Peer Reviewed

Title:
The Role of Capping Protein in Migrating Cells and Neurons

Author:
Sinnar, Shamim A.

Acceptance Date:
2013

Series:
UC San Diego Electronic Theses and Dissertations

Degree:
Ph. D., Biology UC San Diego

Permalink:
http://escholarship.org/uc/item/15r8244q

Local Identifier:
b8127012

Abstract:
Actin capping protein (CP) binds to the barbed ends of actin filaments and inhibits further polymerization. CP is thought to be essential for cell motility, but its role in mammalian migration has not been directly tested. Moreover, CP is widely believed to be absent from filopodia, and a role for CP in filopodia has remained uninvestigated. I have explored the role of CP in migrating cells and in primary hippocampal neurons. I begin this dissertation by reviewing the relevant literature on CP and on filopodia (Chapter One). I then investigate the effects of silencing CP in migrating B16F10 melanoma cells (Chapter Two). I find that depleting CP impairs cell migration. Moreover, CP is unexpectedly detected in filopodia, and CP depletion has dramatic effects on filopodial morphology and dynamics. These effects may be mediated by unchecked actin polymerization resulting from severely reduced capping activity and consequent depletion of monomeric actin. My novel findings suggest that CP may be an important player in filopodial form and function. I also report that silencing CP has dramatic effects on neuronal development (Chapter Three). CP depletion in primary hippocampal neurons accelerates neuronal maturation in vitro without affecting neurite length or number of Stage III neurons; these effects may possibly be mediated by changes in filopodial number. Finally, I outline the outstanding questions raised by my results in Chapters Two and Three and discuss future experiments that could help to address these questions (Chapter Four)

Copyright Information:
All rights reserved unless otherwise indicated. Contact the author or original publisher for any necessary permissions. eScholarship is not the copyright owner for deposited works. Learn more at http://www.escholarship.org/help_copyright.html#reuse
UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Capping Protein in Migrating Cells and Neurons

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Shamim A. Sinnar

Committee in Charge:

Professor Shelley Halpain, Chair
Professor Richard Firtel
Professor Velia Fowler
Professor Richard Klemke
Professor Gentry Patrick
Professor Nicholas Spitzer

2013
The Dissertation of Shamim A. Sinnar is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

__________________________________________________
__________________________________________________
__________________________________________________
__________________________________________________
__________________________________________________
__________________________________________________
__________________________________________________
__________________________________________________

Chair

University of California, San Diego

2013
DEDICATION

To Safa Shama Ali and Nura Shamim Ali, stunning sources of Clarity and Light in my life;
and to S. Nageeb Ali, who makes life so much better.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature page</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>Vita</td>
<td>x</td>
</tr>
<tr>
<td>Abstract of the Dissertation</td>
<td>xii</td>
</tr>
<tr>
<td>Chapter One</td>
<td>1</td>
</tr>
<tr>
<td>Chapter Two</td>
<td>32</td>
</tr>
<tr>
<td>Chapter Three</td>
<td>85</td>
</tr>
<tr>
<td>Chapter Four</td>
<td>97</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 2.1. CP antibodies vary in their immunoreactivity patterns at the leading edge...... 56

Figure 2.2. The 3F2.3 monoclonal antibody immunosignal is excluded from the nucleus and does not colocalize with that of the R26 antibody.......................................................... 60

Figure 2.3. The Millipore AB6017 polyclonal antibody is specific for CP.................................... 62

Figure 2.4. Silencing strategy effectively inhibits CP activity in B16F10 cells.............................. 64

Figure 2.5. Depletion of CP impairs cell migration........................................................................ 66

Figure 2.6. Endogenous CP is detected in filopodia of mammalian cells................................. 68

Figure 2.7. Endogenous CP is detected in filopodia of hippocampal growth cones............... 70

Figure 2.8. CP depletion reduces filopodial length and changes filopodial morphology...... 72

Figure 2.9. F-actin concentration is increased in CP-depleted cells........................................... 74

Figure 2.10. CP depletion reduces filopodial dynamics................................................................. 76

Figure 2.11. A model for how CP in filopodia influences filopodial shape................................. 78

Figure 3.1. CP depletion increases F-actin content and filopodial density in neurons.............. 91

Figure 3.2. CP depletion accelerates neuronal maturation without affecting total neurite outgrowth or neurite number of Stage III neurons......................................................... 93
LIST OF TABLES

Table 2.1. Specifications of various CP antibodies................................................................. 55
ACKNOWLEDGEMENTS

I thank Dr. Shelley Halpain for giving me the opportunity to engage on a fantastic intellectual journey in her laboratory, and for her immense support and guidance which have allowed me to successfully complete my doctoral dissertation.

I thank my committee members, Drs. Richard Firtel, Velia Fowler, Richard Klemke, Gentry Patrick, and Nicholas Spitzer, for their time, their advice, and their encouragement. I thank the members of the Halpain laboratory, past and present, for stimulating conversations and practical assistance.

In particular, I thank Dr. Jean-Michel Saffin for invaluable assistance with the timelapse cell migration experiments discussed in Chapter Two. I also thank Mr. Soroosh Aidun, a dedicated and conscientious undergraduate volunteer, for invaluable assistance with image analysis.

I thank Dr. Alex Mogilner of University of California, Davis, for stimulating discussions about the data contained in Chapter Two, and Drs. Jonathan Cooper and Susumu Antoku of Fred Hutchinson Cancer Research Center for a critical reading of Chapter Two.

I thank my husband, S. Nageeb Ali, for his constant love, support, and honesty. I thank my daughters Safa Shama Ali and Nura Shamim Ali for filling my graduate years with love and laughter. I thank my parents, Abbas and Zehra Sinnar, and my in-laws, Muazzem and Tuhfa Ali, for their constant love and support during my graduate years. Without their help, this dissertation would not have been possible. I thank my siblings, Shirin Sinnar, Imran Maskatia, Dana Smith, and Nausher Ali for their love and encouragement.
I thank the National Science Foundation GK-12 STEM Fellowship (2009-2010) for financial support of my graduate work. Chapter Two, in part, is currently being prepared for submission for publication of the material: Sinnar SA, Saffin JM, and Halpain S.
# VITA

<table>
<thead>
<tr>
<th>Year</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>Bachelor of Science, University of Maryland, College Park</td>
</tr>
<tr>
<td>2001</td>
<td>Doctor of Medicine, Duke University School of Medicine</td>
</tr>
<tr>
<td>2004</td>
<td>Clinical researcher, Stanford University School of Medicine</td>
</tr>
<tr>
<td>2005-2007</td>
<td>Postdoctoral Scholar, Stanford University</td>
</tr>
<tr>
<td>2007-2013</td>
<td>Graduate Researcher, University of California, San Diego</td>
</tr>
<tr>
<td>2013</td>
<td>Doctor of Philosophy, University of California, San Diego</td>
</tr>
</tbody>
</table>

# PUBLICATIONS


FIELDS OF STUDY

Major Field: Neurobiology
Professors Shelley Halpain, Gentry Patrick, and Nicholas Spitzer

Studies in Cell Biology
Professors Shelley Halpain, Richard Firtel, Richard Klemke, Gentry Patrick, Nicholas Spitzer

Studies in Cell Migration
Professors Richard Firtel and Richard Klemke

Studies on the Actin Cytoskeleton
Professors Shelley Halpain and Velia Fowler
ABSTRACT OF THE DISSERTATION

The Role of Capping Protein in Migrating Cells and Neurons

by

Shamim A Sinnar

Doctor of Philosophy in Biology

University of California, San Diego, 2013

Professor Shelley Halpain, Chair

Actin capping protein (CP) binds to the barbed ends of actin filaments and inhibits further polymerization. CP is thought to be essential for cell motility, but its role in mammalian migration has not been directly tested. Moreover, CP is widely believed to be absent from filopodia, and a role for CP in filopodia has remained uninvestigated. I have explored the role of CP in migrating cells and in primary hippocampal neurons. I begin this dissertation by reviewing the relevant literature on CP and on filopodia (Chapter One). I then investigate the effects of silencing CP in migrating B16F10 melanoma cells (Chapter Two). I find that depleting CP impairs cell migration. Moreover, CP is unexpectedly detected in filopodia, and CP depletion has dramatic effects on filopodial morphology and dynamics.
These effects may be mediated by unchecked actin polymerization resulting from severely reduced capping activity and consequent depletion of monomeric actin. My novel findings suggest that CP may be an important player in filopodial form and function. I also report that silencing CP has dramatic effects on neuronal development (Chapter Three). CP depletion in primary hippocampal neurons accelerates neuronal maturation in vitro without affecting neurite length or number of Stage III neurons; these effects may possibly be mediated by changes in filopodial number. Finally, I outline the outstanding questions raised by my results in Chapters Two and Three and discuss future experiments that could help to address these questions (Chapter Four).
CHAPTER ONE

LITERATURE REVIEWS ON ACTIN CAPPING PROTEIN AND ON FILOPODIA

Actin Capping Protein

I. Structure

Capping protein (CP) is a ~60 kD protein that caps the barbed ends of actin filaments with nanomolar affinity and prevents monomer addition (Cooper and Sept, 2008). It is both abundant and ubiquitous in eukaryotic cells (Cooper and Sept, 2008; Wear and Cooper, 2004). For example, the CP concentration in platelets is 2-6 μm (Barkalow et al., 1996; Nachmias et al., 1996). CP is an obligatory heterodimer, composed of one α (either α1 or α2) and one β (either β1 or β2) subunit (Cooper and Sept, 2008). The β1 isoform is muscle-specific; all other tissues, including brain, contain mainly the β2 isoform (Hart et al., 1997). Tissues have varying proportions of the α1 and α2 isoforms, as determined by Western blot (Hart et al., 1997). For example, mouse brain contains slightly more α2 than α1, whereas red blood cells contain 17-fold more α1 than α2 (Hart et al., 1997). It is important to note, however, that these ratios are of individual subunits, and thus the relative proportions of heterodimeric α1β2 versus α2β2 in tissues is unknown.

The three dimensional shape of the CP heterodimer resembles a mushroom in which the “head” interacts with the actin filament (Kim et al., 2010; Wear and Cooper, 2004). The C-termini of the α and β subunits (“tentacles”) lie on top of the head and are essential for interacting with actin (Wear and Cooper, 2004). Interestingly, either tentacle alone can cap, though with greatly reduced affinity (Wear et al., 2003). In vivo, CP may thus bind to actin...
via two distinct sites (Wear et al., 2003). Some investigators have proposed that the initial interaction with actin is mediated by the α tentacle, whereas the β tentacle is required for stabilization of the CP-actin bond (Narita et al., 2006).

II. Immunolocalization

Non-muscle CP has been studied extensively in migrating cells, where it plays a pivotal role in creating branched actin networks in lamellipodia (see below) (Pollard and Borisy, 2003). Consistent with this role, it is detectable at the periphery of epithelial and migrating cells in a punctate distribution (Applewhite et al., 2007; Eddy et al., 1997; Mejillano et al., 2004; Rogers et al., 2003; Schafer et al., 1992; Schafer et al., 1998). Interestingly, in many published images the majority of CP immunoreactivity is away from the cell edge. It is unclear what the bulk of this CP activity does within cells. In one study in fibroblasts, CP localized to dynamic actin spots within the lamella (Schafer et al., 1998). CP is also a component of the dynactin complex (Schafer et al., 1994) and has recently been found to be associated with recycling endosomes as an integral component of the WASH complex (Derivery et al., 2009). Moreover, CP has also been posited to interact with microtubules in regulating neurite outgrowth (Davis et al., 2009). Thus, CP may participate in myriad cellular processes apart from cell migration.

III. Potential regulators of CP

In vitro, PIP2 can reduce capping activity (Schafer et al., 1996). This seems to occur by uncapping of filaments which are already capped, at least with chicken CP (Kim et al., 2007). In one model, CP bound to actin solely by the β tentacle is less stable and wobbles; this wobble exposes a PIP2 binding site on the α subunit (Kim et al., 2007). When PIP2 binds
to this site, it can uncap the actin filament (Kim et al., 2007). Interestingly, CP from different species may interact differently with PIP2: a study in the Pollard laboratory demonstrated that PIP2 could not directly uncap *S. pombe* CP bound to actin (Kuhn and Pollard, 2007). Whether PIP2 regulates CP in vivo remains unknown.

Another regulator of CP is CARMIL (Capping protein, Arp2/3, and Myosin I linker)(Cooper and Sept, 2008). In Dictyostelium, CARMIL interacts with Arp2/3 (Jung et al., 2001), but mammalian CARMIL appears not to do so (Edwards et al., 2013; Yang et al., 2005). CARMIL binds directly to CP and uncaps filaments (Fujiwara et al., 2010; Hernandez-Valladares et al., 2010; Kim et al., 2012). The region responsible for interacting with CP is the C-1 fragment (Yang et al., 2005), also known as the capping protein binding region, or CBR (Edwards et al., 2013). This fragment can also bind to free CP and inhibit capping by CP in vitro (Yang et al., 2005).

CARMIL is a large protein with multiple domains (Hernandez-Valladares et al., 2010) and may have many roles in cells. However, those roles that seem to depend on the interaction with CP are lamellipodia formation and ruffling (Edwards et al., 2013). Thus, CARMIL may inhibit CP in vivo to maintain proper lamellipodial form and function.

CARMIL is one member of a set of proteins which interact with CP via a conserved motif, the capping protein-interacting motif (CPI) (Hernandez-Valladares et al., 2010). These proteins include CD2AP, CKIP-1, and CIN-85 (Hernandez-Valladares et al., 2010). CD2AP has been proposed to recruit CP to the leading edge, and to be necessary for CP-cortactin complexes at the leading edge (Zhao et al., 2013). In this scenario, CD2AP may not inhibit CP, so that CP at the leading edge can still cap barbed ends (Zhao et al., 2013). CKIP-1
overexpression alters cell morphology and actin levels, but it is unclear to what extent these effects are due to CP inhibition (Canton et al., 2005).

Finally, the protein V-1/myotrophin can inhibit CP activity (Cooper and Sept, 2008). V-1/myotrophin binds to the β tentacle of CP and prevents the binding of CP to actin (Bhattacharya et al., 2006). Unlike CARMIL, V-1/myotrophin cannot uncap CP from actin filaments (Bhattacharya et al., 2006).

Some proteins may not interact directly with CP, but functionally inhibit its activity by competing for free barbed ends. These include Ena/VASP (Bear and Gertler, 2009) and formins (Zigmond, 2004). Given that Ena/VASP and CP are both localized to the leading edge of lamellipodia (and perhaps filopodia; see next chapter), functional competition between these proteins may contribute to the distinctive actin architectures of these actin-based structures.

IV. Physiological Role(s) of CP

CP activity has been shown to increase actin-based motility in vitro (Loisel et al., 1999) and in vivo in Dictyostelium (Hug et al., 1995). There are at least two possible reasons why capping of polymerizing filaments paradoxically increases motility. CP could maintain actin monomer supply by preventing unproductive elongation of filaments that do not contribute to cell motility (Carlier and Pantaloni, 1997). Alternatively, CP could shunt monomers from barbed ends to the Arp2/3 complex to increase filament nucleation (as opposed to elongation) (Akin and Mullins, 2008). Some theoretical work supports the former possibility (Hu and Papoian, 2010).
CP’s role in the actin organization of migrating cell lamellipodia has been studied extensively. Lamellipodia are thought to contain a dense, branched, actin network (Pollard and Borisy, 2003). In the dendritic nucleation model for leading edge protrusion, this branched network is created by the actin nucleator Arp2/3, which binds to the sides of pre-existing filaments and nucleates new filaments at an approximately 70 degree angle (Le Clainche and Carlier, 2008; Pollard and Borisy, 2003). New filaments grow only briefly before being capped at their barbed (growing) ends by CP. This allows the network to consist mainly of short filaments that can effectively push against the cell membrane and move the leading edge forward (Le Clainche and Carlier, 2008; Pollard and Borisy, 2003). Thus, capping activity is thought to be essential for leading edge motility.

Indeed, when CP is depleted in migrating cells, normal lamellipodia are absent and leading edge protrusion speed is reduced by half (Mejillano et al., 2004). Moreover, there is a dramatic increase in filopodial density, perhaps secondary to unchecked actin polymerization (Mejillano et al., 2004). Likewise, in Drosophila S2 cells, depletion of CP leads to abnormal (Rogers et al., 2003) or absent (Iwasa and Mullins, 2007) lamellipodia.

Some investigators have reported that the branched lamellipodial network at the leading edge actually contains relatively long filaments which branch less often than previously reported (Vinzenz et al., 2012). These filaments are still stiff enough to push against the membrane because of crosslinkers, but are not necessarily short (Vinzenz et al., 2012). In this scenario, the role of CP is mainly to limit the width of the leading edge so that filaments growing below or above the plane containing high concentrations of actin nucleators are capped and do not consume cellular resources (Vinzenz et al., 2012).
scheme, CP also acts to limit growth of filaments that do not contribute to leading edge motility.

Intriguingly, it has been postulated that net cell migration is dependent not on the lamellipodium, but rather the lamella, an actin-rich region distal to the lamellipodium (Gupton et al., 2005; Ponti et al., 2004). In addition, cells depleted of Arp2/3 and claimed to lack lamellipodia can respond normally to a chemotactic gradient, though they have reduced random motility (Wu et al., 2012). We find that CP depletion significantly reduces cell migration speed (Sinnar et al, unpublished). If lamellipodia are indeed non-essential for cell migration, it may be that CP activity elsewhere in the cell (such as in the lamellum) may contribute to cell migration. Indeed, the bulk of CP immunoreactivity in migrating cells is not at the leading edge (see above).

Besides roles in cell motility, CP has been shown to have important functions in a number of actin-based structures or processes. CP has been shown to be necessary for proper actin cable formation (Amatruda et al., 1992) and endocytosis (Kim et al., 2006) in budding yeast, and for cytokinesis in fission yeast (Kovar et al., 2005). In Drosophila, complete deletion of CP is lethal, whereas reduced CP activity leads to abnormal bristle formation (Hopmann et al., 1996). In mammals, silencing of CP in hippocampal neurons leads to decreased density of dendritic spines, the appearance of abnormal protrusions on spine heads, and electrophysiological abnormalities (Fan et al., 2011).
Filopodia

I. Function

Filopodia are thin, actin-based protrusions found in many cell types, including at the leading edge of migrating cells and in neuronal growth cones (Mattila and Lappalainen, 2008). Filopodia are involved in a plethora of cellular functions associated with sensing and motility (Gupton and Gertler, 2007; Mattila and Lappalainen, 2008). For example, they are integral for cell migration (Arjonen et al., 2011) and wound healing (Mattila and Lappalainen, 2008). In addition, during embryogenesis in both Drosophila and C. elegans, sheets of epithelial cells extend filopodia towards each other to correctly align and zipper together the epidermal cell layer; these processes are known as dorsal closure and ventral closure, respectively (Wood and Martin, 2002).

A similar zippering occurs during the formation of adherens junctions between epithelial cells in keratinocytes (Vasioukhin et al., 2000). Recently, a potential role for filopodia in Sonic Hedgehog (Shh) signaling during development has been discovered (Sanders TA, 2013). During limb bud formation, Shh may be transported along extremely long (up to 150 um) filopodia to partner filopodia containing Shh co-receptors (Sanders TA, 2013).

During neuronal development, filopodia in neuronal growth cones are necessary for sensing and responding to guidance cues and for exerting the mechanical traction necessary for growth cone advance; these roles are crucial for axon pathfinding and thus proper wiring of the nervous system (Dent and Gertler, 2003). In addition, the initiation of neurites, which
are the precursors to axons and dendrites, requires the presence of filopodia (Dent et al., 2007; Kwiatkowski et al., 2007).

Filopodia can also be vital to pathologic processes. For example, filopodial numbers are increased in invasive cancers, and filopodial numbers are correlated with the degree of invasiveness (Khurana and George, 2011). Moreover, proteins that maintain filopodial structure such as fascin and formins are upregulated in cancer, and fascin expression may be related to metastasis (Arjonen et al., 2011). As another example, several pathogenic bacteria and viruses, including HIV, use filopodia as their mode of entry into the cell (Khurana and George, 2011).

Dendritic filopodia are small finger-like protrusions found on developing dendrites of the mammalian nervous system (Fiala et al., 2002). They temporally precede the appearance of dendritic spines (Fiala et al., 2002), which are the sites of most excitatory synapses in the mammalian brain (Calabrese et al., 2006). Some investigators believe dendritic filopodia to be the precursors of spines because of the spatial and temporal correlations between these two structures (Fiala et al., 1998; Ziv and Smith, 1996). Dendritic filopodia are very different in actin organization and molecular composition from filopodia of growth cones and migrating cells. In particular, dendritic filopodia have a loose array of linear and branched actin filaments, are immunopositive for Arp2/3, and lack fascin (Korobova and Svitkina, 2010) (see next section). The rest of this review will not consider these structures.
II. Structure

Basic Actin Biochemistry

Filopodia are composed of actin filaments. Filamentous actin (F-actin) is a polymer of globular actin (G-actin) subunits. These filaments are polarized such that there is a barbed, or growing, end and a pointed, or shrinking, end (Pollard and Borisy, 2003). G-actin subunits non-covalently bound to ATP preferentially associate at the barbed end and undergo hydrolysis to ADP-actin, which then dissociates from the pointed end (Pak et al., 2008; Pollard and Borisy, 2003). These dynamics lead to treadmilling of the F-actin filaments so that the actin filaments move but the subunits themselves are stationary with respect to the cytoplasm. In filopodia, the filaments are oriented so that the barbed ends are pointing toward the filopodial tips (Mattila and Lappalainen, 2008). It should be noted that naked actin filaments nucleate and grow very slowly; in cells, a host of regulatory proteins are responsible for efficient F-actin polymerization and dynamics (Pak et al., 2008; Pollard and Borisy, 2003).

Actin organization

Filopodia are composed of at most a few dozen actin filaments. Filopodia from chicken heart fibroblasts contain between 13-40 filaments (Small, 1981) whereas those from rat superior cervical ganglion cultures contain 15-20 filaments (Lewis and Bridgman, 1992). Indeed, mathematical modeling has shown that given membrane forces, greater than 7-8 actin filaments are needed to maintain the filopodial structure; greater than 30 filaments, on the other hand, severely limits filopodial length because of depletion of G-actin during filopodial growth (Mogilner and Rubinstein, 2005).
Detailed electron micrographs of mammalian filopodia (Lewis and Bridgman, 1992; Svitkina et al., 2003) have been interpreted to mean that all filaments within the filopod extend the entire length from base to tip (and indeed, several reviews implicitly or explicitly support this view (Gupton and Gertler, 2007; Mattila and Lappalainen, 2008; Wear and Cooper, 2004)). However, the filaments in these images are packed so densely that one cannot unambiguously follow individual filaments along their entire length. Thus, it is difficult to say whether every filament runs along the entire length of the filopodium.

Interestingly, a recent study demonstrated that in Dictyostelium filopodia, the actin filaments are rather short with respect to the filopod and do not run along the entire length (Medalia et al., 2007).

F-actin filaments in filopodia are crosslinked by various proteins to give them the necessary rigidity to withstand buckling (Mogilner and Rubinstein, 2005). In many mammalian cells, fascin plays this role. Indeed, depletion of fascin leads to reduced filopodial number; remaining filopodia are long and wavy with loosely-bundled actin in B16F1 melanoma cells (Vignjevic et al., 2006). Other proteins may cooperate with fascin to bundle actin: a very recent study suggests that the formin Daam1 has a non-overlapping role with fascin in bundling filopodial actin (Jaiswal et al., 2013). It should be noted that certain cell types such as epithelial cells lack fascin, and in these cells other proteins such as villin and espin may bundle the filaments in filopodia (Khurana and George, 2011).

Interestingly, recent work in Drosophila suggests that fascin contributes to filopodia formation independent of its actin bundling activity, because mutants which cannot bundle actin can still rescue filopodia formation in mutants lacking endogenous fascin (Zanet et al., 2012).
Interplay between actin and microtubule cytoskeletons

It has long been known that the filopodia of neuronal growth cones are associated with microtubule (MT) filaments (Dent and Gertler, 2003), and indeed coordination between the actin and MT cytoskeletons seems necessary for both neurite initiation (Dehmelt and Halpain, 2004) and axon guidance (Dent and Gertler, 2003; Schaefer et al., 2008). Interestingly, interactions between the actin and MT cytoskeletons have also been demonstrated in B16F1 mammalian melanoma cells (Schober et al., 2007). In this study, microtubules were observed to target filopodia in the lamellipodial wings, and this targeting was correlated with filopodial turning and merging (Schober et al., 2007) (see below for a description of lamellipodia). The authors suggested that such a mechanism might control filopodial density in specific lamellipodial regions.

III. Molecular Composition

Controversy still exists about the core proteins absolutely necessary for filopodia formation. There is strong evidence that the proteins listed below have essential roles in filopodia formation and maintenance.

Ena/VASP Proteins

Ena/VASP proteins localize to filopodial tips (Mattila and Lappalainen, 2008). In mammals, this protein family has three members: EVL, VASP, and Mena (Krause et al., 2003). These proteins can tetramerize and bind and bundle F-actin (Bachmann et al., 1999). In addition, they act as processive polymerases, adding actin subunits to the barbed end of growing actin filaments (Breitsprecher et al., 2011; Hansen and Mullins, 2010). Moreover,
they protect barbed ends from capping by capping protein (CP) and thus allow polymerization to continue (Bear et al., 2002; Hansen and Mullins, 2010).

These functions suggest that in filopodia, Ena/VASP proteins are vital for efficient actin polymerization and maintenance of F-actin filaments. Indeed, neutralization of Ena/VASP proteins in mouse hippocampal neurons leads to shorter and fewer filopodia, whereas enhancement of Ena/VASP activity leads to longer and more filopodia (Lebrand et al., 2004). Likewise, Dictyostelium cells lacking VASP do not contain filopodia (Han et al., 2002). Investigations in mammalian cell lines have demonstrated that antagonism of capping activity is only one role of the Ena/VASP proteins with respect to filopodia formation, and that additional Ena/VASP functions are necessary for filopodia formation (Applewhite et al., 2007; Mejillano et al., 2004).

Despite the strong evidence that Ena/VASP proteins are necessary for filopodia, some studies show that filopodia formation can occur in the absence of Ena/VASP proteins (Bohil et al., 2006; Dent et al., 2007).

**Formins**

Formins can nucleate and elongate linear actin filaments, bundle F-actin, and compete with capping protein to protect barbed F-actin ends from capping (Faix and Rottner, 2006; Gupton and Gertler, 2007). A specific class of formins, the Diaphanous-related formins, are regulated by Rho GTPases (Goh and Ahmed, 2012) and have been studied in filopodia formation.

Mammalian cells contain three known Diaphanous-related formins, mDia1-3 (Goh and Ahmed, 2012). Both mDia1 and mDia2 have been implicated in filopodia formation (Goh
and Ahmed, 2012). Endogenous full length mDia2 localizes to filopodial tips in B16F1 cells (Yang et al., 2007), whereas exogenously expressed mDia1 and mDia2 localize to filopodial tips in different cell types (Faix et al., 2009; Faix and Rottner, 2006).

mDia1 acts downstream of both the Rho GTPases Cdc42 and Rif (Goh and Ahmed, 2012). Silencing mDia1 but not mDia2 in NE115 neuronal cells reduces the number of filopodia induced by overexpression of the Cdc42 effector IRSp53 (Goh et al., 2012). (Filopodia induced by overexpression of IRSp53 differ from canonical filopodia; see below). Furthermore, silencing either mDia1 or mDia2 reduces filopodial numbers (but not length or lifetime) induced by overexpression of constitutively active Rif (Goh et al., 2011). Interestingly, mDia1 but not mDia2 was found to interact with Rif in Rif-induced filopodia (Goh et al., 2011).

On the other hand, B16F1 cells depleted of mDia2 have fewer and abnormal filopodia (Yang et al., 2007), whereas overexpression of mDia2 results in abundant, club-like filopodia, presumably because of enhanced actin polymerization (Block et al., 2008; Yang et al., 2007). The Dictyostelium formin dDia2 is likewise necessary for filopodia formation: cells lacking dDia2 have very short and few filopodia, whereas overexpression results in increased filopodial numbers and length (Schirenbeck et al., 2005).

Based on differences in elongation rates between these two formins, some investigators have suggested that mDia2 primarily nucleates actin filaments whereas mDia1 efficiently elongates preexisting filaments (Faix et al., 2009; Goh and Ahmed, 2012).

Interestingly, a very recent study suggests that the formin Daam1 has an essential role in actin bundling in filopodia (Jaiswal et al., 2013). Unlike the formins mDia1 and mDia2, Daam1 localizes along the entire filopodial shaft (Jaiswal et al., 2013). Silencing of Daam1
leads to wavy, loosely-bundled filopodia, similar to those seen with fascin depletion; silencing both fascin and Daam1 leads to a more severe phenotype than silencing either alone (Jaiswal et al., 2013). These findings suggest that fascin and Daam1 have non-overlapping roles in generating actin bundles in filopodia.

**Fascin**

See above, under *Actin Organization*.

**Myosin 10 (Myo10)**

A relatively new player in filopodial formation is the unconventional myosin Myo10. Unconventional myosins do not form filaments (Sellers, 2000). Myo10 is enriched at filopodial tips and travels back and forth along filopodia (Berg and Cheney, 2002); moreover, overexpression of Myo10 increases filopodial length and number (Berg and Cheney, 2002). Myo10 can apparently induce the formation of substrate-adherent filopodia in an integrin-dependent manner (Zhang et al., 2004) and also induce dorsal filopodia in an integrin-independent manner (Bohil et al., 2006).

Interestingly, dorsal filopodia induced by Myo10 do not require Ena/VASP (Bohil et al., 2006). Myo10 has been suggested to be involved in intrafilopodial transport of several proteins, including integrins, VASP, netrin receptors, and vascular endothelial (VE) cadherin (Kerber and Cheney, 2011).

**Capping Protein (CP)**

Heterodimeric CP caps the barbed ends of actin filaments, thus inhibiting further polymerization (Wear and Cooper, 2004). CP has been reported to be undetectable (Svitkina et al., 2003) or absent (Faix and Rottner, 2006) from filopodia. Indeed, CP knockdown in
B16F1 cells results in abundant filopodia (Mejillano et al., 2004) and reduction of CP activity in *Dictyostelium* results in numerous actin-based filamentous protrusions (Hug et al., 1995). These observations have led to the hypothesis that protection of barbed ends from capping activity is necessary for filopodial initiation (Faix and Rottner, 2006; Mejillano et al., 2004).

Though a reduction in CP activity may be necessary for the emergence of filopodia, theoretical work has suggested that low levels of capping activity are necessary for proper filopodial dynamics (Lan and Papoian, 2008; Zhuravlev and Papoian, 2009). Moreover, other investigators measuring rates of filopodial protrusion have alluded to the potential role of CP in controlling filopodial dynamics (Mallavarapu and Mitchison, 1999). Indeed, we in the Halpain laboratory have consistently detected CP immunoreactivity in filopodia of numerous cell types, and inhibition of CP activity has profound effects on filopodial morphology and dynamics (Sinnar et al, unpublished). Therefore, CP may have an as yet unappreciated role in filopodial function.

### IV. Upstream signaling by Rho GTPases

**Cdc42**

The Rho GTPases (such as Rho, Rac, and Cdc42) have long been appreciated to have essential signaling roles in forming actin-based structures (Ridley, 2006; Tapon and Hall, 1997). These small GTPases are active when bound to GTP and in this state can activate downstream targets (Ridley, 2006). Conversely, Rho GTPase activity is inhibited when GTP is hydrolyzed to GDP (Ridley, 2006). Nearly 20 years ago, two groups demonstrated that Cdc42 activation results in filopodial structures (Kozma et al., 1995; Nobes and Hall, 1995) (of note,
however, at least one study has demonstrated the formation of filopodia in Cdc42-deficient cells (Czuchra et al., 2005)).

The immediate downstream target of Cdc42 with respect to filopodia formation in vivo is unclear. Cdc42 can stimulate actin polymerization via activation of both Arp2/3 (which nucleates branched actin filaments that may subsequently converge into filopodia; see *Mechanisms of Filopodial Genesis*, below) and mDia2 (which nucleates linear actin filaments and has been localized to filopodia) (Ridley, 2006). Interestingly, coexpressing the Arp2/3 activator N-WASP with activated Cdc42 induces very long filopodia (Miki et al., 1998). It is unclear whether this effect is due to activation of Arp2/3 or, as the authors suggest, to the actin-depolymerizing activity of N-WASP that may expose free barbed ends for filopodial polymerization.

Though Cdc42 can activate Arp2/3, filopodia formation has been reported to occur with Arp2/3 knockdown (Beli et al., 2008; Steffen et al., 2006). In addition, a study in *Drosophila* demonstrated minimal disruption of filopodia formation by knocking down the Arp2/3 activator, WASP (Biyasheva et al., 2004). These finding have led some investigators to suggest that mDia2 is the primary downstream target of Cdc42 with respect to filopodia formation (Faix and Rottner, 2006; Ridley, 2006).

Other molecules potentially involved in filopodia formation can also be activated by Cdc42. These include Myosin10 (see above) and IRSp53.

**Rif**

In recent years, several other Rho GTPases have been found to be involved in filopodia formation (Gupton and Gertler, 2007). One of the best characterized is Rif, or Rho
in filopodia (Gupton and Gertler, 2007). Overexpression of the small GTPase Rif induces actin-rich filopodia (Fan and Mellor, 2012). These filopodia differ from those induced by Cdc42; Rif-induced filopodia are longer than those induced by Cdc42, and moreover project from both the apical cell surface and cell periphery, whereas those induced by Cdc42 project only from the cell periphery (Gupton and Gertler, 2007). In addition, Rif-induced filopodia are independent of Cdc42, N-WASP, Mena, and IRSp53, and involve both the formins mDia1 and mDia2 (Goh et al., 2011).

V. Mechanisms of Filopodial Genesis

Two models of filopodia initiation have emerged over the last several years: the convergent elongation model and the tip nucleation model (Yang and Svitkina, 2011). The convergent elongation model proposes that actin filaments from the underlying lamellipodial network merge together in areas with reduced CP activity and increased anti-capping activity; these filaments grow and subsequently become bundled by crosslinkers such as fascin to become filopodia (Svitkina et al., 2003; Yang and Svitkina, 2011). In this scenario, the Arp2/3 complex is vital for filopodial formation because although Arp2/3 nucleates branched lamellipodial filaments, these filaments are the ones that eventually form filopodial filaments (Yang and Svitkina, 2011). The formin mDia2 has an essential role in polymerizing actin filaments within filopodia (Yang and Svitkina, 2011).

Evidence for this model is based on the visualization by time-lapse imaging and platinum replica electron microscopy of emerging filopodial filaments from the underlying lamellipodial filaments (Svitkina et al., 2003). Evidence for this mechanism comes from
studies in both migrating cells (Svitkina et al., 2003) and neuronal growth cones (Korobova and Svitkina, 2008).

Some studies, however, have provided evidence that filopodia formation can occur in the absence of functional Arp2/3 complex (Beli et al., 2008; Steffen et al., 2006; Wu et al., 2012), and this has led to the alternative tip nucleation model (Faix et al., 2009; Faix and Rottner, 2006). In this mechanism, filopodial actin filaments are nucleated de novo by the formin mDia2 (Faix et al., 2009). Formins can nucleate, polymerize, and bundle F-actin (Gupton and Gertler, 2007), and mDia2 is a downstream effector of both Cdc42 and Rif (Faix et al., 2009). In this model, lamellipodial and filopodial formation are distinct and separable in terms of the required molecular components, and importantly, Arp2/3 is not required for filopodial formation.

Given the evidence for and against both models, some investigators have proposed that multiple mechanisms of filopodial formation may exist depending on the cell type or extracellular environment (Gupton and Gertler, 2007), or that filopodial formation occurs using components of both mechanisms (Mattila and Lappalainen, 2008).

**IRSp53**

*Insulin Receptor Substrate p53* and is an I-BAR domain containing protein (Mattila and Lappalainen, 2008). I-BAR domains are capable of curving cell membranes in a convex manner, resulting in protrusions (Ahmed et al., 2010). IRSp53 interacts with a host of proteins potentially associated with filopodia formation; these include Cdc42, the Cdc42 effector WASP, Rac1, the Rac1 effectors WAVE1 and WAVE2, the Ena/VASP family member Mena, the formins mDia1 and mDia2, and the capping protein Eps8 (Ahmed et al., 2010; Goh and Ahmed, 2012). Given its ability to interact with so many key players, IRSp53 has been
suggested to be a key mediator of filopodia formation downstream of Cdc42 by coupling membrane protrusion with actin dynamics (Lim et al., 2008).

Overexpression of IRSp53 results in membrane protrusions suggested to be filopodia (Ahmed et al., 2010). However, not all of these structures contain actin, and in those that do, the actin is loosely-organized and not typical of canonical filopodia (Ahmed et al., 2010; Faix et al., 2009). Moreover, these protrusions are much less dynamic than normal filopodia (Crespi et al., 2012). It is possible that IRSp53 contributes to the formation of canonical filopodia via its ability to curve membranes (Faix et al., 2009), perhaps by creating a submembranous space that actin filaments can polymerize into during filopodia formation (Yang et al., 2009).

Recently, IRSp53 has been suggested to be a key component of filopodial initiation complexes (Vaggi et al., 2011). IRSp53 can bundle actin filaments, but seems to be auto-inhibited (Disanza et al., 2006). However, bundling activity in vitro is enhanced several-fold when IRSp53 is complexed with either the actin barbed-end capper Eps8 (Disanza et al., 2006) or with VASP (Vaggi et al., 2011). Bundling increases filament stiffness and therefore allows polymerization against the cell membrane; indeed, overexpression of IRSp53 with either Eps8 or VASP in vivo increases membrane protrusions (Vaggi et al., 2011). Conversely, removal of IRSp3 and Eps8 decreases filopodia formation downstream of Cdc42 (Disanza et al., 2006). These filopodial initiation complexes are postulated to be a necessary first step for filopodia formation; bundled actin filaments are subsequently elongated by formins or other proteins.
VI. Filopodial Dynamics

Filopodia are dynamic structures which undergo extension and retraction; presumably, this is vital for their ability to sense and respond to environmental cues. Filopodial actin filaments undergo retrograde flow (Mallavarapu and Mitchison, 1999), here defined as the constant rearward movement of material with respect to the substratum (Cramer, 1997). Retrograde flow is thought to depend on myosin II activity (Henson et al., 1999; Lin et al., 1996) and influences filopodial (and cell) protrusion. Specifically, filopodial protrusion depends on the balance between actin assembly at filopodial tips and retrograde flow of the filopodial actin filaments (Mallavarapu and Mitchison, 1999).

In some cells, such as *Aplysia* bag cell neurons, protrusion is driven mainly by changes in retrograde flow (Lin and Forscher, 1995), whereas in other cells such as NG-108 neuroblastoma cells, flow is constant and changes in actin assembly drive filopodial protrusion (Mallavarapu and Mitchison, 1999).

Measured rates of filopodial extension and retraction vary greatly depending on the cell type, experimental conditions, and measurement methodology used (Kress et al., 2007; Lu et al., 1997; Medalia et al., 2007; Miller et al., 1995; Sheetz et al., 1992; Yang et al., 2007). For example, extension rates vary from 0.05 µm/s in hippocampal growth cones (Lu et al., 1997) to 1.0 µm/s in *Dictyostelium* (Medalia et al., 2007).

Several mathematical models of filopodial dynamics have been developed by various investigators (Lan and Papoian, 2008; Mogilner and Rubinstein, 2005; Zhuravlev and Papoian, 2009). Important components of these models underlying filopodial protrusion are G-actin diffusion to filopodial tips and actin assembly at the barbed end; these forces are counteracted by membrane tension (the force of the membrane limiting polymerization) and
retrograde flow (Lan and Papoian, 2008; Mogilner and Rubinstein, 2005). In addition, Mogilner and Rubinstein consider the rate of lamellipodial protrusion as well, since lamellipodial protrusion along the direction of filopodia will act to decrease effective filopodial length (Mogilner and Rubinstein, 2005).

Interestingly, Papoian and colleagues argue that neglecting capping proteins in their model eliminates the macroscopic dynamics that are observed in filopodia experimentally; low levels of CP stochastically capping and uncapping individual filaments are necessary for the filopodia to exhibit macroscopic fluctuations in length (Lan and Papoian, 2008; Zhuravlev and Papoian, 2009). These results may provide some theoretical basis for the existence of CP in filopodia, as we have observed in our experiments (Sinnar et al, unpublished).

Works Cited


CHAPTER TWO

CAPPING PROTEIN IS ESSENTIAL FOR CELL MIGRATION AND FILOPODIAL MORPHOLOGY AND DYNAMICS

ABSTRACT

Capping Protein (CP) is thought to be important for creating branched lamellipodial actin networks and thereby generating protrusive force in migrating cells. However, recent studies have reexamined the role of lamellipodia in cell migration, and suggest that other structures, including filopodia, may be critical as well. CP was postulated to be absent from filopodia, thus its role in filopodial activity has remained unexplored. We observed that silencing of CP impairs cell migration. Moreover, we unexpectedly observed that low levels of CP are detectable in the majority of filopodia. CP depletion decreased filopodial length, altered filopodial shape, and reduced filopodial dynamics. Our results support an expansion of the potential roles that CP plays in cell motility by implicating CP in filopodia as well as in lamellipodia, both of which are important in many types of migrating cells.

INTRODUCTION

Heterodimeric capping protein (CP) binds to actin filament barbed ends with nanomolar affinity in vitro and thus inhibits further polymerization (Wear et al., 2003). Inhibiting CP activity in organisms severely compromises actin-based motility and lamellipodia formation (Hug et al., 1995; Iwasa and Mullins, 2007; Mejillano et al., 2004; Rogers et al., 2003). These studies, among others, have suggested a role for CP in lamellipodial form and function at the leading edge of migrating cells (Le Clainche and Carlier, 2008; Pollard and Borisy, 2003).
In one model, termed the dendritic nucleation model, CP is thought to limit the growth of actin filaments nucleated by the Arp2/3 complex at the leading edge (Pollard and Borisy, 2003). Capping activity thus creates short and stiff filaments that can then efficiently resist backward pressure from the cell membrane and allow the cell to move forward (Le Clainche and Carlier, 2008; Pollard and Borisy, 2003). In addition, capping of actin filaments may maintain actin monomer supply by preventing elongation of unproductive filaments and therefore facilitate ongoing polymerization (Carlier and Pantaloni, 1997; Hu and Papoian, 2010).

Recent work, however, has questioned the necessity of lamellipodia for cell migration (Edwards et al., 2013; Gupton et al., 2005; Wu et al., 2012). Fibroblast cells in which lamellipodium formation was inhibited following tropomyosin injection moved faster than did control cells (Gupton et al., 2005). In another study, cells which lacked lamellipodia because of absence of the Arp2/3 complex underwent normal chemotaxis, though haptotaxis and random motility were impaired (Wu et al., 2012). The authors suggested that these cells employed a “filopodia-based protrusion system” to locomote (Wu et al., 2012).

Finally, cells with silenced CARMIL protein demonstrate abnormal lamellipodia and impaired cell migration (Liang et al., 2009). However, the defective migration in these cells can be substantially rescued without rescuing the lamellipodial phenotype, leading the authors to suggest that lamellipodia are not essential for cell migration in this setting (Edwards et al., 2013). Although CP has a vital role in creating the lamellipodial actin networks thought essential for protrusion and migration, the necessity of CP in mammalian cell migration has not been directly tested.
Filopodia are thin, actin-based protrusions found in many cell types, including the leading edge of migrating cells and neuronal growth cones (Mattila and Lappalainen, 2008). They are integral for both chemosensing and motility (Gupton and Gertler, 2007; Mattila and Lappalainen, 2008). Vertebrate filopodia are composed of 15-30 actin filaments (Lewis and Bridgman, 1992; Small, 1981) and can range from a few micrometers (Mattila and Lappalainen, 2008) to over a hundred micrometers in length (Sanders TA, 2013).

A number of actin-interacting proteins localize to filopodia and seem to have essential functions in filopodial initiation and maintenance. These include Ena/VASP (Han et al., 2002; Lebrand et al., 2004; Mattila and Lappalainen, 2008), diaphanous formins (Faix et al., 2009; Goh and Ahmed, 2012; Yang et al., 2007), fascin (Vignjevic et al., 2006), and myosin 10 (Berg and Cheney, 2002; Bohil et al., 2006).

There are two main models for how filopodia in migrating cells emerge, but both models posit that a relative absence of CP allows filopodia to initiate and grow (Faix and Rottner, 2006; Svitkina et al., 2003). CP has been reported to be undetectable in filopodia (Svitkina et al., 2003) and most current models for filopodial structure and function do not include a role for CP (Faix et al., 2009; Gupton and Gertler, 2007; Mattila and Lappalainen, 2008; Wear and Cooper, 2004). Interestingly, however, some investigators have suggested that CP within filopodia may have an important role in controlling filopodial dynamics (Mallavarapu and Mitchison, 1999; Zhuravlev and Papoian, 2009).

We report here that CP is essential for mammalian cell migration. Moreover, CP is detectable in filopodia of multiple cell types, including melanoma cells and primary hippocampal neurons. Consistent with previous reports, depletion of CP greatly increases filopodial numbers. Strikingly, silencing CP also has significant effects on filopodial
morphology and dynamic behavior. Specifically, filopodia in CP-depleted cells are shorter, visibly altered in shape, and grow more slowly than do those in control cells. We explore potential mechanisms to explain these findings, and suggest that while a decrease in capping activity stimulates emergence of filopodia, CP activity within filopodia is nonetheless necessary for their proper form and function.

MATERIALS AND METHODS

Reagents.

Plasmids. We created shRNA to CPβ based on the T1 sequence from Mejillano et al, 2004 (CCTCAGCGATCTGATCGAC) (Mejillano et al., 2004). We cloned this sequence into pSuper vector (a kind gift from Dr. T Wittman, Scripps Research Institute) that contained the polymerase-III H1-RNA promoter (Brummelkamp et al., 2002). We also created a Scramble sequence (GCACCCGTCTTCAACAGGT) and cloned it into pSuper. eGFP-tagged versions of the α2 and β2 subunits of CP as well as CP holoprotein were generous gifts of Dr. D. Schafer, U. Virginia. RFP-LifeAct was a generous gift from Dr. R. Truant, McMaster University, Canada.

Antibodies. A polyclonal antibody against the CP β2 subunit was purchased from Millipore (AB6017). This antibody was selected for the main experiments because of its superior sensitivity over a panel of polyclonal and monoclonal antibodies (see below, A Detailed Examination Of CP Antibodies) and was used at a dilution of 1:200 in B16F10 and U2-OS cells and a dilution of 1:50 in primary hippocampal neurons.

For antibody testing, the Anti-CAPZB (Cat. No. HPA031531) and Anti-CAPZA2 (Cat.No. HPA007470) polyclonal antibodies were purchased from Sigma. The R26B polyclonal
antibody was a generous gift from Dr. D. Schafer, U. Virginia. The 3F2.3 monoclonal antibody was purchased from the U. Iowa Developmental Studies Hybridoma Bank.

Secondary antibodies (Alexa 488, Alexa 568, or Alexa 647) and Alexa Fluor 568 or 647-phalloidin were purchased from Life Technologies and used at a dilution of 1:500.

**Cell Culture.**

B16F10 cells were a generous gift from Dr. S. Dowdy (UC-San Diego), and U2-OS cells were a generous gift from Dr. G. Yeo (UC-San Diego). Both cells lines were cultured in DMEM supplemented with 10% FBS. Primary hippocampal neurons were cultured as described (Calabrese and Halpain, 2005).

B16F10 cells were transfected with Scramble or shRNA to CP for 5 days using Lipofectamine 2000 (Life Technologies) in 24-multiwell plates. For live-cell imaging experiments, cells were co-transfected with RFP-LifeAct to visualize the actin cytoskeleton. After 4 days, cells were trypsinized and replated onto glass coverslips that had been sequentially precoated with poly-L-ornithine (0.1 mg/mL overnight; Sigma) and laminin (5 μg/mL for 3-4 hours; Life Technologies) in preparation for imaging.

For antibody staining, cells on glass coverslips were fixed in 3.7% formaldehyde in PBS with 8% sucrose (w/v) for 20 min at 37°C. Cells were permeabilized in 0.2% Triton in phosphate-buffered saline (PBS) for 5 min, blocked with 2% BSA for 45 min, incubated with primary antibody for 1 hr at RT, washed with PBS, and incubated with secondary antibody or phalloidin for 45 min at 37°C. After washing, coverslips were mounted with Aquamount (Thermo Scientific), dried overnight, and imaged.
**Image Acquisition and Analysis.**

Images for all fixed cell analyses and some live cell analyses (filopodial dynamics and cell migration) were acquired on an Olympus IX70 inverted microscope mounted with a Yokogawa CSU-X1 Spinning disk using either a 60x (PlanApo, 1.42 NA) or 20x (UplanApo, 0.8 NA) oil objective or 20x dry air objective. For live imaging, samples were kept in a humid chamber at 35°C containing 5% CO2. For movies of filopodial dynamics, images were acquired every 5 sec for 2 minutes.

For cell migration experiments, cells were imaged using DIC imaging every 5 min for 6 hrs. Cell tracking was accomplished using the Manual Tracking plugin in ImageJ. Live-cell imaging to study cell morphing was done on a Nikon Eclipse TiE inverted confocal microscope. Images were either acquired every 20 sec for 15 min using a 20x dry air objective.

For measurement of filopodial length, the entire actin bundle was quantified regardless of its position relative to the lamellipodium since filopodia, retraction fibers, and microspikes are dynamically interchangeable (Svitkina et al., 2003). Measurements were based on the methods of Vignjevic et al 2006 (Vignjevic et al., 2006). For the fractional length protruding beyond the cell margin (Fig. 4A), the length protruding beyond the cell edge was divided by the total length.

For lamellipodial measurements, the segmented line function in ImageJ was used to manually measure lamellipodial perimeter. CP-depleted cells often had abnormal lamellipodia and for these cells the perimeter of the cell region containing filopodia was measured.
For analysis of protrusion rates, filopodial lengths were measured every 5 sec until they disappeared or merged with a neighboring filopodium. A filopodium was considered pausing if its length did not change by more than 2 pixels between frames.

Statistical analysis was done using GraphPad Prism. Student’s t-test or one-way ANOVA was used for all statistical analyses except for comparison of filopodial morphologies (Figure. 2.8), for which the binomial test was used.

A Detailed Examination of CP Antibodies.

ABSTRACT

We determined how well a panel of commercial and non-commercial antibodies to capping protein detected CP in intact mammalian cells. We report that these antibodies varied widely in their patterns of immunofluorescence in mammalian cells. Of the antibodies tested, the Millipore AB6017 antibody had the best signal: noise ratio and reliably detected CP at the leading edge of migrating cells. Surprisingly, though the R26B and 3F2.3 antibodies are raised to the same CP sequence and though both detect CP by Western blot, 3F2.3 did not produce the expected immunosignal at the leading edge of migrating cells. Moreover, the immunosignal from the 3F2.3 antibody seemed to be excluded from the nucleus.

INTRODUCTION

Capping protein is a heterodimer consisting of one α and one β subunit (Cooper and Sept, 2008). Invertebrates have only one isoform of each subunit, whereas vertebrates have two somatic α isoforms (α1 and α2) and two β isoforms (β1 and β2) (Cooper and Sept, 2008). The β1 subunit predominates in muscle (skeletal and cardiac), whereas the β2 subunit is
ubiquitous in other tissue types (Hart et al., 1997). Tissues have varying ratios of the α1 and α2 isoforms (Hart et al., 1997), but the relative proportion of heterodimeric α1β2 and α2β2 remains unknown. However, an antibody to the β2 subunit should theoretically recognize all heterodimeric CP in non-muscle tissue regardless of the α subunit.

The CP heterodimer takes on a mushroom shape, with the mushroom “head” binding to and capping the actin filament at its barbed end (Kim et al., 2010; Wear and Cooper, 2004). The C-terminal regions of the α and β subunits, known as “tentacles,” lie on top of the mushroom, and are thought to be mobile and flexible in solution (Wear and Cooper, 2004). These tentacles are proposed to “find” and interact with the barbed end of actin (Wear and Cooper, 2004). The N-terminal regions of the subunits form the stalk of the mushroom and do not contact the actin filament (Wear and Cooper, 2004).

We tested several CP antibodies to find one that would faithfully detect CP in mammalian cell lines and primary hippocampal neurons. Here we report the immunostaining properties of several commercial and non-commercial antibodies.

MATERIALS AND METHODS

Reagents.

Plasmids. eGFP-tagged versions of the α2 and β2 subunits of CP were a generous gift of Dr. D. Schafer, U. Virginia.

Antibodies. The AB6017 polyclonal antibody was purchased from Millipore. Antibodies were used at a dilution of 1:200 in B16F10 and HeLa cells, and at a dilution of 1:50 in primary hippocampal neurons. Secondary antibodies (Alexa 488, Alexa
568, or Alexa 647) and Alexa Fluor 568 or 647-phalloidin were purchased from Life Technologies and used at a dilution of 1:500.

**Cell Culture.**

B16F10 cells were a generous gift from Dr. S. Dowdy (UC-San Diego) and HeLa cells were a generous gift from Dr. J. Lykke-Andersen (UC-San Diego). Both cell lines were cultured in DMEM supplemented with 10% FBS. Cells were plated on glass coverslips that had been sequentially precoated with poly-L-ornithine (0.1 mg/mL overnight; Sigma) and laminin (5 µg/mL for 3–4 hours; Life Technologies).

For antibody staining, cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) with 8% sucrose (w/v) for 20 min at 37°C. Cells were permeabilized in 0.2% Triton in PBS for 5 min, blocked with 2% BSA for 45 min, incubated with primary antibody for 1 hr at RT, washed with PBS, and incubated with secondary antibody and phalloidin for 45 min at 37°C. After washing, coverslips were mounted with Aquamount (Thermo Scientific), dried overnight, and imaged.

For live cell extraction experiments, cells were incubated in 1% Triton-X detergent in PEM buffer (100 mM PIPES, pH 6.9, 1 mM MgCl₂, 1 mM EGTA, and 4% PEG [MW 40,000]) for 5 min at RT prior to fixation. All other steps were the same as above except that for cells undergoing live extraction, the post-fixation permeabilization step was omitted.

**Image Acquisition and Analysis.** Images were acquired on an Olympus IX70 inverted microscope mounted with a Yokogawa CSU-X1 Spinning disk using a 60x (PlanApo, 1.42 NA) oil objective.
RESULTS and DISCUSSION

We examined the staining properties of several CP antibodies (Table 2.1). The R26B polyclonal and 3F2.3 monoclonal antibodies have been extensively used to detect CP in mammalian cells (Applewhite et al., 2007; Davis et al., 2009; Fan et al., 2011; Kitanishi et al., 2010; Korobova and Svitkina, 2010; Mejillano et al., 2004; Svitkina et al., 2003), and so we initially tested these. However, we found that for various reasons (see below), these antibodies were unsuitable for reliably detecting CP. Thus, we tested some newer commercial antibodies that were advertised to work well for immunofluorescence (AB6017 from Millipore, Anti-CAPZB and Anti-CAPZA2 from Sigma).

In some publications detailing CP immunostaining, investigators have extracted cell membranes using detergent before fixation (Korobova and Svitkina, 2010; Mejillano et al., 2004). Though this live-extraction procedure is primarily used to prepare the actin cytoskeleton for correlative light and electron microscopy (Svitkina and Borisy, 1998), it presumably also removes soluble CP. We employed this method in some of our experiments because we found that it was necessary to optimally detect the immunosignal at the leading edge using certain antibodies.

Published Western blots purport each of the antibodies we tested to be specific for CP (Kitanishi et al., 2010; Schafer et al., 1994b),
http://www.sigmaaldrich.com/catalog/product/sigma/hpa031531?lang=en&region=US,
http://www.millipore.com/catalogue/item/ab6017). We found that the immunosignal from all antibodies tested increased with overexpression of the appropriate CP subunit (α2 or β2; data not shown).
The antibodies we tested differed dramatically in their detection of CP at the leading edge of migrating B16F10 melanoma cells (Fig. 2.1). Strikingly, whereas all the polyclonal antibodies demonstrated a relative enrichment at the leading edge, the monoclonal 3F2.3 antibody did not (Fig 2.1 A, B). We could not detect signal at the leading edge using the 3F2.3 antibody regardless of whether we used (Fig. 2.1B) or did not use (Fig. 2.1A) the live extraction procedure.

It should be noted that even the polyclonal antibodies differed somewhat with respect to their immunoreactivity (ir) patterns at the leading edge. All polyclonal antibodies tested showed a non-continuous, punctate, pattern of CP ir at the leading edge; this is consistent with images in other publications (Applewhite et al., 2007; Rogers et al., 2003). The Millipore and Sigma Anti-CAPZA2 antibodies yielded a higher density of puncta than did the R26B and Sigma Anti-CAPZB antibodies (compare Fig 3.1B and 3.1F with Fig. 3.1C and 3.1D). We found that the R26B antibody, which has been used extensively in other publications (Applewhite et al., 2007; Fan et al., 2011; Korobova and Svitkina, 2010; Mejillano et al., 2004), had an unsuitably low signal: noise ratio in our hands (data not shown).

With the Millipore antibody, we were able to detect a good immunosignal in non-live-extracted cells, whereas the other antibodies seemed to work better in live-extracted cells. Live extraction, however, considerably decreased the immunofluorescence signal of CP, by 20-60% depending on antibody (data not shown; in Fig. 2.1, the contrast of the live-extracted images has been stretched to aide visibility of the leading edge).

It is also unclear whether live extraction preserves every aspect of native architecture. For example, live extraction noticeably changed the ir pattern of the Sigma Anti-CAPZB antibody (compare Fig. 2.1D and 2.1E). After live extraction, the CP ir signal
became much more concentrated in the nuclear region, which may not reflect CP localization in intact, live cells. Furthermore, we found that live extraction considerably distorted the architecture of some delicate cell types, such as growth cones of primary hippocampal neurons (data not shown). Thus, the Millipore antibody had a distinct advantage in that it produced a better signal: noise ratio using a more conventional fixation protocol.

Interestingly, the signal from the 3F2.3 monoclonal antibody seemed to be relatively excluded from the nucleus in many cell types, including B16F10 cells (Fig. 2.1A and B), HeLa cells (Fig. 2.2A), and primary neurons (data not shown). This is extremely surprising given that the exact same sequence of chicken CP β2 was used to generate the 3F2.3 monoclonal and R26B polyclonal antibodies (Table 2.1). A magnified view from cells co-stained with the 3F2.3 and R26B antibodies shows that remarkably, the signals from these two antibodies do not colocalize (Fig 2.2B; white would mark colocalization between magenta and green).

Many commercial and non-commercial CP antibodies, including the R26B and Millipore ones that we tested, are raised to the C-terminus of the β2 subunit. The β C-terminus is thought to be mobile and flexible in solution (Wear and Cooper, 2004), and thus might seem accessible to an antibody. However, the β C-terminus is likely hidden when CP is actually bound to actin because the C-terminus fits into a hydrophobic cleft in the actin filament (Kim et al., 2010). Thus, it is unclear whether and how CP antibodies that recognize the β2 C-terminus detect CP that is bound to actin at the time of fixation.

To investigate this issue, we specifically tested two antibodies that detect the N-terminal regions of the CP subunits. Specifically, Sigma Anti-CAPZA2 detects the N-terminus of the α2 subunit and Sigma Anti-CAPZB detects the N-terminus of the β2 subunit (Table 2.1). Both of these antibodies yielded approximately similar ir patterns at the leading edge of
B16F10 melanoma cells (Fig. 2.1D and 2.1F), although as stated previously, the puncta density was higher for the Anti-CAPZA2 antibody than for the Anti-CAPZB one.

In conclusion, it is quite remarkable that different CP antibodies, some ostensibly raised to the same sequence, yield different ir patterns. The reasons for these differences are unknown, but might possibly reflect different pools of CP. Besides interacting with actin filaments, CP has been reported to be a member of the dynactin (Schafer et al., 1994a) and WASH (Derivery et al., 2009) complexes and to interact with microtubules (Davis et al., 2009). In addition, there may be physiologically relevant phosphorylation sites in CP (Canton et al., 2005). Therefore, the antibody ir patterns we observe may point to as yet unknown properties of CP.
MAIN CHAPTER RESULTS

**CP depletion impairs mammalian cell migration.** To understand the roles of CP in cell migration, we first employed a silencing strategy in B16F10 cells. We used a previously published shRNA sequence (Mejillano et al., 2004) and created a scrambled construct to use as control. In addition, after testing several commercial and non-commercial antibodies, we determined that a polyclonal antibody from Millipore (AB6017) recognizing the β2 subunit of CP is both sensitive and specific for immunostaining of multiple cell lines and primary hippocampal cultures.

The immunostaining intensity using this antibody increased dramatically with CP overexpression (Fig 2.3 A) and decreased with CP silencing in B16F10 cells (see Fig 2.4A). In addition, the signal decreased considerably with preadsorption by holoprotein (Fig 2.3B).

Transfecting B16F10 cells with CP shRNA effectively inhibited CP activity. In one representative experiment, CP immunoreactivity (CP-ir) following 5d of shRNA treatment was decreased by 80% compared to that in untransfected neighboring cells (Fig. 2.4 A, B). As reported previously (Mejillano et al., 2004), CP silencing led to a robust, more than two-fold increase in filopodial density (Fig. 2.4 C,D). These structures were immunopositive for fascin (data not shown), thus confirming that they were indeed filopodia, as has been reported previously (Mejillano et al, 2004).

To test whether CP is required for mammalian cell migration, we used time lapse imaging to measure rates of cell migration in control and CP-depleted cells. The average cell migration rate of Scramble-transfected cells was 0.68 µm/min, a value similar to those reported previously for a related cell type, B16F1 cells (Hotulainen et al., 2005; Yang et al., 2007). Silencing CP reduced rates of cell migration by more than half, from 0.68 µm/min to
0.31 µm/min (Fig 2.5A). It also reduced the net area traversed by cells over 6 hours by 67% (Fig 2.5B). Representative tracks of Scramble-transfected and CP-depleted cells are shown in Fig 2.5C and 2.5D, respectively. These results demonstrate that CP is indeed critical for migration of mammalian cells. Our results are consistent with a prior study in Dictyostelium (Hug et al., 1995) in which cells underexpressing CP moved 35% slower than did control cells.

**CP is detected in filopodia of multiple cell types.** As expected, we clearly detected CP at the leading edge of migrating B16F10 mouse melanoma cells (Fig 2.6B, arrowhead). This pattern of CP-ir is similar to that observed previously by other investigators (Applewhite et al., 2007; Rogers et al., 2003; Schafer et al., 1998; Yang et al., 2007). CP has also been shown to be associated with sites of actin assembly in the lamella (Schafer et al., 1998), and we detected CP-ir in the lamella as well (Fig. 2.6B, arrow).

Interestingly, though CP’s role has been studied most extensively at the leading edge, the majority of CP-ir was not at the leading edge but instead in the cell body (Figs. 2.4A and 2.6A); this distribution persisted even after extracting soluble CP from live cells with detergent before fixation (data not shown). This pattern of immunoreactivity is similar to that appearing in published images of endogenous CP in various cell types (Rogers et al., 2003; Schafer et al., 1998).

CP has been shown to be an integral component of both the dynactin complex (Schafer et al., 1994a) and the WASH complex (which associates with recycling endosomes) (Derivery et al., 2009). Moreover, CP may interact with microtubules in neurons (Davis et al., 2009). Therefore, CP may fulfill other cellular roles besides leading edge motility.
Unexpectedly, we also clearly detected low levels of CP-ir in a punctate distribution along filopodia of B16F10 cells (Fig. 2.6C,D). This was surprising because CP has been postulated to be absent from filopodia (Faix and Rottner, 2006; Svitkina et al., 2003), and indeed, the relative absence of CP is thought to be important for filopodia to emerge from the dendritic network (Svitkina et al., 2003; Wear and Cooper, 2004).

Out of 311 filopodia examined from B16F10 cells, 79% had at least one CP punctum, and 52% had at least two puncta. On average, there were 2.4 CP puncta detected per filopodia. The majority of filopodia (91%) had CP puncta along the shaft. If one assumes that CP only interacts with the barbed ends of actin, this observation suggests that actin filament lengths are not uniform within filopodia. Only a minority of filopodia (36%) had any CP-ir detectable at the tip, which may be due to the possibility that very few filaments span the entire length of the filopodium, and that the probability of detecting immunoreactivity decreases as the number of actin filaments decreases.

To confirm whether filopodial CP-ir was a general phenomenon, we examined another cell line (U2-OS osteosarcoma cells; Fig 2.6E), as well as growth cones in primary hippocampal neuronal cultures (Fig 2.7). CP-ir was clearly detected in the filopodia of both of these cell types.

**CP depletion reduces filopodial length and changes filopodial shape.** Interestingly, filopodial length was significantly decreased by one third in CP-depleted cells (Figs. 2.4D and 2.8A). A frequency histogram comparing shRNA-transfected and Scramble-transfected cells shows that CP depletion results in selective loss of longer filopodia and a higher proportion
of short filopodia (Fig 2.8B). These novel findings imply that CP depletion directly or indirectly reduces filopodial length.

Besides dramatically reducing filopodial length, other effects of CP depletion on filopodial morphology were apparent. First, nearly the entire length of individual filopodia in CP-depleted cells appeared to be protruding beyond the cell membrane (Fig. 2.8C, D; see Materials and Methods for details on measurements). In contrast, filopodia from Scramble-transfected cells often had much of their length embedded in the cell lamellipodia (Fig. 2.8C, D).

Second, the shapes of filopodia from CP-depleted cells were visibly altered (Fig. 2.8E, F). In Scramble-transfected filopodia, the majority of filopodia had a cone-like or tapered appearance, with a smaller percentage having a more rod-like or uniform appearance (Fig. 2.8E, F). However, in shRNA-transfected filopodia, the majority of filopodia had a rod-like appearance (Fig. 2.8E, F). In addition, a significant fraction of filopodia had a “cattail” appearance, in which the base was visibly thinner than the shaft and tip regions (Fig. 2.8E).

These types of filopodia were rarely seen in Scramble-transfected cells. Of note, a similar filopodial morphology (“clublike filopodia”) has been described with formin overexpression (a manipulation expected to decrease relative capping activity)(Yang et al., 2007). These results demonstrate that CP activity is essential for normal length and morphology of filopodia.

**CP depletion increases cellular and filopodial F-actin concentration.** Strikingly, depleting CP caused a significant increase in F-actin concentration inside cells, as measured by phalloidin staining (Fig.2.9A). This increased staining was especially evident at cell margins.
at low magnification. At higher magnification (Fig. 2.9A, inset, and Fig. 2.9B), it was clear that
the phalloidin staining of individual filopodia was also dramatically increased in CP-depleted
cells.

Quantification of phalloidin intensities demonstrated more than two-fold increases
in F-actin concentrations globally in CP-depleted cells (Fig. 2.9C) and within individual
filopodia (Fig. 2.9D). These data suggest that filopodia from CP-depleted cells have a greater
number of F-actin filaments than do those from Scramble-transfected cells. In other words,
decreased capping leads to greater actin polymerization.

**CP depletion reduces filopodial dynamics.** To understand whether CP depletion has
consequences for filopodial function, we examined filopodial behavior using time-lapse
imaging. The average protrusion rate for Scramble-transfected filopodia was 95 nm/s, which
is similar to that reported previously for B16F1 cells (Yang et al., 2007) (Fig 2.10A). However,
filopodia from CP-depleted cells had significantly reduced protrusion rates, on average only
61 nm/s (Fig. 2.10A). In addition, filopodia from shRNA-transfected cells spent significantly
less time growing and more time pausing (Fig. 2.10B). Thus, we conclude that CP depletion
reduces filopodial dynamics, and could therefore have functional consequences for cell-
based activities that require proper filopodial function.

**DISCUSSION**

CP is an essential player in creating the actin architecture of lamellipodia, which in
turn has been thought necessary for cell protrusion and migration (Le Clainche and Carlier,
2008; Pollard and Borisy, 2003). CP is necessary for actin-based motility in a cell-free system
in vitro (Loisel et al., 1999) and in Dictyostelium (Hug et al., 1995). Moreover, mammalian cells depleted of CP have decreased lamellipodial protrusion (Mejillano et al., 2004). However, recent work has questioned the need for a normal lamellipodium and lamellipodial dynamics for actual cell migration (Edwards et al., 2013; Gupton et al., 2005; Wu et al., 2012). In addition, the requirement for CP in mammalian cell migration has not been established.

We report here that depleting CP impairs mammalian cell migration. Therefore, CP is necessary for proper locomotion. Even in cells with impaired lamellipodia, CP may still play an essential role in generating normal motility. Some investigators have proposed that the lamella, the region behind the lamellipodium, drives net cell displacement (Gupton et al., 2005; Ponti et al., 2004). CP is present in the lamella (see Fig. 2.6A,B) and has been shown to associate with sites of actin assembly in this region (Schafer et al., 1998). Thus, CP function in the lamella may be vital for cell motility.

In addition, cells without normal lamellipodia are postulated to use a filopodia-based system to locomote (Wu et al., 2012). We find that CP is present in filopodia, and is required for normal filopodial form and dynamics (see below). Filopodia have important sensing and possibly adhesion roles in cell migration (Arjonen et al., 2011; Mattila and Lappalainen, 2008), and it is possible that CP’s role in filopodia is also necessary for proper cell migration.

Though capping protein (CP) is postulated to be absent from filopodia (Faix and Rottner, 2006; Mejillano et al., 2004; Svitkina et al., 2003), we clearly detected CP in filopodia from multiple mammalian cell types, including B16F10 melanoma cells and primary hippocampal neurons (Figs. 2.6 and 2.7). This finding led us to investigate a possible role of
CP in filopodial function, and we found that silencing CP dramatically changed filopodial morphology (Figs. 2.8 and 2.9) and dynamics (Fig. 2.10).

Filopodia from CP-depleted cells showed multiple alterations in shape and behavior. Filopodia in CP-depleted cells were significantly shorter than those from control cells and had a more uniform (as opposed to tapered) morphology. Moreover, a significant fraction of them had a cattail-like appearance. Filopodia from CP-silenced cells had two-fold increased F-actin content compared to filopodia from scramble-transfected cells. Finally, filopodia from CP-depleted cells had a decreased protrusion rate and spent less time growing and more time pausing.

Our findings yield new insights into filopodial structure and formation. The punctate distribution of CP along filopodia (as opposed to CP localizing exclusively to filopodial tips) suggests that actin filaments within filopodia are not necessarily of uniform length (Fig. 2.11). Instead it suggests that individual filaments may grow to different lengths before being stochastically capped by CP. Filopodia in which relatively few filaments extend the entire length from base to tip have a tapered appearance, whereas filopodia that contain actin filaments of equal lengths appear to be uniform.

In control cells, there seem to be roughly equal numbers of tapered and uniform filopodia. In cells depleted of CP, decreased capping of individual filaments within filopodia would allow all of the filaments to grow uniformly. Thus, there may be a dramatic increase in the proportion of uniform filopodia, offset by a concomitant decrease in the proportion of tapered filopodia.

Reducing CP also induced the emergence of cattail filopodia, which have a thicker tip and a thinner base. One explanation for this morphology is that some filopodial filaments
may normally sever or depolymerize from their pointed ends and begin to treadmill. In control filopodia, CP caps these treadmilling filaments and retrograde flow moves them out of filopodia; hence, they are transient structures and not frequently detected. Fewer than one percent of control filopodia fell into this category (Fig. 2.8F).

In CP-depleted filopodia, however, these filaments may persist and thus become easily detected. Indeed, in time lapse imaging of knockdown cells, we sometimes observed uniform filopodia morphing into cattail filopodia, indicating a temporal relationship between these categories. Of note, cattail filopodia are somewhat similar in appearance to the “clublike” filopodia observed after formin overexpression (Yang et al., 2007). Increased formin activity would be expected to decrease relative capping activity, similar to the effect of CP silencing.

Why are filopodia from CP-depleted cells remarkably shorter than those from control cells? This observation may seem counterintuitive if one assumes that a decrease in capping allows actin filaments to polymerize for longer periods and attain greater lengths. However, unchecked polymerization may consume a limiting cellular factor, such as monomeric G-actin. CP silencing induces a 2.5-fold increase in filopodial density (Fig. 2.4C). Moreover, individual filopodia from CP-silenced cells probably contain an increased number of actin filaments, as evidenced by their 2-fold increase in phalloidin intensity (Fig. 2.9).

The burst of actin polymerization due to increased filopodial density and increased F-actin content in individual filopodia conceivably depletes cellular G-actin and limits filopodial length. Indeed, mathematical modeling has suggested that growth of filopodia composed of greater than ~30 filaments would be limited by G-actin diffusion from the base to the growing filopodial tip (Mogilner and Rubinstein, 2005). Moreover, CP is thought to be
essential for maintaining an adequate supply of actin monomers for actin-based motility (Carlier and Pantaloni, 1997; Loisel et al., 1999; Pollard and Borisy, 2003). Thus, we suggest that inhibiting CP activity in cells counterintuitively reduces filopodial length due to depletion of actin monomer.

One might expect that artificially increasing cellular levels of monomeric actin by overexpressing G-actin would reverse the length phenotype. However, we found that overexpressing G-actin and depleting CP simultaneously did not increase filopodial length (data not shown). It is possible that increasing G-actin levels globally fails to increase actin concentration sufficiently at filopodial tips; actin polymerization in filopodia would still be limited by inadequate actin supply.

Why does a greater part of individual filopodia from CP-depleted cells extend beyond the cell margin (Fig. 2.8C, D)? A possible explanation takes into account relative rates of retrograde flow, which exists in filopodia as well as in lamellipodia (Mallavarapu and Mitchison, 1999). Reduced F-actin polymerization at filopodial tips in CP-depleted cells (as evidenced by decreased filopodial length) would be expected to reduce retrograde flow of filopodia. Thus, a greater fraction of the filopodial length protrudes beyond the lamellipodia.

Why are filopodia from CP-depleted cells less dynamic than those from control cells? One possibility is that limiting levels of G-actin reduce polymerization at filopodial tips and thus decreases protrusion rate. Likewise, filopodia spend less time growing and more time pausing. Interestingly, theoretical work has suggested that stochastic capping of individual actin filaments is necessary for macroscopic filopodial dynamics (Zhuravlev and Papoian, 2009), and this mechanism may also contribute to our observation of reduced filopodial dynamics in CP-depleted cells.
Consistent with other studies, we observe a dramatic increase in filopodial density upon inhibition of capping activity (Fig. 2.4). We therefore agree with other investigators that a relative decrease in capping activity seems necessary for filopodia to emerge (Mejillano et al., 2004; Svitkina et al., 2003). We suggest, however, that low levels of CP within filopodia are essential for normal filopodial morphology and dynamic behavior. It is possible that the filopodial functions of CP, in addition to other potential roles like microtubule regulation and membrane trafficking (Davis et al., 2009; Derivery et al., 2009), may contribute to the critical role of CP in cell migration in vivo.

ACKNOWLEDGEMENTS

In particular, I thank Dr. Jean-Michel Saffin for invaluable assistance with the timelapse cell migration experiments discussed in Chapter Two. I also thank Mr. Soroosh Aidun, a dedicated and conscientious undergraduate volunteer, for invaluable assistance with image analysis.

I thank Dr. Alex Mogilner of University of California, Davis, for stimulating discussions about the data contained in Chapter Two, and Drs. Jonathan Cooper and Susumu Antoku of Fred Hutchinson Cancer Research Center for a critical reading of Chapter Two.

I thank the National Science Foundation GK-12 STEM Fellowship (2009-2010) for financial support of my graduate work. Chapter Two, in part, is currently being prepared for submission for publication of the material: Sinnar SA, Saffin JM, and Halpain S.
Table 2.1. Specifications of various CP antibodies. Several antibodies to CP were tested for their ability to reliably detect CP at the leading edge of migrating cells and primary hippocampal neurons. The clonality and source of these antibodies, as well as the sequence used to raise them, are listed.

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Clonality</th>
<th>Source</th>
<th>Sequence used to raise antibody</th>
<th>Example publications using this antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>R26</td>
<td>polyclonal</td>
<td>J. Cooper/D. Schafer laboratories (Schafer et al., 1994b)</td>
<td>27 C-terminal AA from chicken β2 subunit (^1)</td>
<td>(Korobova and Svitkina, 2010; Mejillano et al., 2004)</td>
</tr>
<tr>
<td>3F2.3</td>
<td>monoclonal</td>
<td>J. Cooper/U. Iowa Developmental Studies Hybridoma Bank</td>
<td>27 C-terminal AA from chicken β2 subunit (^1)</td>
<td>(Davis et al., 2009; Kitanishi et al., 2010)</td>
</tr>
<tr>
<td>AB6017</td>
<td>polyclonal</td>
<td>Millipore</td>
<td>C-terminal region of human β2 subunit (AA unspecified) (^2)</td>
<td></td>
</tr>
<tr>
<td>Anti-CAPZB</td>
<td>polyclonal</td>
<td>Sigma</td>
<td>82 N-terminal AA from human β2 subunit (^2)</td>
<td></td>
</tr>
<tr>
<td>Anti-CAPZA2</td>
<td>polyclonal</td>
<td>Sigma</td>
<td>99 AA near N-terminus of human α2 subunit (^3)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)The relevant portions of chicken and mouse/rat CP β2 are 96% identical and 100% similar. 
\(^2\)Human, mouse, and rat β2 are 100% identical. 
\(^3\)The relevant portions of human and mouse/rat α2 are 96% identical and 99% similar.
Figure 2.1. CP antibodies vary in their immunoreactivity patterns at the leading edge. (A,B) The polyclonal Millipore antibody but not the 3F2.3 monoclonal antibody localizes to the leading edge of B16F10 melanoma cells. This pattern occurs in cells fixed without live extraction (A) and those whose membranes have been extracted by detergent before fixation (B). (C) The R26B antibody shows signal at the leading edge. Arrowheads point to leading edge, and scale bars are 10 μm.
Figure 2.1. CP antibodies vary in their immunoreactivity patterns at the leading edge, continued. (D-F) The Sigma anti-CAPZB (D), and Sigma anti-CAPZA2 (F) antibodies show signal at the leading edge. The IR from the Sigma anti-CAPZB antibody becomes concentrated in the nucleus after live extraction (D) but not without live extraction (E). Arrowheads point to leading edge, and scale bars are 10 µm.
Figure 2.2. The 3F2.3 monoclonal antibody immunosignal is excluded from the nucleus and does not colocalize with that of the R26 antibody. (A) The signal from the 3F2.3 antibody seems to be excluded from the nucleus in HeLa cells. Comparisons with the Millipore (top) and R26B (bottom) antibodies are shown. Asterisks point mark the nucleus. (B) A magnified view of the boxed region in (A) demonstrates little colocalization between the signals from the R26B and 3F2.3 antibodies. Scale bars are 10 µm.
Figure. 2.3. The Millipore AB6017 polyclonal antibody is specific for CP. (A) The signal from the Millipore antibody increases with overexpression of eGFP-tagged CP α2 and β2 subunits in B16F10 melanoma cells (compare top row, untransfected, with bottom row, transfected). The inset in the top row is a contrast-enhanced image that shows that CP IR is indeed present in the expected distribution in a non-transfected cell. (B) Mean CP IR decreases with preadsorption of antibody with holoprotein. -, no preadsorption; +, plus preadsorption. 30 cells were analyzed in each category. Scale bar: 20 µm.
Figure 2.4 Silencing strategy effectively inhibits CP activity in B16F10 cells. (A) Silencing CP in B16F10 cells for 5d robustly decreases CP IR in silenced cells compared to neighboring non-transfected cells. Arrowhead points to transfected cell. (B) In one representative experiment, silencing CP for 5d results in 80% decrease of CP IR. For each category, CP fluorescence intensities were normalized to those of untransfected cells on the same coverslip, imaged and processed in an identical manner. eGFP, cells transfected with eGFP only; CTL, cells transfected with a Scramble-construct; KD, cells transfected with shRNA against CP. (C) An example of a Scramble-transfected cell (left) and a CP-depleted cell (right). Cells are stained with phalloidin to visualize F-actin. (D) Filopodial density is increased more than two-fold in CP-depleted cells. 47-69 cells were analyzed across 3 different experiments and include 1600-3200 filopodia/condition. Scale bars: 10 µm.
Figure 2.5. Depletion of CP impairs cell migration. (A) The average migration speed of CP-depleted cells is reduced by 54%. (B) The average net area traversed by CP-depleted cells over 6 hrs is reduced by 67%. Area traversed was calculated for each cell by multiplying the greatest absolute difference between x-coordinates by the greatest absolute difference between y-coordinates. (C,D): Representative migration tracks from Scramble-transfected (C) and CP-depleted (D) cells. 34-36 cells from 6 independent experiments were analyzed for each group.
Figure 2.6. Endogenous CP is detected in filopodia of mammalian cells. (A) B16F10 melanoma cell stained with antibody to endogenous CP. Note substantial CP signal in cell body. (B) A magnified view of the boxed region in (A). CP is present at the leading edge (arrowhead) and in the lamella (arrow). (C) Enlarging and increasing the contrast of the boxed region in (B) shows that CP IR is detected in filopodia (C1). Further magnification of the boxed region in C1 shows that CP IR is detected both along the length (top arrowhead) and at the tip (bottom arrowhead) of filopodia (C2-C4). (D) An example of CP IR along the length and tip of filopodia in a different B16F10 cell. (E) CP IR is detected along filopodia of U2-OS cells. Scale bars: A and B, 10 µm; C-E, 2 µm.
Figure 2.7. Endogenous CP is detected in filopodia of hippocampal growth cones. (A,B) Neuronal growth cones from primary hippocampal neurons are immunopositive for CP (A) and have CP IR detectable along the shaft and at tips of individual filopodia (B). Images in (A) and (B) are from two different neurons. Scale bar, 5 μm.
Figure 2.8. CP depletion reduces filopodial length and changes filopodial morphology. (A) Filopodial length is significantly shorter in CP-depleted cells. Length was quantified for the entire actin bundle, as per Svitkina et al 2003, and averaged per cell. 47-69 cells were analyzed across 3 different experiments and include 1600-3200 filopodia/condition. (B) A frequency histogram comparing Scramble-transfected and shRNA-transfected cells shows that longer filopodia are selectively lost in CP-depleted cells. (C) Depletion of CP increases the fraction of filopodial length which protrudes beyond the cell margin. Filopodial values were averaged per cell, and 30-35 cells were analyzed across three independent experiments. (D) An example of a Scramble-transfected cell (left) and a CP-depleted cell (right). Note how filopodia are more embedded in the Scramble-transfected cell. (E) Categories of filopodial shapes found in Scramble-transfected and CP-depleted cells. See text for category descriptions. (F) CP depletion significantly decreases the proportion of tapered filopodia and increases the proportion of uniform filopodia. In addition, there is an emergence of a new class, named cattail filopodia, in CP-depleted cells. 325-355 filopodia were quantified for each group and represent two separate experiments. Scale bar: 2 µm.
Figure 2.9. F-actin concentration is increased in CP-depleted cells. (A) F-actin concentration as determined by phalloidin staining is visibly increased in a CP-depleted cell (left) compared to an untransfected cell (arrowhead, right). Inset shows that saturated phalloidin staining represents individual filopodia with increased F-actin content. (B) Close up of filopodia in a different Scramble-transfected cell (left) and CP-depleted cell (right). These two phalloidin-stained cells were imaged under identical conditions and are displayed with identical contrast. (C and D) Average F-actin concentration is increased globally in CP-depleted cells (C) as well as in individual filopodia from CP-depleted cells (D). 50-80 cells (C) or 180-340 filopodia (D) from the same two independent experiments were analyzed. Phalloidin intensities were normalized to the average intensity of Scramble-transfected cells (C) or of filopodia from Scramble-transfected cells (D). Scale bars: A, 20 µm; B, 5 µm.
**Figure. 2.10. CP depletion reduces filopodial dynamics.** (A) Filopodial protrusion rates are decreased by 35% in CP-depleted cells. (B) Filopodia from CP-depleted cells spend significantly less time growing and more time pausing. 51-56 filopodia from 13-14 movies were analyzed for each group.
Figure. 2.11. A model for how CP in filopodia influences filopodial shape. (A) Stochastic capping of individual actin filaments within filopodia results in a distribution of filament lengths and consequently a tapered appearance (left). In filopodia with reduced capping activity, all filaments span the entire length of the filopodia, giving the filopodium a uniform appearance (middle). In CP-depleted cells, cattail filopodia are present, in which there is a greater density of actin filaments at the tip of the filopodia than at the base (right). (B) A possible mechanism for the creation of cattail filopodia. In cells depleted of CP, the majority of filopodia are of the uniform variety (left). Actin filaments that sever or begin to depolymerize from their pointed ends (middle) are normally capped and cleared away by retrograde flow. However, decreased capping within filopodia from CP-depleted cells results in these filaments treadmilling and persisting (right).
Works Cited


CHAPTER THREE

CAPPING PROTEIN ACCELERATES NEURONAL MATURATION WITHOUT AFFECTING NEURITE OUTGROWTH

ABSTRACT

We have shown that silencing actin capping protein (CP) in a mammalian cell line has robust effects on cell migration and filopodial morphology and dynamics. In order to explore the effects of CP depletion in a more physiological system, we turned our attention to primary hippocampal neurons. We report that silencing CP in developing neurons increases cellular F-actin and filopodia number, results similar to those obtained in transformed mammalian cells. Moreover, depleting CP accelerates neuronal maturation without affecting total neurite outgrowth or neurite number of Stage III neurons.

INTRODUCTION

Acting capping protein (CP) is a ~60 kD heterodimeric protein which binds to the barbed ends of actin filaments and thus prevents further polymerization (Cooper and Sept, 2008). CP is a major regulator of actin within migrating cells and is thought to be essential for cell motility (Pollard and Borisy, 2003). Silencing CP in mammalian cells increases filopodial density and decreases lamellipodial protrusion (Mejillano et al., 2004). Moreover, we find that depleting CP in mammalian cells affects filopodial morphology and dynamics (Sinnar et al, unpublished).

Less is known about the role of CP in mammalian neurons. Developing neurons are extremely rich in actin, and CP may thus be an important regulator of morphology and function in these cells. One study has suggested that depleting CP in young hippocampal
neurons affects neurite length and growth cone morphology (Davis et al., 2009). Another study has shown that overexpressing CP in cortical neurons decreases filopodial density and delays neuronal maturation (Dent et al., 2007).

Dissociated cultures of hippocampal neurons are well-suited for studies of neuronal development, since these cells progress through a well-characterized series of developmental stages (Dotti et al., 1988). When first plated, these cells are round and compact, but they soon begin to extend neurites. One of these neurites becomes the axon while the rest develop into dendrites (Dotti et al., 1988). Another advantage of dissociated cultures is that they more readily lend themselves to experimental manipulation and imaging than do other model systems. We explored the function of CP in developing neurons by silencing this protein using shRNA. We found that depleting CP increased F-actin content within neurons, a feature similar to that observed after silencing CP in a transformed mammalian cell line (Sinnar et al, unpublished). Moreover, depleting CP accelerated neuronal maturation without affecting neurite length or number of Stage III neurons.

MATERIALS AND METHODS

Reagents.

*Plasmids.* We created shRNA to CPβ based on the T1 sequence from Mejillano et al, 2004 (CCTCAGCGATCTGATCGAC) (Mejillano et al., 2004). We cloned this sequence into pSuper vector (a kind gift from Dr. T Wittman, Scripps Research Institute) that contained the polymerase-III H1-RNA promoter (Brummelkamp et al., 2002). We also created a Scramble sequence (GCACCCGTCTTCAACAGGT) and cloned it into pSuper. eGFP-tagged versions of the
α2 and β2 subunits of CP were generous gifts of Dr. D. Schafer, U. Virginia. RFP-LifeAct was a generous gift from Dr. R. Truant, McMaster University, Canada.

**Antibodies.** A polyclonal antibody against the CP β2 subunit was purchased from Millipore (AB6017) and was used at a dilution of 1:50. Secondary antibodies (Alexa 488, Alexa 568, or Alexa 647) and Alexa Fluor 568 or 647-phalloidin were purchased from Life Technologies and used at a dilution of 1:500.

**Cell Culture.**

Primary rat hippocampal neurons were cultured as described (Calabrese and Halpain, 2005). Neurons were electroporated with Scrambled or CP-specific shRNA at the time of plating using an Amaxa Rat Neuron Nucleofector kit (Lonza AG). Neurons were grown on poly-L-lysine-coated coverslips in 24-multiwell plates for 72 hr and then fixed and immunostained as described in Chapter One.

**Image Acquisition and Analysis.**

Images were acquired on an Olympus IX70 inverted microscope mounted with a Yokogawa CSU-X1 Spinning disk using a 20x (UplanApo, 0.8 NA) oil objective. For quantification of neuronal stages, the following definitions were used: Stage I neurons had no neurites whose lengths were greater than the cell body diameter; Stage II neurons had at least one neurite whose length was greater than the cell body diameter; and Stage III neurons had at least one neurite whose length was more than twice that of the cell body diameter.

Neurite length and number were quantified using the NeuronJ plugin for ImageJ.
Statistical analysis was done using GraphPad Prism. Student’s t-test or one-way ANOVA was used for all statistical analyses except for comparison of Stages (Fig. 2), for which the binomial test was used.

RESULTS

Silencing CP in primary hippocampal neurons increases cellular F-actin and filopodial density. To determine the effects of silencing CP in primary hippocampal neurons, we depleted CP using a previously published shRNA sequence (Mejillano et al., 2004) and created a scrambled construct to use as control. In one representative experiment, CP immunoreactivity was decreased by ~60% 72h after electroporation (Fig. 3.1A). Strikingly, F-actin content was increased nearly 2-fold in CP-depleted neurons, as measured by phalloidin staining (Fig. 3.1B, C). Moreover, a large proportion of CP-depleted neurons seemed to be covered with filopodia, leading to a “spiky” or “hairy” appearance (Fig. 3.1C). The findings of increased F-actin and filopodial density following CP depletion are consistent with the effects of silencing CP in cell lines and Dictyostelium ((Hug et al., 1995; Mejillano et al., 2004), Sinnar et al, unpublished).

CP depletion accelerates neuronal maturation without affecting neurite length or number of Stage III neurons.

Our visual impression was that there was a higher proportion of more mature neurons in cultures depleted of CP than in cultures expressing Scrambled shRNA. To quantify this impression, we scored neurons by stage (Fig. 3.2A, B). Primary neurons in culture progress through a number of stereotyped stages as they establish polarity (Dotti et al.,
Stage I neurons are more or less symmetric and have not extended any neurites (Fig. 3.2A, left). Stage II neurons have extended one or more neurites, but none of these is long enough to be considered an axon (Fig. 3.2A, middle). Stage III neurons have one major process, the axon, and multiple minor processes (Fig. 3.2A, right).

In line with our visual impression, cultures depleted of CP had a significantly higher proportion of Stage III neurons and a lower proportion of Stage I neurons 72h after electroporation (Fig. 3.2B). Strikingly, this maturation occurred without other detectable changes in neurite length or number of Stage III neurons. Specifically, the total neurite outgrowth, longest neurite length, and total neurite number were similar between control cells and those depleted of CP (Fig. 3.2C-E).

DISCUSSION

Our data show that depleting CP in developing neurons accelerates neuronal maturation. Interestingly, it has been reported that an increase in neuronal actin capping activity, either by overexpression of CP or by treatment of cells with Cytochalasin D, inhibits neuronal maturation (Dent et al., 2007). In our case, the opposite manipulation (CP silencing) leads to the opposite result (accelerated maturation) and thus our findings are consistent with this earlier observation. In the earlier study, excess capping activity was found to inhibit filopodia formation, and the authors show that neurite initiation depends on the presence of filopodia (Dent et al., 2007). It is therefore possible that excessive filopodia formation in our case somehow enhances neurite initiation and maturation.

It is surprising that accelerated maturation occurs without a change in neurite outgrowth or number of Stage III neurons (Fig. 3.2). Interestingly, a prior study demonstrates...
that manipulation of Ena/VASP in developing neurons leads to changes in filopodial and neurite number without affecting total neurite length or length of the longest neurite (likely to be the axon) (Lebrand et al., 2004). Ena/VASP is a protein found at the leading edge of lamellipodia and at filopodial tips, and is thought to protect free barbed ends by competing with CP (Bear and Gertler, 2009; Bear et al., 2002).

In this study, increasing Ena/VASP activity (and thus reducing CP activity) increased filopodial and neurite numbers, whereas inhibiting Ena/VASP (and thus increasing capping activity) decreased filopodial and neurite numbers (Lebrand et al., 2004). A later study by the same authors showed that inhibiting Ena/VASP impaired neuronal maturation (Kwiatkowski et al., 2007); this result is also consistent with ours.

Interestingly, recent work by the Dent laboratory suggests that CIP4 (Cdc42-interacting protein), an F-bar family protein, may both functionally interact with CP and have a role in neurite outgrowth (Saengsawang et al., 2012; Saengsawang et al., 2013). Neurons overexpressing CIP4 have reduced filopodial density of Stage I neurons, inhibited neurite outgrowth, and reduced axon length. Conversely, neurons lacking CIP4 have an increased filopodial density, accelerated outgrowth, and increased axon length (Saengsawang et al., 2012).

In a follow-up study, the authors show that overexpressing the β subunit of CP significantly decreases CIP4 localization at the leading edge of Stage I neurons (Saengsawang et al., 2013). However, the significance of this finding is unclear, because (1) only the β subunit, and not the CP heterodimer, was overexpressed, and (2) the effects of overexpressing the CP β subunit on neurite outgrowth were not determined.
Figure 3.1. CP depletion increases F-actin content and filopodial density. (A) In one representative experiment, CP immunoreactivity 72h after electroporation with CP shRNA is reduced by 60%. (B) In these same cells, F-actin content as measured by phalloidin staining is increased nearly 2-fold. For (A) and (B), 19-26 cells per condition were analyzed. (C) Representative images of Scramble-transfected (left) and CP-depleted (right) neurons stained with phalloidin. Scale bar: 30 µm.
Figure 3.2. CP depletion accelerates neuronal maturation without affecting total neurite outgrowth or neurite number of Stage III neurons. (A) Primary hippocampal neurons progress through a series of stereotyped stages in culture. See text for definitions. (B) CP-depleted cultures contain a significantly higher proportion of Stage III neurons and a significantly lower proportion of Stage I neurons after 72 h in culture. 380-460 neurons/condition across 3 experiments were analyzed. (C-E) Total neurite outgrowth (C), longest neurite length (D), and total neurite number (E) of Stage III neurons are not significantly different between control and CP-depleted cultures. 120-140 Stage III neurons across 3 different experiments were analyzed.
Works Cited


CHAPTER FOUR

OPEN QUESTIONS AND FUTURE DIRECTIONS

The observations detailed in Chapters One and Two raise several questions about the mechanisms underlying the phenotypes we observe. In this chapter we explore some directions our research could take to address these questions.

Chapter Two.

We find that depleting CP in B16F10 melanoma cells results in a dramatic increase in filopodial density. Compared to filopodia from control cells, filopodia from CP-depleted cells have dramatically altered lengths, shapes, and actin content, and emerge not just from the leading edge, but from the dorsal aspect of the cell as well. The question arises, are these structures still filopodia? How alike are they to conventional filopodia in terms of their molecular composition?

We believe that the structures are indeed filopodia because they are immunopositive for fascin (data not shown), as has been observed previously (Mejillano et al., 2004). Moreover, they have also been shown to be positive for VASP (Mejillano et al., 2004). Filopodia, however, contain numerous other proteins such as formins (Faix and Rottner, 2006) and Myosin X (Bohil et al., 2006), and it would be important to determine whether filopodia from CP-depleted cells also have these proteins in the expected distribution. These studies could also shed light on whether the molecular composition of filopodia in CP-depleted cells is similar to or different from that in control cells.

One explanation for the (perhaps counterintuitive) reduction in filopodial length following silencing of CP is that unchecked actin polymerization depletes monomeric actin. Expressing eGFP-actin does not rescue filopodial length (data not shown). It may be that
overexpression of G-actin increases cellular actin levels globally but does not increase actin concentration sufficiently at filopodial tips to contribute to filopodial growth. To test this idea, one could determine whether and where G-actin is specifically increased following eGFP-actin expression.

One method to quantitate G-actin and F-actin in intact cells is to rely on the molecule DNase I, which is thought to bind with much higher affinity to G-actin than to F-actin (Blikstad et al., 1978; Heacock and Bamburg, 1983). Life Technologies markets fluorescent DNase I conjugates which it claims can selectively bind to G-actin in cells. However, there have been some concerns about how specific DNase I is for G-actin versus F-actin (Morrison and Dawson, 2007), and thus it is unclear whether DNase I can serve as a specific reporter for G-actin levels in the cell.

Our data show that CP is required for cell migration. However, given that both lamellipodia (Pollard and Borisy, 2003; Ridley et al., 2003) and filopodia (Arjonen et al., 2011; Mattila and Lappalainen, 2008) are thought to be important for cell migration, it is difficult to determine to what extent CP dysfunction in filopodia contributes to the overall migration defect. One question that thus remains is: are there functional defects that can be specifically attributed to CP depletion in filopodia?

Filopodia are thought to be integral for detecting and responding to environmental cues (Dent and Gertler, 2003; Gupton and Gertler, 2007; Mattila and Lappalainen, 2008). To directly explore how the chemosensing roles of filopodia may be affected by CP depletion, one could use a growth cone turning assay. It has been reported that the actin content within filopodia, and possibly the number of filopodia, increase on the side of the growth cone responding to a positive guidance cue (Dent and Gertler, 2003; Dent et al., 2011). We
could locally inactivate CP within growth cone filopodia and assess whether the filopodia continue to respond normally to guidance cues via timelapse imaging. We could also determine the effects of acute CP inhibition on filopodial morphology and dynamics.

One technique to inactivate CP with high temporal and spatial resolution is micro-CALI (chromophore-assisted laser inactivation) (Jay, 2000). This method relies on introducing non-function blocking antibodies to the protein of interest (in this case, CP) into the cell (Jay, 2000). These antibodies are conjugated to a dye such as malachite green (Jay, 2000). Laser irradiation of the dye generates free radicals which damage the protein bound to the antibody without affecting surrounding structures (Jay, 2000). Thus, micro-CALI offers a method to specifically and acutely decrease CP activity and monitor the immediate effects. Micro-CALI has been used to determine the effects of molecules such as Myosin V (Wang et al., 1996) and vinculin and talin (Sydor et al., 1996) on filopodial dynamics.

Another outstanding question raised by our data is: how is directional migration affected by CP depletion? Directional migration entails responding to either soluble (chemotaxis) or substrate-bound (haptotaxis) chemoattractants, and is moreover thought to rely on proper filopodial function. Interestingly, cells seem to use different machineries for random migration, haptotaxis, and chemotaxis; indeed it has been shown that cells with depleted Arp2/3 have defective random motility and haptotaxis but not chemotaxis (Wu et al., 2012). It will thus be important to parse out what forms of migration are affected by CP depletion by using transwell assays to assess chemotaxis and haptotaxis. Interestingly, our preliminary results suggest that haptotaxis is not affected by CP depletion (data not shown).

It may also be informative to expand our analysis of random cell migration by quantifying persistence. We determined that the net area traversed was significantly
reduced in CP-depleted cells; however, it may be that the total distance travelled by CP-depleted cells is similar to or even exceeds that travelled by control cells if the former exhibit a large degree of back-and-forth or non-productive movement. Furthermore, it would be useful to assess whether cells depleted of CP have difficulty in maintaining cell polarity during movement. Both persistence and polarity could be quantified from timelapse image sequences.

Though cell migration is inhibited in CP-depleted cells, an interesting question is whether the invasive abilities of malignant cells is enhanced by the increased filopodial density. In addition, our qualitative impression is that the number of invadopodia, the actin-based structures associated with invasion and metastasis in cancer cells (Ridley, 2011; Yamaguchi and Condeelis, 2007), may be increased in cells depleted of CP.

One could use a variety of invasion assays (Machesky, 2008) to determine whether CP depletion in B16F10 melanoma affects the invasive ability of these cells. Either a positive or negative result would be informative. Increased invasiveness could imply that increased filopodial numbers or altered filopodial morphology confer an advantage to metastasizing cells. On the other hand, an inhibition of invasive activity could justify investigating CP as a potential marker for metastasis.

CP is perhaps the most widely-studied metazoan actin capping protein (Cooper and Sept, 2008), but by no means is it the only protein with capping activity. Gelsolin is a calcium-dependent protein which both caps and severs F-actin (Kwiatkowski, 1999) and Eps8 is an actin capping protein that is also involved in Rac activation (Disanza et al., 2004). Interestingly, both gelsolin and Eps8 are present in axonal filopodia (Gallo, 2013). Moreover,
Eps8 can interact with IRSp53 to bundle F-actin and induce membrane protrusions (Vaggi et al., 2011).

Though gelsolin overexpression does not rescue the filopodial phenotype caused by CP depletion (Mejillano et al., 2004), it would be informative to determine whether Eps8 could functionally compensate for inadequate levels of CP, or whether Eps8 silencing and CP silencing produce similar phenotypes. Of note, depletion of Eps8 in neurons dramatically increased the number of axonal filopodia (Menna et al., 2009).

It would be of extreme interest to correlate CP location and concentration with filopodial behavior in timelapse imaging. Our efforts to do so have been hindered by the absence of a suitable live probe for CP. Overexpressing eGFP-tagged versions of either the α or β subunits of CP, or both concurrently, results in a cytosolic expression pattern that does not coincide with the localization pattern of endogenous CP as detected by antibody staining of fixed tissues (data not shown).

One final question raised by the data presented in Chapter One is: is filopodial severing affected by CP depletion? We often observe in timelapse imaging that filopodia that seem to be adherent to the underlying substratum often sever from the cell as the cell moves away. It would be interesting to determine whether the incidence of filopodial severing is affected by CP depletion, especially because we posit that filopodia without CP may have an increased number of severed or depolymerizing actin filaments (See Fig. 1.11). We could address this question by analyzing the filopodial severing incidence in control and CP-depleted cells in timelapse sequences.
Chapter Three.

It has been shown that inhibiting filopodia formation by overexpression of CP or inhibition of Ena/VASP activity delays neuronal maturation (Dent et al., 2007; Kwiatkowski et al., 2007; Lebrand et al., 2004). Conversely, restoring filopodia by overexpressing Myosin X in cells that lack Ena/VASP rescues the maturation defect (Dent et al., 2007). From these data it has been concluded that filopodia are required for neurite initiation (Dent et al., 2007). Our data demonstrate that reducing CP levels both qualitatively increases filopodial numbers and accelerates neuronal maturation, and is thus consistent with previous reports. In total, this data raise the very interesting question: what is the mechanism linking filopodial numbers and neuronal maturation?

In both invertebrates (O'Connor et al., 1990) and mammals (Dent et al., 2007), neurites can sometimes arise from dilated filopodia; thus, one could imagine that an increased number of filopodia could directly result in an increased number of neurites; increased neurite number could then possibly account for accelerated neuronal maturation. However, it is unclear whether neurites in vivo normally arise by dilation of filopodia. Moreover, we found that neurite number of Stage III neurons was not affected by CP depletion. Thus, it is unclear how increased filopodial numbers could account for accelerated maturation. It is also striking that both in our case and with inhibition of Ena/VASP (Kwiatkowski et al., 2007; Lebrand et al., 2004), changes in neuronal maturation occurred without changes in total neurite outgrowth or axon length.

One way to investigate the process of neuronal maturation is via extended timelapse imaging. Following neurons from Stage I to Stage III can lead to insights into how neurite initiation (the transition from Stage I to Stage II) and axon specification and outgrowth (the
transition from Stage II to Stage III) are affected, if at all, by the increased numbers of filopodia. Neurons transfected with a fluorescent molecule conjugated to LifeAct can allow visualization of the actin cytoskeleton over time (Riedl et al., 2008).

Ultimately, the most interesting question with regard to CP depletion in young neurons relates to axon guidance, or the ability of neurons to correctly find their synaptic targets. Does CP depletion affect the ability of young neurons (and specifically, of the growth cones at the tips of neurites) to successfully respond to positive and negative guidance cues? To address this issue, one could perform growth cone turning assays with young neurons depleted of CP.

Assays using microfluidics chambers may produce more precise and defined gradients of soluble molecules than the traditional creation of gradients using micropipettes (Pujic et al., 2008; Wang et al., 2008). In addition, the former can also create gradients of bound molecules (Wang et al., 2008). One could also use microfluidics chambers to quantify axon outgrowth (Shi et al., 2010); such chambers offer the additional advantage of isolating the growth cone from the cell body for pharmacological manipulations.

Guidance could be affected by either defects in growth cone motility or defects in growth cone chemosensing abilities, and the experiments described above do not necessarily distinguish between these two possibilities. However, determining the requirement for CP in axon guidance would help to address a major gap in our understanding regarding the function of actin filament capping in growth cone function (Dent et al., 2011).
Works Cited


