAP2 to bind G-actin in this assay, though Necab1 itself has a substantial inhibitory effect.

## Materials and Methods

*Molecular Cloning*

The pCI-HA-CAP2-N304 clone was constructed by using the forward primer *#31* and the reverse primer *#109* (**Appendix A**) in a PCR reaction, using pCI-HA-CAP2 full-length as a template. pCI-HA-CAP2-C232 was constructed using the forward primer *#108* and the reverse primer *#32* (**Appendix A**) in a as described earlier. The PCR fragments were subsequently digested with EcoR1 and Xho1 and ligated into pCI-HA cut with the same restriction enzymes.

*Functional Immunoprecipitation*

The immunoprecipitations were performed as desribed in Chapter Two, except that the anti-HA antibody was not preconjugated to Protein-A beads. Briefly, HEK293 cells were transfected as described in Chapter Two. Cells lysates were prepared in RIPA buffer, and 200-300 µg of total protein extract were brought up to a volume of 250 µl of RIPA, and 250 µl of RIPA containing either 6 mM

$CaCl_2$, or 6 mM EGTA was added for a total volume of 500 µl. Extracts were then incubated with Protein A beads and precleared for 30 min. Precleared extracts were then incubated with the anti-HA (12CA5) antibody for 1 hr, and immune complexes were collected by the addition of Protein A beads, and incubated for another hour. Immune complexes were then washed 4× for 10 min each with 500 µl of RIPA buffer containing either 3 mM $CaCl_2$ or 3 mM EGTA, boiled off in 1× sample buffer and analyzed via Western blot analysis using a monoclonal anti-HA, anti-MYC, and anti-Actin antibody. All immunoprecipitations were carried out at 4°C.

*Actin Polymerization Assays*

The Actin Polymerization Biochemistry Kit (#BK003 Cytoskeleton Inc.) was used to study the effect of CAP2 and Necab1 on actin polymerization using pyrene-labeled actin (10% of total actin). GST protein preparations were performed as described in Chapter Two, except that proteins were concentrated, and buffer exchanged into Storage Buffer (10 mM Tris, pH 8.0, 25 mM NaCl, 1 mM DTT + 10% glycerol). Recombinant Necab1 used in this study was cleaved free of GST. Prior to performing actin polymerization assays, lyophilized G-actin was resuspended in Buffer A (5 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.01% $NaN_3$, and 0.2 mM ATP) and incubated overnight at 4°C to ensure full depolymerization of G-actin. The next day G-actin was centrifuged at 150,000 × g for 1 hr at 4°C to remove any nucleating centres. A Varian Eclipse fluorescence spectrophotometer was set at the following conditions to measure polymerization: excitation wavelength at 365 nm with a 10 nm slit bandwidth; emission wavelength set at 407 nm wavelength with a 10 nm slit bandwidth, and the PMT voltage set at medium. The concentration of G-actin to protein studied, was set at a 1:1 ratio. 4 µM G-actin (10% pyrene labelled) was incubated with 4 µM of GST, GST-CAP2, Necab1, GST-CAP2 + Necab1, or Storage Buffer alone with the addition of Supplementary Buffer (3.45 mM Tris, pH 8.0, 0.262 mM ATP, 0.262 $CaCl_2$), so that a final low salt buffer was constituted (5 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 4.8 mM NaCl, 1.9% glycerol). This 400 µl mixture was allowed to incubate and equilibrate at 25°C for 30 min. After incubating for 30 min, 40 µl of 10× Actin Polymerization Buffer (500 mM KCl, 20mM $MgCl_2$, 10 mM ATP) was mixed and transferred to a 500 µl quartz fluorometric cuvette and the

kinetics of the reaction was measured by reading the fluorescence (arbitrary units) every minute for a total of 55 min at 25°C. The time required to mix the proteins with Actin Polymerization Buffer and transfer to cuvette was noted, and a standard time was allotted from the point of mixing, to the point of fluorometer reading. Raw data was entered into Microsoft Excel, where the Fluorescence (arbitrary units) was plotted against time (minutes).

*In Vivo Phosphorylation Assays*

HEK293 cells were transfected via Lipofectamine 2000 in 60 mm dishes, 24 hr prior, with the pCI-HA constructs of CAP2, CAP2-N304, and CAP2-C232 as described in Chapter Two. Cells were rinsed twice with phosphate-free Dulbecco's modified Eagle's medium (Life Technologies) containing 10% FBS, and subsequently phosphate starved in the same media for 15 min. Cells were once again rinsed with phosphate free media, plated in1.2 ml of media, then $^{32}PO_4$ ( 10 mCi/ml, in $H_20$; Amersham Pharmacia Biotech Inc.) was added directly to the cells at a final concentration of 0.3 µCi/ml, and incubated at 37°C with gentle agitation every 30 min for 4 hr. After incubation, cells were rinsed twice with ice cold 1×PBS and scraped off in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM KCl, 1% IGEPAL CA-630 (NP-40), 10% glycerol) containing a protease inhibitor cocktail (Complete Mini, Roche), and briefly sonicated on ice. Extracts were then clarified by centrifuging at 12,000 × g for 10 min at 4°C. 300 µg of protein were then brought up to final volume of 500 µl with RIPA, and IPs were performed as described in Chapter Two. Cell extracts samples (25 µg) and IPs were then separated on SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was then exposed to autoradiography film for five days and subsequently developed. Western blot analysis using the anti-HA antibody was then performed on the same membrane to ensure the proper expression and immunoprecipitation of fusion proteins.

## Results

### Necab1 Inhibits CAP2 Actin Binding

In order to understand the functional purpose of the Necab1-CAP2 interaction, an immunoprecipitation was performed to study the effect calcium had on the interaction, and what effect Necab1 had on the actin binding properties of CAP2. The IPs were performed as in Chapter Two; HA-Necab1 and MYC-CAP2 were co-expressed in mammalian cells, and HA-tagged proteins and complexes were immunoprecipitated using the anti-HA antibody. As **Figure 17** demonstrates, the addition of 3 mM $CaCl_2$ to the RIPA buffer did not have a substantial effect on the interaction between Necab1 and CAP2 (first two lanes of IP). Furthermore, no actin is pulled down in the complex with Necab1 and CAP2, suggesting that when the proteins interact, Necab1 prevents CAP2 from binding actin in a calcium independent manner. Necab1 itself does not demonstrate any actin binding properties, with or without the presence of $Ca^{2+}$ (lanes three and four of IP). As a control for actin binding, CAP2 bound actin, and is not affected by $Ca^{2+}$ (lanes five and six of IP). The *Extracts* lane reveals that all proteins are properly expressed. Therefore Necab1, independent of $Ca^{2+}$, interacts with CAP2 and prevents actin binding.

### CAP2 is a Potent Inhibitor of Actin Polymerization

To further study whether CAP2 and/or Necab1 can affect actin dynamics, actin polymerization assays were performed. These assays are based on the enhanced fluorescence that occurs when pyrene labelled G-actin polymerizes into pyrene F-actin. As the pyrene labels come in closer contact with each other, an increase in fluorescence is observed. An important factor in the equilibrium between G and F-actin is the Critical Concentration (CC), described as the actin monomer concentration below which actin will not polymerize. By changing conditions of ionic type and strength, this affects the CC. For example at low ionic strength the CC is high, but upon addition of $Mg^{2+}$ and KCl, the CC decreases substantially. Thus, increasing ionic strength, can induce the polymerization of actin. This increase in fluorescence can be followed over time by the use of a flourescent spectrophotometer.

**Figure 17. Functional Immunoprecipitation of Necab1 with CAP2 and Actin.** Bindings were performed in a similar manner as described in **Figure 8B**. HEK293 cells extracts expressing HA- and/or MYC-tagged fusion proteins were prepared in RIPA buffer. Protein extracts were then prepared in the presence of 3 mM $CaCl_2$ ($Ca^{2+}$ +) or containing 3 mM EGTA ($Ca^{2+}$ -) and incubated with anti-HA antibody. Immune complexes were then subsequently collected by the addition of Protein-A beads. Complexes were washed extensively with RIPA buffer containing 3 mM $CaCl_2$ or 3 mM EGTA and boiled off in sample buffer. Protein extracts samples (*Extracts*) along with immunoprecipitations (*IP*) were subjected to SDS-PAGE, and western blot analysis was performed using *anti-HA* (12CA5), *anti-MYC* (9E10), or *anti-actin* antibodies. Presence (+) and absence (-) of constructs used is denoted. Arrows to the right of blots indicate bands that correspond to indicated fusion proteins, while other bands represent the heavy chain of the anti-HA antibody. Note: the curving of protein bands is an artifact of the gel.

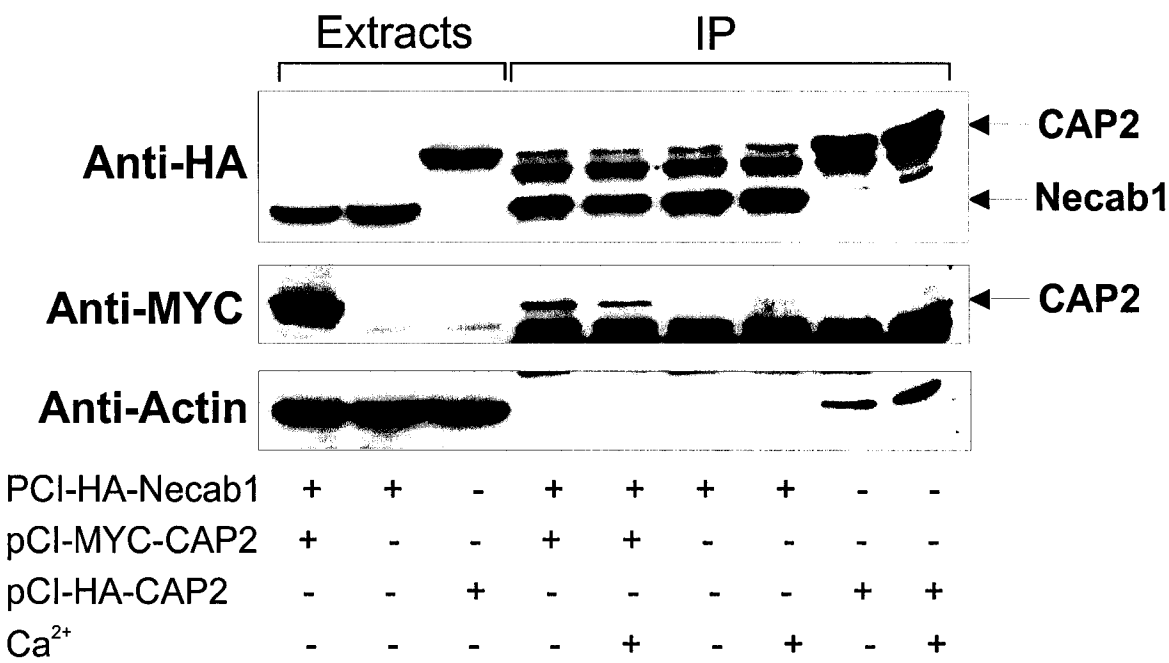



**Figure 17.**

Recombinant proteins of GST-CAP2 and Necab1 (GST-cleaved) were produced to study their effect on actin polymerization, and whether any cooperative or inhibitory interactions would occur. As **Figure 18** demonstrates, addition of *Buffer* alone (Storage Buffer) demonstrates a typical curve of actin polymerization. This also verified that Storage Buffer alone did not alter the kinetics of polymerization, in addition to it having no increase in fluorescence prior to the addition of Actin Polymerization Buffer (results not shown). What effect the addition of proteins would have on actin polymerization at a ratio of 1:1 G-actin was then studied. GST alone, as a control, had a small effect on limiting the rate of actin polymerization and was used to standardize the background. Conversely, GST-CAP2 was a potent inhibitor of actin polymerization, as demonstrated by the lack of fluorescence. Surprisingly, the addition of Necab1, resulted in a substantial decrease in the polymerization of actin. This is an unexpected result as we are unaware of any actin binding motifs within Necab1, and no actin binding was demonstrated in immunoprecipitational analysis. Furthermore, Necab1 does not seem to alter the ability of CAP2 to inhibit actin polymerization when both proteins are studied together in the assay. Thus, results demonstrate that CAP2 inhibits the formation of F-actin, and this effect is not alleviated by the addition of Necab1.

**CAP2 is a Phosphorylation Target**

Little information is known about how CAPs are regulated, and whether or not CAPs are phosphorylated. Thus, *in vivo* phosphorylation assays, wherein HEK293 cells were transfected with different HA-CAP2 constructs, and subsequently cultured in the presence of radioactive ortho-phosphate were performed. Cells were transfected with HA-tagged full-length CAP2, an N-terminal mutant expressing the first 304 amino acids (*CAP2-N304*), and a C-terminal mutant expressing the last 232 amino acids (*CAP2-C232*). After labelling, immunoprecipitations were performed on extracts, proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and exposed to autoradiography film. Surprisingly, wild-type CAP2 and CAP2-C232 are phosphorylated, while CAP2-N304 is not (**Figure 19A**). Interestingly, the C-terminal deletion mutant seems to be highly phosphorylated when compared to wild-type CAP2. As a control, immunoprecipitation from cells extracts expressing

**Figure 18. Actin Polymerization Assays of CAP2 and Necab1.** Different GST-tagged fusion proteins or buffer alone were incubated with pyrene labelled G-actin in a 1:1 ratio and actin polymerization was induced by the addition of actin polymerization buffer. The subsequent increase in fluorescence was measured every minute in a fluorescence spectrophotometer for a total of 55 min and data was plotted as *Fluorescence* (a.u.) against *Time* (minutes) using Microsoft Excel.

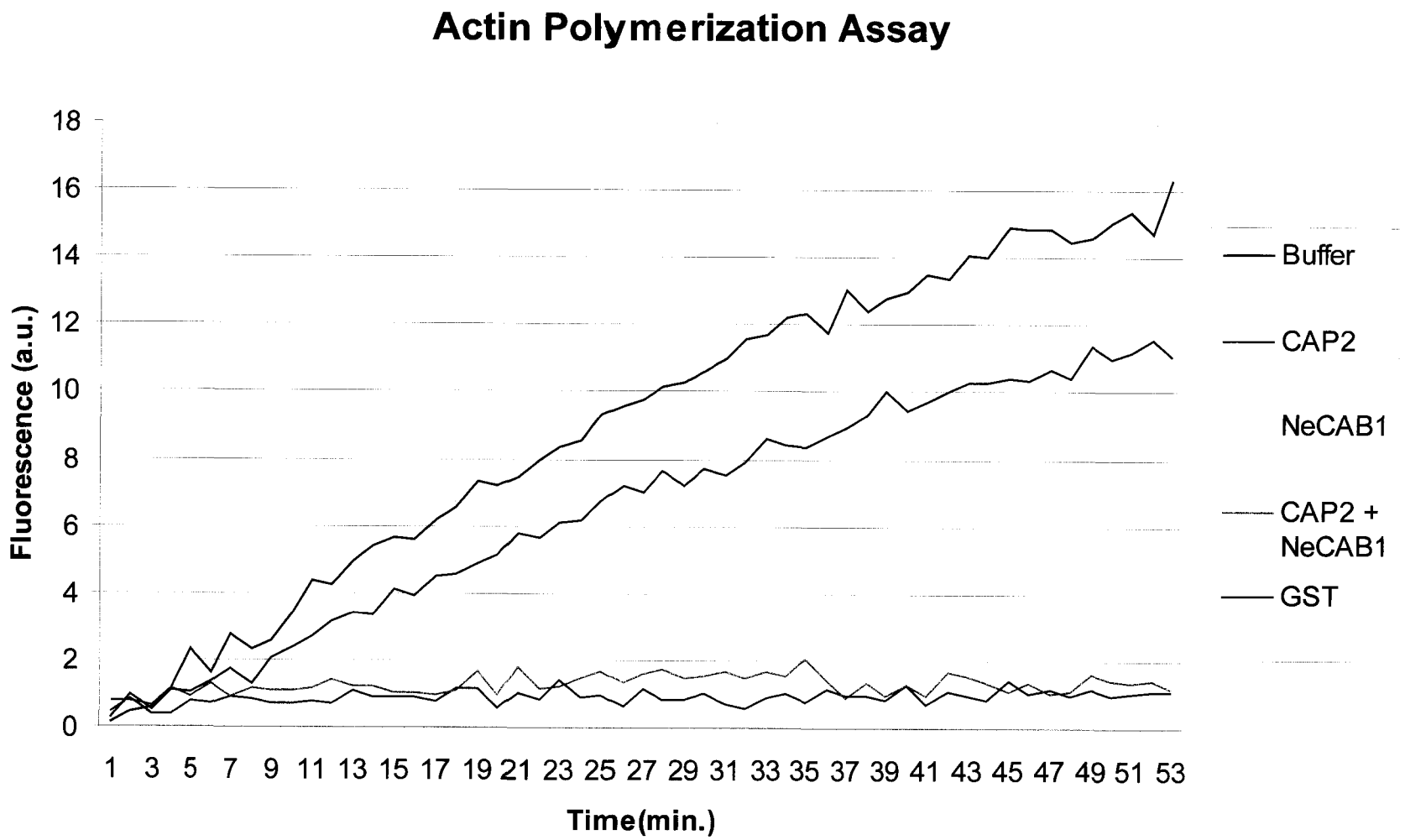
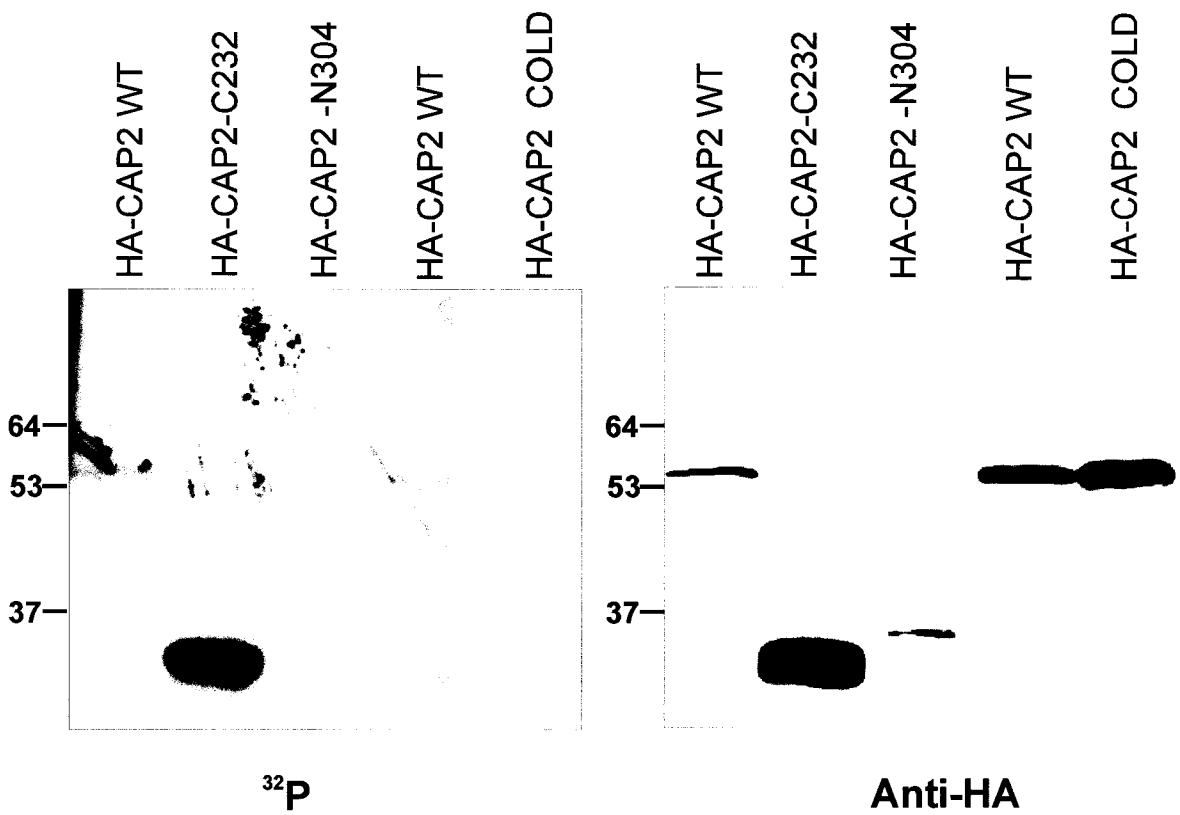



**Figure 18.**

**Figure 19. CAP2 is phosphorylated within the C-terminus and likely occurs within a highly conserved consensus sequence.** ***A***, *in vivo* labelling of cells expressing different HA-tagged CAP2 constructs with $^{32}$P ortho-phosphate, demonstrates that CAP2 is phosphorylated within the C-terminus. Labelled cell extracts expressing the empty vector pCI-HA (*HA*), pCI-HA-CAP2 WT (*wild-type*), pCI-*HA-CAP2-C232*, pCI-*HA-CAP2-N304*, or unlabelled wild-type CAP2 *(HA-CAP2 COLD*) were immunoprecipitated using anti-HA (12CA5) conjugated Protein-A beads. Precipitated proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and exposed to autoradiographic film for five days ($^{32}P$). Western blot analysis was subsequently performed using *Anti-HA* antibodies on the membrane to confirm the immunoprecipitation of the indicated constructs. Approximate protein standard sizes are indicated in kDa. ***B,*** the highly conserved consensus sequence of human CAP2 that is present within the C-terminus of all CAPs. Residues that are underlined represent those that are conserved at greater than 80% in all CAPs. The serines that are highlighted and in bold, represent residues that are highly predicted to be phosphorylation sites by the NetPhos 2.0 phosphorylation prediction program (out of 1.000). The first serine in grey is conserved in all vertebrate CAPs and has score of 0.942, while the white residue is conserved in the majority of all CAPs and has a score of 0.995. The amino acid residue numbers of CAP2 are illustrated next to the amino acids.

A




B

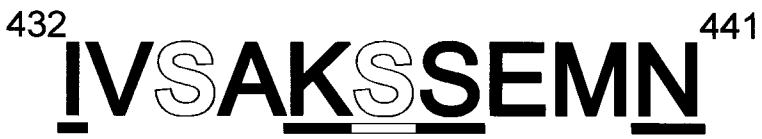



**Figure 19.**

HA-CAP2 which were not labelled with radioactive phosphate, do not appear on the autoradiograph (*HA-CAP2 COLD*). Western analysis reveales that all constructs are immunoprecipitated. Since the phosphorylation assays revealed that CAP2 was phosphorylated in the C-terminus, the last 232 amino acids of CAP2 were analyzed for any conservation among CAP proteins by performing a Clustal W alignment (Hubberstey and Mottillo, 2002), and also searched for consensus phosphorylation sites using NetPhos 2.0. A single motif within the C-terminal 232 amino acids of CAP2 was identified. This consensus sequence is highly conserved within all CAPs, and contains one serine (S-437) that is conserved from yeast to mammals, and a second serine (S-434) conserved in all vertebrates, and both are highly predicted to be phosphorylation sites (0.995 and 0.942 out of 1.000; respectively) (**Figure 19B**). Thus, it has been revealed that CAP2 is phosphorylated, and a highly conserved consensus sequence within the C-terminus where this phosphorylation may occur has been identified.

## Discussion

### Necab1 is a Potential Effector of CAP2

In order to gain insight into a functional purpose for the Necab1 - CAP2 interaction, immunoprecipitations were performed that studied the role $Ca^{2+}$ had on the interaction, and furthermore, if Necab1 modulated CAP2's binding of actin in any manner. Binding studies demonstrate that when CAP2 is bound to Necab1, no actin binding occurs, and that this inhibition is independent of $Ca^{2+}$. Perhaps this is one manner in which CAP2's actin binding is regulated. This role for Necab1 may act as an additional control for CAP2 during actin dependent processes that occur specifically in neuronal cells of the cerebral cortex and hippocampus.

Actin polymerization assays demonstrated that CAP2 is a potent inhibitor of F-actin formation. This is in agreement with other CAPs, as yeast and *Dictyostelium* also inhibits the polymerization of actin (Freeman et al., 1995; Gottwald et al., 1996). CAP2 may have a similar function *in vivo*, as it does *in vitro;* that is to regulate the polymerization of F-actin. This function has been proposed for the *Drosophila* CAP homologue (Baum et al., 2000). The significance of this is that CAP activity would be

essential in determining when and where actin filaments are formed, and agrees with the idea formulated in Chapter Three. This role for CAP has been proposed in *Drosophila* epithelial cells, where it acts to control the apical formation of actin filaments synergistically with the Ableson Tyrosine Kinase (Abl) (Baum and Perrimon, 2001).

To further characterize the possible synergistic and/or inhibitory role that Necab1 and CAP2 have on actin dynamics, additional actin polymerization assays were performed. The concomitant addition of Necab1 and CAP2 in these assays did not differ from that of CAP2. Though this suggests that Necab1 does not have an effect on CAP2 actin binding in these *in vitro* assays, this scenario is not ruled out *in vivo*. Reasons for this apparent contradiction exist: the effector role of Necab1 may be regulated, and a signal that is only apparent *in vivo* is required; other proteins may be needed to manifest this effect of Necab1; the self-association of Necab1 may be a preferred state, and would need to be alleviated before it binds CAP2; and we are unaware of the kinetics for the Necab1 - CAP2 interaction, as a 1:1 ratio of proteins used in the polymerization assay is not the proper stochiometry seen *in vivo*. We suggest that Necab1 acts *in vivo* as an effector to regulate the actin sequestering property of CAP2, and thus its ability to inhibit actin filament formation. A substantial inhibition of actin polymerization was also demonstrated by Necab1. We are unaware of any cryptic actin binding motifs within Necab1 which would explain the inhibition of filament formation. Though, this may explain why the ability of Necab1 to alleviate CAP2 actin binding was not demonstrated in these assays.

**CAP2 May Exist in a Cell Signalling Cascade**

In order to understand how CAPs in general may be regulated, *in vivo* phosphorylation assays were performed demonstrating that CAP2 is phosphorylated within the C-terminus. The only other known form of CAP regulation has been demonstrated in *Dictyostelium*, where $PIP_2$ inhibits actin binding, and is dependent upon the N-terminus of CAP (Gottwald et al., 1996). In a separate study, Benali *et al.* studied the function of *Drosophila* CAP by performing overexpression studies in eye disc. The disruption of CAP expression in eye disc perturbed the pattern of eye differentiation, while overexpression of CAP had no discernable effect, suggesting that CAP is regulated (Benlali et al.,

2000). Due to the inhibitory role of $PIP_2$ in *Dictyostelium*, Benali *et al.* overexpressed the C-terminus of CAP in hopes of alleviating the possible inhibition. Surprisingly, no apparent phenotypes were observed, suggesting that $PIP_2$ regulation is not conserved within the C-terminus (Benlali et al., 2000), and furthermore, that a regulatory domain exists within the C-terminus. This agrees with our analysis on the last 232 amino acids of CAP2, wherein a domain that exists within all known CAPs, and is a consensus sequence for phosphorylation has been identified.

Serine(S)-434 of CAP2 is conserved in all vertebrate CAPs and has a phosphorylation potential of 0.942 out of 1.000. Interestingly, S-434 is predicted phosphorylation site for protein kinase C (PKC). PKC is a family of phospholipid-dependent serine/threonine kinases, wherein some members are calcium dependent due to a $C_2$ domain. PKC is involved in a wide-array of cellular processes, and more recently has been implicated in the organization of the actin cytoskeleton (Keenan and Kelleher, 1998). A role for PKC has also been demonstrated during $Ca^{2+}$ mediated exocytosis in chromaffin cells. In chromaffin cells, the actin cytoskeleton acts as a barrier at the plasma membrane for the release of vesicles. PKC, via phosphorylation, was demonstrated to activate a cascade leading to the subsequent disassembly of cortical F-actin, and an increase in the release of vesicles (Rose et al., 2001). A PKC isoform is also implicated in the outgrowth of neurites. $PKC_\epsilon$ contains an F-actin binding domain, which is crucial for its activation in neurite outgrowth through an unknown upstream signal (Zeidman et al., 2002). PKC is also implicated in signalling the disassembly of actin stress fibers and the subsequent formation of membrane ruffles in smooth muscle cells via the down-regulation of Rho (Brandt et al., 2002). Interestingly, the identified consensus sequence in CAP2 is just upstream of its actin binding motif. Thus, it would be intriguing to propose that phosphorylation of CAP2 by PKC, may regulate its actin binding properties.

A second consensus site for phosphorylation, S-437, exists within the C-terminus of CAP2 and is highly conserved from yeast to mammals. Only *Candida albicans*, and *Lentinula edodes* do not fit the pattern, suggesting that they may have lost this possible phosphorylation site during evolution. S-438 is also a well conserved serine, though it is not a predicted phosphorylation site. At this site yeast *S. cerevisiae* and *S. pombe*, and *Candida* contain a threonine instead of a serine; still constituting a

residue for phosphorylation. Thus, either of these two residues may be a manner in which CAPs in general are regulated. Studies are underway to determine if they do play a role in CAP phosphorylation. No current proof exists for CAP involvement in a Rho GTPase family signalling cascade, even though it is a common manner in which actin binding proteins are regulated. For example, mAbp1, the mammalian homologue of yeast Abp1, which has been shown to interact with yeast CAP, acts in a Rac1 signalling cascade (Kessels et al., 2000). Cofilin, which has recently been demonstrated to bind human CAP1 (Moriyama and Yahara, 2002), acts through Rho, Rac, and Cdc42 GTPases (Bamburg et al., 1999; Luo, 2002). Thus, it would be interesting to propose that the CAPs also act through the Rho family of GTPase, and these studies are currently underway.

*In vivo* labelling studies suggest that phosphorylation of CAP2-C232 is higher than that of full-length CAP2. This is evident in two instances. Firstly, with the two sets of wild-type CAP2, the level of phosphorylation is the same, but the amount immunoprecipitated differs. Secondly the amount of CAP2-C232 phosphorylated is substantially higher than wild-type CAP2, even though more CAP2-C232 is pulled down. This suggests that a potential regulatory domain of CAP2 phosphorylation exists within the N-terminus. All CAPs contain dimerization domains mapped to the N-terminus, and a second one within the C-terminus. The N-terminus can also interact with the C-terminus of CAP, suggesting that an intramolecular interaction occurs (Hubberstey and Mottillo, 2002). The increased phosphorylation observed in CAP2 due to deletion of its N-terminus, is reminiscent of the autoinhibition that is observed in Neuronal-Wiscott-Aldrich Syndrome Protein (N-WASP). N-WASP is an activator of the ARP2/3 complex, wherein ARP2/3 itself is a weak inducer of actin nucleation and polymerization. Binding of N-WASP accelerates the activity of ARP2/3, leading to the *de novo* nucleation and branching of filaments (Prehoda and Lim, 2002). Intramolecular interactions within N-WASP normally block the activation region of the ARP2/3 complex. Upon cellular stimuli, Cdc42 and $PIP_2$ bind N-WASP, relieving the inhibitory conformation, and an active N-WASP can then act upon ARP2/3 (Pufall and Graves, 2002). Thus, a similar autoinhibition mechanism may exist within CAPs, wherein its autoinhibitory conformation prevents phosphorylation within the C-terminus. Likewise, this could regulate actin binding, as phosphorylation may result in activating or deactivating this property.

# CHAPTER FIVE

## General Conclusions and Future Studies

This thesis characterizes a novel protein, Neuronal Calcium Binding Protein 1 (Necab1), and a role for the human CAPs in neuronal function. Results demonstrated that Necab1 is preferentially expressed in brain, specifically in the regions of the cerebral cortex and hippocampus where CAP2 is also present at high levels. Immunoprecipitation studies in addition to two-hybrid analysis, demonstrated that Necab1 is capable of dimerizing independent of calcium. Immunolocalization studies revealed that both Necab1 and CAP2 co-localize to neurite processes. Furthermore, CAP2 is potent inhibitor of actin polymerization *in vitro*, and does not co-localize with F-actin in neurons. Immunoprecipitation studies demonstrated that Necab1 may be an effector of CAP2. Additionally, the following statements can be concluded that were initially proposed in the Thesis Objectives:

1. Necab1 is a novel binding partner of the human CAPs.
2. Necab1 and CAP2 are physiological binding partners that interact via coiled-coils and Necab1 is a potential SNARE effector.
3. CAP2 and Necab1 are cytosolic proteins which also display a membrane localization.
4. Necab1 functions to prevent CAP2 actin binding, and CAP2 is regulated at the level of phosphorylation.

Therefore, results presented in this thesis put forth the notion of a neuronal role for CAP2 and Necab1.

The Cyclase Associated Proteins, are highly conserved actin binding proteins that are thought to link rearrangements in the actin cytoskeleton to cellular signalling events, as is evident with yeast CAP. A role for CAPs is also evident in vesicle trafficking; CAP mutants in both yeast and *Dictyostelium* are deficient for endocytosis (Noegel et al., 1999; Wesp et al., 1997). Furthermore, yeast SNC1, a homologue of the neuronal v-SNARE synaptobrevin, complements deletions associated with the C-terminus of yeast CAP (Gerst et al., 1992). Finally, yeast CAP interacts with a multitude of proteins involved in endocytosis such as Abp1, Rvs167, and Sla1p (Drees et al., 2001).

The exact nature of CAPs function during vesicle trafficking still remains uncertain, but a general functional role for CAPs in regulating the actin cytoskeleton is evident. Research in a wide array of organisms poses a role for CAPs as determinants in actin filament formation. Study of *Drosophila* CAP reveals that it inhibits the polymerization of actin and regulates cell polarity, and a similar role was also demonstrated in yeast (Baum et al., 2000). Furthermore, *Drosophila* CAP limits the spatial formation of actin filaments in epithelial cells (Baum and Perrimon, 2001), and in a separate study, acts to prevent actin filament formation during the cell shape changes in eye discs (Benlali et al., 2000). Similarly, overexpression of *Arabidopsis* CAP in suspension-cultured tobacco cells, resulted in the loss of actin filaments (Barrero et al., 2002). Thus, human CAPs may have analogous roles during vesicle trafficking and neuronal plasticity; that is, determining when and where actin filaments are formed during the dynamics of these processes. We have focussed our attention on characterizing the function of the human CAPs and their role in neuronal processes.

The involvement of the actin cytoskeleton in neuronal processes is strikingly apparent, though many of the actual molecular players involved still remain elusive. The actin cytoskeleton has an active role during synaptic transmission in regulating the pool of reserve vesicles (Doussau and Augustine, 2000), and also the actual release of neurotransmitter, where it acts to restrict the fusion of vesicles (Doussau et al., 2000; Humeau et al., 2002; Morales et al., 2000). Furthermore, actin has been implicated in recycling synaptic vesicles, where filaments are thought to propel endocytosed vesicles back to the releasable pool (Shupliakov et al., 2002).

Studies have also identified actin as the ideal candidate for the morphological changes that occur during synaptic plasticity. These changes occur in excitatory neurons during long term potentiation (LTP). Specifically, actin dynamics are essential to LTP as F-actin depolymerization drugs in hippocampal neurons impaired its induction and maintenance (Krucker et al., 2000). Furthermore, the disruption of F-actin in young hippocampal neurons, completely inhibited the formation of dendritic spines, while no effect was observed on mature neurons, thereby suggesting that dynamic actin is needed for spine formation, and a stable actin cytoskeleton for the maintenance of mature synapses (Zhang and Benson, 2001). A real-time view of actin-mediated morphological changes was observed

during LTP, as its induction resulted in the remodelling of both pre- and postsynaptic actin of hippocampal neurons. In addition, these actin dependent changes resulted in the formation of new active synapses, capable of forming synaptic connections (Colicos et al., 2001). Thus, it is evident that the dynamics of the actin cytoskeleton are essential to neuronal function.

**Future Experiments**

The SNAREs involved in synaptic transmission can be regulated in different manners, such as through the Rab GTPase Rab3a, the syntaxin 1a effector nSec1, and through the phosphorylation of kinases such as CaMKII, PKA, and PKC (Lin and Scheller, 2000). The interaction of Necab1 with syntaxin 1and the homology of its central coiled-coil to an R-SNARE motif, suggests that it may be an effector of the neuronal SNARE complex. Necab1 is unlikely to be a true SNARE protein, as it lacks a transmembrane domain, and its cellular localization is mainly cytosolic. In accordance, Necab1 would not be able to directly mediate fusion in any type of SNARE complex, as its R-SNARE would displace that of synaptobrevin (in agreement with the 3Q:1R SNARE model), and disrupt the prerequisite for a minimum of two transmembrane bound SNAREs during vesicle fusion (Scales et al., 2002). This is further proof that Necab1's role is an effector of the neuronal SNAREs, rather than a true SNARE itself.

Necab1's calcium binding properties in addition to its restricted expression in pyramidal neurons of layer 4 of cerebral cortex, and the CA2 region of the hippocampus (Sugita et al., 2002), suggests a function during LTP. LTP mechanistically occurs when the neurotransmitter glutamate is released from the presynapse and binds glutamatergic receptors AMPA and NMDA (Malenka and Nicoll, 1999). AMPA receptors provide inward current of ions during low periods of stimulation. Under strong periods of stimulation the postsynaptic neuron is depolarized, activating the voltage-dependent NMDA receptors which causes an influx of $Ca^{2+}$ ions into the dendritic spine. This rise of intracellular $Ca^{2+}$ is the actual trigger for LTP; activating different signal transduction molecules such as CaMKII and PKC, which carry out the actual molecular functions (Malenka and Nicoll, 1999). Thus, Necab1 may respond to the rise of intracellular $Ca^{2+}$, and modulate some function of the exocytic process in either the pre- or postsynapse.

To further understand a role for Necab1 in synaptic transmission and plasticity, additional studies are required. Understanding the stochiometry of Necab1's $Ca^{2+}$ binding will be essential to understanding its function, especially if it acts in response to the influx of $Ca^{2+}$ during LTP. For example, the Synaptotagmins have different $Ca^{2+}$ binding affinities attributed to specific functions (Sudhof, 2002). The possibility that Necab1 through its R-SNARE, acts as a mediator of SNARE complex formation, could be verified through competitive bindings. Additionally, x-ray crystallography would deduce the structure of Necab1, fortifying its ability to associate with SNAREs, since their structures are already known. Furthermore, cracked-cell assays would elucidate a functional role for Necab1 in exocytosis, in which recombinant Necab1 is introduced into PC12 cells, and the subsequent release of norepinephrine is measured. This protocol has been used regularly to study either the inhibitory or stimulatory effect that a protein has on $Ca^{2+}$ mediated neurotransmitter release (Earles et al., 2001; Shin et al., 2002). Necab1's localization within the pre and/or postsynapse would strengthen a role in synaptic plasticity. Once verified, functional studies of overexpressing Necab1, or the introduction of Necab1 antibodies in hippocampal neurons could elucidated a role in LTP. Understanding how Necab1 is regulated will also be pertinent to elucidating its function. For example, determining if Necab1 is phosphorylated, via *in vivo* phosphorylation assays and subsequent phospho-amino analysis, and deducing which kinase, could shed light on its role. Verifying that Necab1 is a signal responsive molecule could provide a link between extracellular signalling events (*i.e.* those during LTP) and the SNARE-dependent exocytic machinery, as the mechanisms are currently poorly understood. Also, determining what purpose Necab1 dimerization serves will be important in understanding its function.

Similar to Necab1, we have determined CAP2 to be differentially expressed in the brain, exhibiting highest levels in the cortex and hippocampus, which suggests a specialized role in these regions. Firstly, this implies that Necab1 and CAP2 may have similar functions in these regions. Secondly, CAP2 has a more specified role than a ubiquitous actin sequestering protein. CAP2's multiple interaction domains, in addition to being phosphorylated, provides a manner that CAPs in general could respond to extracellular signals and impart an effect on the actin cytoskeleton. This is in contrast to the ubiquitous G-actin sequestering protein β-thymosin, which does not have any protein-

protein interaction domains, nor has any active role in filament assembly/disassembly (Pollard et al., 2000).

The role for CAP2 that we propose would be similar to that of the actin regulatory protein N-WASP. N-WASP integrates cell signalling events through $PIP_2$ and Cdc42, activating the ARP2/3 complex to form *de novo* polymerization of actin filaments, and thus resulting in actin-dependent morphological changes in a cell (Prehoda and Lim, 2002). In Chapter Four, it was hypothesized that CAP2's regulation occurred through an autoinhibitory mechanism, equivalent to that of N-WASP, indicating another similarity, and further confirmation that CAP2 may respond to cell signalling events. Furthermore, WASP auto-inhibition is relieved and thus activated upon phosphorylation (Cory et al., 2002). Therefore, through phosphorylation, CAP2 may be regulated in a similar manner. Determining the kinase that phosphorylates CAP2 will be imperative to further understanding its function. As mentioned in Chapter Four, PKC is a good candidate for this kinase. In addition to its effects on the actin cytoskeleton, PKC also has a role in synaptic plasticity. Inhibitors of PKC block LTP and increased activity of PKC enhances synaptic transmission (Malenka and Nicoll, 1999). This, in addition to Necab1's putative role discussed above, poses the interesting possibility that CAP2 may also have a role in synaptic plasticity.

It is apparent that the morphological changes during synaptic plasticity are dependent on the actin cytoskeleton and an important regulator of actin dependent changes within the brain is LIMK1. It exerts its effects by phosphorylating and inactivating cofilin, thus inhibiting the subsequent turnover of actin filaments. The importance of this relationship is evident in LIMK1 knockout mice, which exhibit abnormalities in dendritic spine morphology, synaptic transmission, fear responses, spatial learning, and enhanced LTP (Meng et al., 2002). These phenotypes are a direct result of a hyperactive cofilin and an abnormal actin cytoskeleton. Interestingly, human CAP1 has been recently implicated in playing a co-operative role with cofilin in the turnover of actin filaments. The interaction between cofilin and CAP1 is dependent on actin and similar results were reported with human CAP2 (Moriyama and Yahara, 2002). Thus, this provides further evidence that the human CAPs may play a role in the actin-dependent morphological changes during synaptic transmission and plasticity. It was proposed that

Necab1 acts to inhibit CAP2 actin binding, in addition, Necab1 may also prevent the interaction between hCAPs and cofilin, and thus the role in actin filament turnover.

The recent paper that identified the association between CAP1 and cofilin, proposed that it serves to accelerate the turnover of actin filaments via CAP1's ability to recycle cofilin and actin (Moriyama and Yahara, 2002). The *in vivo* function for cofilin in treadmilling of actin filaments through its ability to sever filaments and bind G-actin is well accepted, and clearly evident in overexpression studies which demonstrate the formation of cofilin actin rods/bundles (Minamide et al., 2000; Pfannstiel et al., 2001). This is in contrast to overexpression studies with *Arabidopsis* CAP which revealed reduced F-actin filaments and mitosis defects (Barrero et al., 2002); in our own studies, overexpression of CAP2-GFP in mammalian cells via a recombinant adenoviral vector, demonstrated severed actin filaments and also a reduced mitotic activity (Mottillo *et al.,* American Society for Cell Biology Poster Presentation, 2001). Thus, these results dispel the idea that CAPs have an active role in filament formation, but rather, agree with our premise that CAPs act negatively to regulate the formation of F-actin filaments. We do not dismiss the interaction between cofilin and the human CAPs; but alternatively suggest that CAPs act to regulate the availability of monomers to proteins that rearrange or assemble F-actin structures. Thus, determining the spatial and temporal formation of actin filaments. This idea is further supported by immunofluorescence studies in Chapter Three, where CAP2 and F-actin did not co-localize. Furthermore, the ability of hCAPs to recycle actin (*i.e.* exchange ADP for ATP) (Moriyama and Yahara, 2002) would nonetheless fit in with our model, as this would be a fundamental function of CAPs if they are to limit F-actin formation and aid in the subsequent delivery of monomers.

CAP2's role in neurite extensions discussed in Chapter Three, in addition to the current idea that CAP2 functions during synaptic plasticity, puts forth the idea that CAP2 has a general role in regulating actin dynamics during neuronal function. The putative role in neurite outgrowth can be assimilated to the role that CAPs play in cell elongation, suggested in cotton (Kawai et al., 1998), *Drosophila* (Benlali et al., 2000), and *Arabidopsis* (Barrero et al., 2002), and hyphal formation in *Candida albicans* (Bahn and Sundstrom, 2001). Furthermore, similar mechanisms have been suggested during bud formation in yeast and neuritogenesis in vertebrates (da Silva and Dotti, 2002).

Interestingly, yeast deficient for *cap* undergo abnormal cell budding (Fedor-Chaiken et al., 1990; Field et al., 1990), is further suggestive of a plausible role in neurite extension. This premise also has implications during neuronal rewiring, as the retraction of synapses and neurites is an actin-dependent process (Luo, 2002) and CAP2 could be involved in this. In accordance with its functional role in the brain, CAP2 would be responsive to signalling cascades involving neurite extensions and synaptic plasticity, in which we have proposed that PKC may be one manner of eliciting this signal. In accordance with our suggested neuronal role for hCAPs and their ability to respond to neuronal signalling, *Drosophila* CAP has been recently demonstrated to be responsive to axon guidance signalling via the Slit/Robo repulsive signal. This function of CAP occurred in collaboration with the tyrosine kinase Abl (Wills et al., 2002). A general role for both Necab1 and CAP2 in neuronal function is further supported by the observation that both are only present in vertebrates, organisms in which the neuronal system is most developed and advanced.

Studies that merit further examination of CAP2 include its manner of regulation, in which we have proposed an autoinhibitory mechanism and also phosphorylation. Determining domains necessary for CAP2 autoinhibition can be elucidated by deletion mutant analysis combined with activity assays (*i.e.* actin binding). One such domain that merits examination is the RLE motif (*i.e.* dimerization domain) that has already been mutated. Additionally, PKC was suggested as a candidate for CAP2 phosphorylation, *in vitro* phosphorylation assays would determine if PKC is indeed the kinase that phosphorylates CAP2. Also performing site directed mutagenesis on the conserved serine residues within the C-terminus of CAP2 (S-434, S-437, S-438) and subsequent *in vivo* labelling studies will determine if either of these sites are phosphorylated. Overexpression studies in hippocampal neurons and cracked-cell assays as those suggested for Necab1, would also verify a role for human CAPs in both neurotransmitter release and synaptic plasticity. Developing a dominant negative form of CAP2 with respect to actin binding, along with overexpression studies, would allow elucidation of its function. This dominant negative form may exist in CAP2 phosphorylation, thus making mutants that act as either pseudophosphorylated (*i.e.* S to E), or nonphosphorylatable (*i.e.* S to A), accompanied by overexpression studies will prove essential in further understanding human CAP function.

Understanding the signalling pathway(s) that act upon CAP2 will also be essential to further determining its role. *In vivo* phosphorylation assays in conjunction with growth factor treatment could elucidate such signalling cascades. For example, determining if CAP2 phosphorylation changes in response to PDGF (Rac pathway), bradykinin (Cdc42 pathway), or PMA (PKC and Rac pathway). Further studies that look at the role that Necab1 plays in the interaction between CAP2 and cofilin merits research, and can be elucidated through competitive binding studies. Also, characterization of the mammalian role that Abl has in CAP function will need to be verified.

In conclusion, this study suggests that CAP2 is a regulator of actin filament formation, likely responding to neuronal signalling events to determine when and where actin filaments are formed. It is proposed that CAPs are regulatory molecules that function to control actin polymerization, and the dynamics of the actin cytoskeleton in response to extracellular signals. The functional link between CAP2 and Necab1 may be dependent on the morphological and functional changes that occur during synaptic plasticity. Studies are currently underway to determine the specific functional roles that Necab1, CAP2 and actin play in neuronal plasticity and actin reorganization.

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## APPENDIX A

Table 2. Primers used for amplification of PCR products.

| # | Primer Name | Sequence 5' - 3' |
|---|---|---|
| 31 | LEXCAP2-F | TTGAATTCATGGCCAACATGCAGGGACTGG |
| 32 | LEXCAP2-R | TTCTCGAGTTAGGCCATAATTTCTGCAGG |
| 52 | GAPDH-F | TCAATGGAAATCCCATCACCAT |
| 53 | GAPDH-R | CTCCTTGG AGGCCATGTGGGCC |
| 93 | CIP1SEQ1-F | AGAATTCCCTGGAATGTGCC |
| 97 | HCIP5A-F | TTCTCGAGATGGAAGATTCCCAGGAGACATCG |
| 99 | HCIP-R | TTCTCGAGCTAGTTGTTCAGGATCCACCACGA |
| 108 | HACAP2C232-F | TTGAATTCCTTTTCGAGAATGAAGCAAAAAAAG |
| 109 | HACAP2N304-R | TTCTCGAGTTAGGTGGGAGATTGAGTTTGCCC |
| 123 | GSTCAP2-F | TTGAATTCGGATGGCCAACATGCAGGGACTG |
| 124 | VPSTIPBAM-F | TTGGATCCCCATGGAAGATTCCCAGGAGACA |
| 125 | VPSTIPNOT-F | TTGCGGCCGCTAATGGAAGATTCCCAGGAGAC |
| 127 | VPN284-R | TTGCGGCCGCTATCCACTTTGGGAGGAAGCAC |
| 128 | VPN250-R | TTGCGGCCGCTAGTGAGATTTAGTATTCCCT |
| 129 | VPN200R | TTGCGGCCGCTAAAACTGAGGGCTGTTTGG |
| 130 | VPSTIP-R | TTGCGGCCGCTAGTTGTTCAGGATCCACCACG |
| 136 | VPC156-F | TTGCGGCCGCCAAACAGCCCTCAGTTTAATG |
| 137 | LEXSTIP-F | TTCTCGAGTTATGGAAGATTCCCAGGAGAC |
| 138 | VPC100-F | TTGCGGCCGCACATCATGCTTGTGCAGCGG |
| 142 | VPC226NOT-F | TTGCGGCCGCACTTGGGCGAGTATGAGAATG |
| 153 | IL228231KR-F | AACCAGTGGATGACCCAGAAAAATAGACGCCAGAAATTAATTGAT |
| 154 | IL228231KR-R | ATCAATTAATTTCTGGCGTCTATTTTTCTGGGTCATCCACTGGTT |

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**E. P. Mottillo**, J.M. Sullivan, V. Patel, W. Lepeak, L.A. Elferink, and A.V. Hubberstey. (2002) Necab1: A novel human neuronal protein that interacts with Cyclase Associated Proteins (CAPs). 42nd American Society for Cell Biology Annual Meeting, San Francisco, December 14-18, 2001. Mol.Biol. Cell 13, 452a.

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**Published Paper:**

A.V. Hubberstey and **E. P. Mottillo**. (2002) Cyclase Associated Proteins: CAPacity for linking signal transduction and actin polymerization. FASEB Journal 16, 487-499.