KINETIC ANALYSIS OF TRUNCATION AND ELONGATION MUTANTS OF THE CapG SEVERING MUTANT

By

ANDREA ROEBUCK

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2006

To my mother, whose presence here today exemplifies perseverance.

ACKNOWLEDGMENTS

I would first like to extend my thanks to my mentor (whose patience is great) and to my supervisory committee for their thoughtful input. Members of my lab should be commended for their support. I thank my family for their encouragement and love.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS ix

ABSTRACT x

CHAPTER

1 INTRODUCTION 1

Actin 1
Actin-Regulatory Proteins 2
Origins of the Name CapG 3
Characterizing CapG 3
Evolution/Sequence Comparison 4
CapG Sequence, Structure and Function 4
Regulation of CapG 5
Cellular Distribution 6
Membrane Ruffling 6
Mutations to CapG 6
Severing Mutant Mutations 7

2 MATERIALS AND METHODS 9

Site-Directed Mutagenesis 9
Protein Expression and Purification 9
Actin Purification 10
Kinetic Assays 11
Subcritical Actin Monomer Fluorescence Assay 11
Monomer Sequestration Assay 12
Capping Assay 12
Severing Assay 12

3 RESULTS 14

Purification of CapG Mutants 14
Structure-Function Analysis 14
Effects of Actin Monomer Binding by CapG Mutants 14
Capping 16
Severing 17

4 DISCUSSION 21

Role of the S1-S2 Linker Length on Function 21
Structural Determinants of Capping and Severing 21
Comparison between CapG Severing Mutant and New Mutants 23

5 CONCLUSION 28

LIST OF REFERENCES 29

BIOGRAPHICAL SKETCH 32

LIST OF TABLES

Table page

2-1. Primer design for CapG severing mutants.....13

4-1. Functional activities of CapG and its mutants.....24

LIST OF FIGURES

Figure page

1-1 Sequence analysis of CapG, gelsolin, villin, fragmin, and severin 8

3-1 Protein gels of the CapG severing mutant and its triplicate mutants 17

3-2 Monomer Binding of CapG Severing Mutant and its triplicate mutants 18

3-3 Capping activities of CapG Severing Mutant triplicate mutants.....19

3-4 CapG Severing Mutant triplicate mutants lack severing activity.....20

4-1 Structure of the CapG severing mutant. 25

4-2 Possible model of filament capping by gelsolin.....26

4-3 Sequence of events during severing of actin by fully activated gelsolin 27

LIST OF ABBREVIATIONS

Å	Angstrom
ATP	Adenosine 5'-triphosphate
˚C	Degree Celsius
cDNA	Complementary deoxyribonucleic acid
Cys	Cysteine
DNA	deoxyribonucleic acid
DEAE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)] tetraacetic acid
g	gravity
h	hour
IPTG	isopropyl-1-thio-*β*-D-galactoside
kb	kilobase
kDa	kilodalton
λ_{em}	wavelength of emission
λ_{ex}	wavelength of excitation
L	liter
LB	Luria-Bertani broth
µ	micro
m	meter
min	minute
M	molar
n	nano
PCR	polymerase chain reaction
pH	hydrogen ion concentration
pyrene	*N*-(1-pyrenyl) iodoacetamide
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

KINETIC ANALYSIS OF TRUNCATION AND ELONGATION
MUTANTS OF THE CapG SEVERING MUTANT

By

Andrea Roebuck

May 2006

Chair: Frederick Southwick
Major Department: Medical Sciences—Molecular Genetics and Microbiology

Numerous proteins help regulate the actin cytoskeleton network, one of several systems that necessitate cellular movement. One such regulator is CapG, whose main role is to cap the barbed ends of actin filaments, thereby preventing actin monomers from additional polymerization at the barbed end. Other family members in the gelsolin superfamily (to which CapG belongs) possess the ability to sever actin filaments. CapG was mutated in two separate locations based on family-member sequence homology, to create a mutant that could both sever and cap filamentous actin (the CapG severing mutant). In this work, site-directed mutagenesis was employed to change the critical domain I-II linker region of the CapG severing mutant by three amino acids. One insertion and two deletion mutants were created.

The three mutants (CapG severing mutant +AAA, –KHV, and –GGV) were then subject to three types of kinetic assessments: monomer binding, capping, and severing of pyrene-labeled actin. The monomer binding curve of CapG severing mutant +AAA was

most similar to its parent CapG severing mutant. However its affinity for actin monomers and for mutants –KHV and –GGV was lower than that for CapG severing mutant and resembled wild type CapG. All three mutants were still capable of capping the barbed ends of actin filaments. However, all three mutants lost the ability to sever filamentous actin. These observations demonstrate that length and charge interactions in the domain I-II linker are critical for proper severing function, and that alterations in length are less detrimental to the capping function of the CapG severing mutant.

CHAPTER 1
INTRODUCTION

Cell movement is essential for such activities as chemotaxis, wound healing, pathogen invasion, and immune function (Marx, 2003). The actin cytoskeleton and actin-regulatory proteins play vital roles in generating the forces and shape changes required for cell movement.

Actin

Actin is a globular protein that is separated into two lobes by a cleft that forms the ATP-binding site (Bettinger et al., 2004). ATP-bound actin monomers (globular or G-actin) can assemble into filaments (filamentous or F-actin), associated with the hydrolysis of ATP. Actin filaments are composed of two strands that twist around one another to form a double right-handed helix. These filaments are further organized by accessory proteins into diverse structures that carry out actin's cytoplasmic functions. There are three isoforms of actin in higher eukaryotes; α-actin is muscle specific, whereas β- and ɣ-actin are found in all cell types (Bettinger et al., 2004).

Actin assembly from G-actin to F-actin occurs in a three-step process. The first step is also the rate-limiting step (called nucleation or the lag phase). At least three actin monomers need to aggregate for nucleation to begin. After the initial setup, the growth or elongation phase occurs as subunits rapidly add to the nucleated filament ends. The final step is the steady-state phase, when a balance exists between the rate of new subunits adding to the filament ends and the rate of subunits leaving the ends. A value known as the critical concentration designates the concentration of free subunits left in

solution once the steady state is reached. Below this value actin will depolymerize and above the critical concentration it will polymerize (Oosawa and Asakura, 1975).

Once assembled into a filament, actin has two distinct ends: a barbed end and a pointed end. The pointed end is known historically for the arrow-like appearance of myosin heads bound along the filament (Alberts et al., 2002). The barbed end is also called the plus end, and is the more dynamic of the two ends; meaning that monomers will grow and depolymerize faster at this end than at the pointed or minus end.

Actin-Regulatory Proteins

A myriad of proteins are dedicated to actin regulation. These include proteins that nucleate, bind monomers, side bind, bundle/cross-link, cap and sever actin (Ayscough, 1998). Actin binding-proteins are also found in the nucleus (Bettinger et al., 2004). I focused on the capping and severing functions of actin regulatory proteins. When one end of the actin filament is capped, no further polymerization or depolymerization occurs at that end. A protein that severs binds alongside the actin filament and inserts itself between adjacent subunits to weaken the noncovalent bonds of the filament, until it severs and binds to one of the barbed ends (Ferrary et al., 1999).

Both capping and severing abilities play essential roles in cell motility. When a protein severs an actin filament, new ends are created as old filaments are dismantled. Consider a macrophage in fierce pursuit of a bacterium. Inside the macrophage is an actin network that is continuously chopped up by severing proteins and redistributed to form new positions that the macrophage must assume to catch its bacterial prey.

A model that illustrates the importance of capping is cellular necrosis. When a cell dies, both G-actin and F-actin pour into the extracellular space. Conditions in this space (ionic strength, pH, and temperature, etc.) are conducive for rapid polymerization of the

actin monomer pool which leads to increased viscosity and potential damage to the surroundings. Proteins that cap actin stop this elongation, reduce viscosity, and thwart uncontrolled polymerization (Burtnick et al., 1997).

Gelsolin, an 82 kDa protein that contains six domains and has the ability to cap and sever actin filaments, was discovered in 1979 by Yin and Stossel. Gelsolin along with related proteins including villin, severin, adseverin, fragmin and CapG, constitute the gelsolin superfamily.

Origins of the Name CapG

My study focused on CapG. This gelsolin family member was discovered in 1986 by Southwick and DiNubile. CapG has also been called macrophage capping protein (MCP), gCap39, and *Myc* basic motif homolog-I (Mbh1). Then in 1994 under a suggestion from the Genome Data Base organizers at Johns Hopkins University Medical School, researchers agreed on the name CapG. The "Cap" denotes the protein's ability to cap the barbed ends of actin filaments, but not to sever them. The "G" signifies that the derived amino acid sequence most closely resembles gelsolin (Mishra et al., 1994). Due to this relatedness, gelsolin will be compared alongside CapG for most of the discussion.

Characterizing CapG

CapG has a molecular weight of 38 kDa. There are three 14 kDa repeat subdomains, as compared to gelsolin which contains six of these 14 kDa repeats (Mishra et al., 1994). CapG has a stokes radius of 3.0 nM, and its isoelectric point is 6.6, migrating as a single polypeptide (Southwick and DiNubile, 1986).

According to Mishra et al. (1994) the CapG gene is 16.6 kb with 10 exons and 9 introns. The open reading frame is 6.9 kb with 9 exons, 8 introns, and 3 splice sites that

are nearly identical to the human gelsolin gene. The proximal short arm of chromosome 2 houses the CapG gene.

Evolution/Sequence Comparison

In 1994 Mishra et al. asked whether gelsolin and/or villin may have arisen from CapG gene duplication. However the pathway to this evolutionary jigsaw appears to be more complex than simple CapG gene duplication. One clue to the complexity of this issue is derived from sequence comparisons. When CapG is compared to other gelsolin family proteins, the sequence identity varies depending on the region of the protein as well as its origin (i.e., mammalian vs. other). The first three domains of mammalian gelsolin show a 49% sequence identity to CapG. However, the last three domains are only 16% identical, thus casting doubt on the possibility of CapG gene duplication (Dabiri et al., 1992). Mammalian villin also showed this sequence identity discrepancy with a 41% identity in the first three domains, and only a 10% identity in the last three domains (Dabiri et al., 1992). Other family members with three domains include fragmin (*Physarum polycephalum*) and severin (*Dictyostelium discoideum*), that show sequence identity to CapG of 30% and 25%, respectively (Dabiri et al., 1992).

CapG Sequence, Structure and Function

CapG possesses three domains. According to Yu et al. (1991) domain I is gelsolin's equivalent for actin monomer binding. CapG domain I is dependent on Ca^{2+} for actin binding where as gelsolin does not share this dependency. Gelsolin has 6 domains, referred to as G1-G6. In gelsolin's N-terminal half there is an actin side-binding site (domains II and III), and a monomer binding site (domain I), but domain I and half of domain II are required for severing actin filaments (Yu et al., 1991).

In 1991 Prendergast and Ziff probed for candidate factors to interact with the c-Myc oncoprotein. They found CapG and therein a distantly related basic/helix-loop-helix (B/HLH) DNA-binding motif. The real catch was the potential nuclear localization signal dubbing CapG a nuclear protein. In 1993 this fact was solidified by Onoda, Yu and Yin who discovered that CapG was indeed both a nuclear and cytoplasmic protein. Another 1993 paper by Onoda and Yin showed that CapG could be phosphorylated and that this phosphorylated form (enhanced by okadaic acid) of CapG was abundant in the nucleus, perhaps providing some sort of subcellular regulation. CapG's size of 38 kDa places it on the border line of being actively transported or passively diffused into the nucleus which becomes hampered and inefficient as protein size approaches 20-40 kDa (Van Impe et al., 2003). They also reported that CapG did not possess the nuclear localization signal typified by other nuclear actin-binding proteins (i.e., supervillin and zyxin). Severin and fragmin were found to contain Rev-like nuclear export signals in their N-terminus which controlled their passage from the nucleus to cytoplasm.

In terms of function, CapG has the ability to cap the barbed ends of actin filaments. It should again be noted that CapG unlike its other family members is not capable of severing actin filaments.

Regulation of CapG

Capping activity in CapG requires micromolar concentrations of Ca^{2+}. CapG can dissociate from actin filament ends readily either by a decrease in Ca^{2+} levels to submicromolar concentrations (Southwick and DiNubile, 1986) or by increasing phosphoinositide 4,5-bisphosphate concentrations (Yu et al., 1990). Gelsolin uncapping

requires both increases in phosphoinositides and a decrease in Ca^{2+} concentrations (Yu et al., 1990).

Cellular Distribution

With the exception of platelets, CapG is found in nearly all cells usually at low concentrations of approximately 0.05% of the total protein. However, CapG is one of the most abundant cytoplasmic proteins in macrophages and dendritic cells, where it accounts for 0.9-1% of the total cytoplasmic protein (Dabiri et al., 1992; Parikh et al., 2003). Yu et al. (1990) also purified CapG from human plasma where it appeared to be a minor component. Gelsolin also has a broad tissue distribution and is found in platelets as well as smooth and skeletal muscle cells (Dabiri et al., 1992).

Membrane Ruffling

Loss of CapG affects cell movement (Witke et al., 2001). Macrophages from CapG knockout mice demonstrate decreases in phagocytosis and membrane ruffling. In 2003, Parikh et al. once again studied CapG knockout mice with their impaired membrane ruffling and found the null mice to be more susceptible to infection by the food borne pathogen *Listeria monocytogenes*, but demonstrated normal susceptibility to *Salmonella enterica* Serovar Typhimurium infection. This suggested that patients with unexplained listeriosis may be suffering from a CapG deficiency, although no current CapG defects in humans have been described (Parikh et al., 2003).

Mutations to CapG

In 1995 Southwick reported a series of gain-of-function mutations to CapG which rendered the protein capable of severing actin filaments. First the sequences of CapG, gelsolin, villin, severin and fragmin were scrutinized for consensus sequences likely to confer severing function (Figure 1-1). Two CapG sequences were found to be divergent.

Thus mutations to convert the divergent stretches to gelsolin sequences were performed and a gain-of-function resulted. The resultant mutant CapG ^{124}GFKHV^{128} and ^{84}LDDYLGG^{90} still did not sever as well as gelsolin (gelsolin required one-fiftieth the concentration to cause severing comparable to the ^{124}GFKHV^{128} and ^{84}LDDYLGG^{90} mutant). The LDDYLGG region is known to be the central region of a long α-helix that interacts with the subdomain 1 and 3 cleft of the actin monomer (Southwick, 1995).

Further mutations were performed to CapG ^{124}GFKHV^{128} and ^{84}LDDYLGG^{90} again as dictated by sequence divergence in other gelsolin family members. Amino acids 129-137 of CapG were made identical to gelsolin. The new mutant CapG ^{124}GFKHVVPNEVVVQR^{137} and ^{84}LDDYLGG^{90} proved to be a more potent severer than the original gain-of-function by 3-4 fold (Zhang et al., unpublished).

Severing Mutant Mutations

My contribution to understanding how CapG's structure relates to its kinetic functions was to perform truncation and addition mutations to the already mutated CapG ^{124}GFKHVVPNEVVVQR^{137} and ^{84}LDDYLGG^{90} (here called CapG severing mutant). One addition and two deletions were made in sets of three amino acid changes to the linker region between domains I and II that is believed to hold spatial significance to the severing ability previously bestowed upon this mutant. It was determined that three amino acid changes would be sufficient to alter this linker's length, and might be expected to alter CapG severing mutant's function as reflected by kinetic analysis experiments.

Mutations performed in my studies were as follows:

- CapG severing mutant ^{15}KYQEGGVESGFKHVV
- Deletion of –KHV ^{115}KYQEGGVESGF____V
- Addition of +AAA ^{115}KYQEGGVESAAAGFKHVV
- CapG severing mutant ^{115}KYQEGGVESGFKHVV
- Deletion of –GGV ^{115}KYQE____ESGFKHVV

I postulated that these mutations would not alter the ability of CapG severing mutant to cap the barbed ends of actin filaments. However, given the complexity of the severing mechanism, I proposed that the mutations would modify the proteins ability to sever actin filaments.

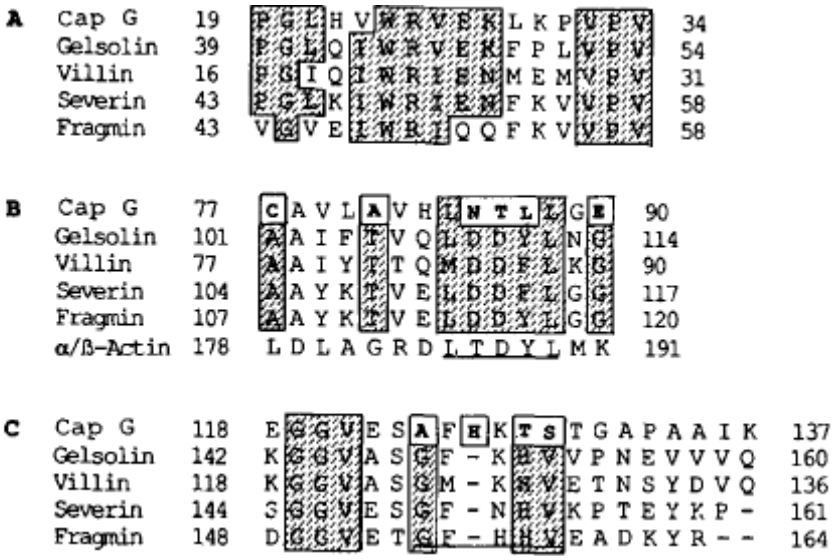


Figure 1-1. Sequence comparisons of CapG, gelsolin, villin, fragmin, and severin. A) amino acid sequences were compared within the region previously suggested to be important for severing. The shaded areas show regions of high identity. Two regions (in B and C) revealed deviation of the CapG sequence (boldface letter in unshaded blocks from the consensus sequences shared by the severing proteins. Gaps are designated by dashes. The region of identity to the α- and β-actin sequence in underlined in B. The region highly conserved among the severing proteins, but different in CapG, is underlined in C. (Southwick, F.S. 1995. Gain-of-function mutations conferring actin-severing activity to human macrophage cap G. *J. Biol. Chem*. 270:45-8. Figure 1, page 46.)

CHAPTER 2
MATERIALS AND METHODS

Site-Directed Mutagenesis

Human CapG cDNA was cloned into pET12a at *NdeI* and *SalI* polylinker sites as previously described by Dabiri et al. (1992). Mutants were generated by Stratagene's QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. PCR melting, annealing, and elongation temperatures used were 95, 55, and 68˚C respectively.

Protein Expression and Purification

Clones in BL21(DE3) were grown in 1L LB at 37˚C containing 50 µg/ml carbenicillin to an A_{600} = 0.6 and induced with 0.5 mM isopropyl-1-thio-β-D-galactoside (IPTG). Upon induction CapG severing mutants –KHV and –GGV were grown for 3 h at 25˚C. CapG severing mutant +AAA remained at 37˚C for 3 h post-induction. To harvest cells, centrifugation was carried out at 5,000 x *g* for 5 min at 4˚C. Pellets were resuspended in 10 ml lysis buffer (10 mM Imidazole, 1 mM EGTA, 1 mM DTT, 1X mini cocktail protease inhibitor tablet from Roche Applied Science, Mannheim, Germany) and then freeze thawed three times to lyse cells. 100 µg/ml lysozyme and 10 µg/ml DNaseI was added and samples shaken for 30 min at 25˚C before sonication at 80% power for 3 min. The cell lysates were then centrifuged at 40,000 x *g* for 30 min at 4˚C and resultant supernatant and pellet fractions were analyzed on Coomassie Blue stained SDS-polyacrylamide gels.

The supernatants were loaded onto DEAE ion-exchange columns (Amersham Biosciences). If necessary samples were concentrated in a 9 kD Apollo 20 ml high performance centrifugal spin concentrators (Orbital Biosciences, LLC, Topsfield, MA) for 15 min at 170,000 X *g*, 4˚C, and repeated until a volume of 5 ml was attained before being loaded onto a HiPrep desalting column (Amersham Biosciences).

Coomassie Blue stained SDS-polyacrylamide gels were employed to analyze the proteins purity. Pure fractions were pooled and stored in 30% ethylene glycol. The method of Bradford was used to obtain protein concentrations using BSA standards (Bradford, 1976).

CapG severing mutant was grown and harvested following the same protocol as the mutants except that CapG severing mutant was grown at 37˚C for 2 h post-induction.

Actin Purification

Actin was purified from skeletal muscle (rabbit, porcine, chicken) and conjugated to *N*-(1-pyrenyl) iodoacetamide at actin's Cys 374 as previously described (Young et al., 1990). Briefly, actin was made into an acetone powder (Spudich and Watt, 1971; Pardee and Spudich, 1982) by mincing muscle and extracting it with 1L 0.1 M KCl, 0.15 M potassium phosphate, pH 6.5 before being filtered through cheesecloth, stirred in 2L 0.05 M $NaHCO_3$ and then cheesecloth filtered again. This extraction was followed by the addition of 1L 1 mM EDTA, pH 7.0 and then two extractions of 2L of distilled H_2O. The last five extractions were performed with 1L acetone. The filtered residue was air dried in glass evaporating dishes overnight in a fume hood. The resulting acetone powder was extracted in Buffer-G (5 mM Tris, 0.1 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM DTT, 0.01% Sodium Azide, pH 8.0) and filtered through cheese cloth before being ultracentrifuged for 40 min at 4˚C, 28,000 X *g*. The supernatant was slowly stirred at room temperature for

1 h while adding 2 µl/ml $MgCl_2$ and 52 mg/ml KCl to polymerize the actin, and followed by centrifugation for 3 h at 4˚C, 49,300 x *g*. The actin pellet was resuspended in a minimal volume of Buffer-G and dialyzed against fresh Buffer-G for 3 days to depolymerize the filaments. Residual F-actin was removed by ultracentrifugation for 45 min at 4˚C, 49,300 x *g*. The supernatant was loaded onto a S-200 Column equilibrated with Buffer-G and eluted with Buffer-G. Purified monomeric actin was labeled with pyrene, *N*-(1-pyrenyl) iodoacetamide, according to standard procedures (Kouyama and Mihashi, 1981). The critical concentration was determined before each assay and found to be between 0.01 µM and 0.08 µM. (Cooper et al., 1983).

Kinetic Assays

Subcritical Actin Monomer Fluorescence Assay

Increasing amounts of protein were added in a glass cuvette with monomeric actin just below the critical concentration at 300 nM G-Actin in S1 buffer (10 mM imidazole, 100 mM KCl, 0.4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.5 mM DTT, pH 7.6). The solution was mixed 5X after addition of protein to ensure the formation of protein-actin complexes. These complexes are weakly fluorescent due to the conformation change of the actin molecule labeled with pyrene. Fluorescent intensity of these protein-actin complexes was measured and once the intensity values stabilized, additional CapG mutant protein was added to the solution, mixed 5X, and the intensity was measured again. The final intensity values were plotted against protein concentration. Fluorescence was monitored on a HORIBA Jobin Yvon FluoroLog 3 spectrofluorometer (Edison, NJ) with λ_{ex} = 366 nm and λ_{em} = 385 nm while maintaining a slit length at 5.0 mm. Data was collected using DataMax Software (HORIBA Jobin Yvon Edison, NJ).

Monomer Sequestration Assay

2 μM Actin was polymerized in S1 buffer (10 mM imidazole, 100 mM KCl, 0.4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.5 mM DTT, pH 7.6) using gelsolin capped F-actin nuclei, molecular ratio 1:20 (gelsolin to actin, final concentration of F-actin seeds of 0.1 μM). Assembly rates of the pointed end were measured in the presence of increasing concentrations of capping protein, 3 μM G-actin, and 200 μl 2XP buffer (20 mM imidazole, 0.2 mM KCl, 4 mM $MgCl_2$, 2 mM ATP, 2 mM DTT, pH 7.4). The capping protein sequestered monomers away from the G-actin pool resulting in a decrease in fluorescence of pointed end filament assembly. Fluorescent intensity was observed with respect to time on the FluoroLog 3 (same settings as described before).

Capping Assay

2 μM Actin was allowed to polymerize overnight in S1 buffer (10 mM imidazole, 100 mM KCl, 0.4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.5 mM DTT, pH 7.6) at 25˚C in the dark. F-Actin was diluted 1:20 below the critical concentration in S1 buffer to 100 nM in a glass cuvette. F-Actin was mechanically sheared 5X with an extended length p200 pipette tip (Fisher Scientific, Suwanee, GA) to create new barbed and pointed ends that rapidly depolymerized as shown by a decrease in fluorescent intensity. Increasing amounts of capping protein were added to the buffer which capped the barbed ends of the actin filaments and retained fluorescent intensity. Fluorescence was monitored over time on the FluoroLog 3 (spectrofluorometer settings as described before).

Severing Assay

2 μM Actin was allowed to polymerize in S1 buffer (10 mM imidazole, 100 mM KCl, 0.4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.5 mM DTT, pH 7.6) using gelsolin

capped actin filament molar ratio 1:200 (gelsolin to actin). F-Actin was diluted 1:20 below the critical concentration in S1 in buffer to 100 nM in a glass cuvette and mixed gently 5X to avoid shearing filaments (and keep fluorescence at a steady intensity) before adding capping protein while mixing another 5X. Severing proteins create new barbed and pointed ends that depolymerize below the critical concentration resulting in decreased fluorescent intensity. Fluorescence was observed with respect to time on the FluoroLog 3. Spectrofluorometer settings were the same.

Table 2-1. Primer design for CapG severing mutants.

+AAA—foward

					Δ	Δ	Δ						
119	120	121	122	123				124	125	126	127	128	129
119	120	121	122	123	124	125	126	127	128	129	130	131	132
GGT	GGT	GTG	GAG	TCA	GCG	GCG	GCG	GGA	TTT	AAA	CAC	GTG	GTT
G	G	V	E	S	A	A	A	G	F	K	H	V	V
Gly	Gly	Val	Glu	Ser	Ala	Ala	Ala	Gly	Phe	Lys	His	Val	Val

+AAA—reverse

AAC CAC GTG TTT AAA TCC CGC CGC CGC TGA CTC CAC ACC ACC

-KHV—forward (delete K126 H127 V128)

							Δ	Δ	Δ					
119	120	121	122	123	124	125	126	127	128	129	130	131	132	133
GGT	GGT	GTG	GAG	TCA	GGA	TTT	AAA	CAC	GTG	GTT	CCG	AAC	GAA	GTT
G	G	V	E	S	G	F	K	H	V	V	P	N	E	V
Gly	Gly	Val	Glu	Ser	Gly	Phe	Lys	His	Val	Val	Pro	Asn	Glu	Val

-KHV—reverse

AAC TTC GTT CGG AAC CAC GTG TTT AAA TCC TGA CTC CAC ACC ACC

-GGV—forward (delete G119 G120 V121)

							Δ	Δ	Δ						
112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127
CGG	GGC	CTC	AAG	TAC	CAG	GAA	GGT	GGT	GTG	GAG	TCA	GGA	TTT	AAA	CAC
R	G	L	K	Y	Q	E	G	G	V	E	S	G	F	K	H
Arg	Gly	Leu	Lys	Tyr	Gln	Glu	Gly	Gly	Val	Glu	Ser	Gly	Phe	Lys	His

-GGV—reverse

GTG TTT AAA TCC TGA CTC TTC CTG GTA CTT GAG GCC CCG

CHAPTER 3
RESULTS

Purification of CapG Mutants

All mutants were purified as outlined in Materials and Methods. CapG severing mutant +AAA was first passed through a DEAE column and analyzed on Coomassie Blue stained SDS-polyacrylamide gels. Fractions 42 to 61 were concentrated in a 9 kD Apollo 20ml high performance centrifugal spin concentrator before being loaded onto a large HiPrep desalting column. The protein was found to be of 99% and 96% purity and collected from fractions 5-7 and 10-11, respectively (Figure 3-1A). CapG severing mutant –GGV was found to be of 99% and 90% purity and collected from fractions 17-19 and 20-24, respectively, of a DEAE column (Figure 3-1B). CapG severing mutant –KHV was collected from pooled fractions 32-37 from a DEAE column and found to be of 98% purity (Figure 3-1C).

Structure-Function Analysis

Effects of Actin Monomer Binding by CapG Mutants

We first assessed the ability of the mutant protein to bind monomeric actin. A subcritical actin monomer fluorescence assay (which operates based on change in fluorescence intensity of a subcritical concentration of pyrenyl actin; refer to Materials and Methods), was performed on all mutant and wild type proteins. The conformational change in actin upon polymerization is readily detected by the sulfhydryl reagent *N*-(1-pyrenyl) iodoacetamide. This probe was found to display a fluorescence spectrum more sensitive than previously reported probes, and has since been the standard for

kinetic assessment (Kouyama and Mihashi, 1981). When pyrenyl actin undergoes polymerization a 20 fold increase in fluorescence is observed (Kouyama and Mihashi, 1981). However, in the subcritical monomer assay where no polymerization occurs, only a 2 to 3 fold increase in fluorescence was seen. This small rise in fluorescence reflects a conformational change in monomeric actin as capping protein-actin complexes are formed (Southwick and DiNubile, 1986; Young et al., 1990 and 1994).

In Figure 3-2B CapG severing mutant +AAA reaches peak fluorescence at 2000 nM. The K_D (dissociation) was determined to be 1000 nM. CapG severing mutant –GGV achieved saturation at 5000 nM (Figure 3-2C) yielding a K_D of 2,500 nM. CapG severing mutant has a higher affinity for actin monomers with a K_D of 150 nM (Figure 3-2A), than the +AAA and –GGV mutants. Wild type CapG has a K_D reported to be 1000 nM (Young et al., 1990).

For reasons that are unknown, and in contrast to the other mutants, the –KHV mutant was not amenable to study using the subcritical actin monomer fluorescence assay. To overcome this I employed a second assay known as the monomer sequestration assay to determine the monomer binding affinity in this mutant. For this monomer sequestration assay, gelsolin is incubated with actin (1:20) which caps the barbed end of the actin filament leaving only the pointed ends open for elongation. G-actin is added to the reaction cuvette above the critical concentration of the pointed end allowing polymerization at that end. When –KHV mutant is added it cannot cap the pointed end, therefore any reduction in the rate of pointed end assembly would result from its ability to bind and prevent actin monomers from adding to the filament (often termed monomer sequestration). Figure 3-2D shows that very high amounts of CapG severing mutant

–KHV are required to inhibit actin assembly. 3000 nM is required to completely sequester actin monomers and the K_D is estimated to be 1,500 nM. The affinity of this mutant for actin monomers is markedly reduced when compared to the primary severing mutant (Figure 3-2A). All mutants, +AAA, –GGV and –KHV displayed this weaker binding affinity with a K_D of 1,000 nM, 2,500 nM and 1,500 nM respectively.

Capping

The ability of the mutants to cap barbed ends of actin filaments was assessed via a capping assay. In this assay, actin was diluted below the critical concentration and mechanically sheared as described in Materials and Methods to create free barbed ends that rapidly depolymerize. Pointed ends are also created, however, the dynamics at the pointed end are considered negligible in comparison to the more dynamic barbed end. Capping protein added in increasing amounts to the dilute actin will cap the barbed ends and retain fluorescence as depolymerization halts.

In Figure 3-3A CapG severing mutant +AAA begins to slow depolymerization at 2.5 nM. The addition of 10 nM, 20 nM and 100 nM shows similar retention of fluorescence as the +AAA mutant caps the barbed ends. Note the saturation value of capping protein will never be a perfectly straight line due to the slight depolymerization occurring at the pointed end. CapG severing mutant –GGV began capping monomers at 0.5 nM (Figure 3-3B). Good fluorescence retention is observable at concentrations of 12 nM.

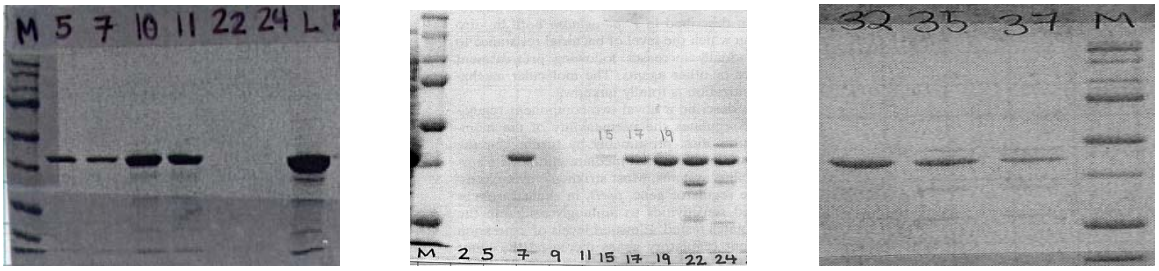
CapG severing mutant +AAA and –GGV proved to be most similar to wild type CapG with half maximal capping values of 5 nM and 3 nM respectively. Wild type CapG is reported by Southwick (1995) to have a half maximal capping value of 0.5 nM. CapG severing mutant also has a half maximal capping value of 0.5 nM (Zhang et al.,

unpublished). CapG severing mutant –KHV was found to have a much higher half maximal capping value between 20 to 50 nM (Figure 3-3C) perhaps due to steric charges (reviewed in Discussion section).

Severing

In order to ascertain whether CapG severing mutant's ability to sever was affected by altering the length of the domain I-II linker, a severing assay was performed (refer to Materials and Methods for a detailed description). Gelsolin seeded actin was diluted below the critical concentration with care taken in mixing to avoid mechanically shearing the F-Actin (fluorescence will stay constant). When the severing protein is added to the reaction cuvette the filaments will sever creating new barbed and pointed ends that will depolymerize resulting in a decrease in fluorescence.

Figure 3-4A, B, and C reveal that all CapG severing mutants +AAA, –GGV and –KHV respectively, did not exhibit any severing activity as observed by the lack of fast depolymerization despite addition of high amounts of protein. Figure control, CapG severing mutant, displays this rapid depolymerization.



A B C

Figure 3-1. Protein gels of the CapG severing mutant and its triplicate mutants. A) +AAA. B) –GGV. C) –KHV. Coomassie Blue stained SDS-polyacrylamide gels showing purified protein migrating at 38 kDa. M is protein marker, L is the column load, and numbers denote protein fractions.

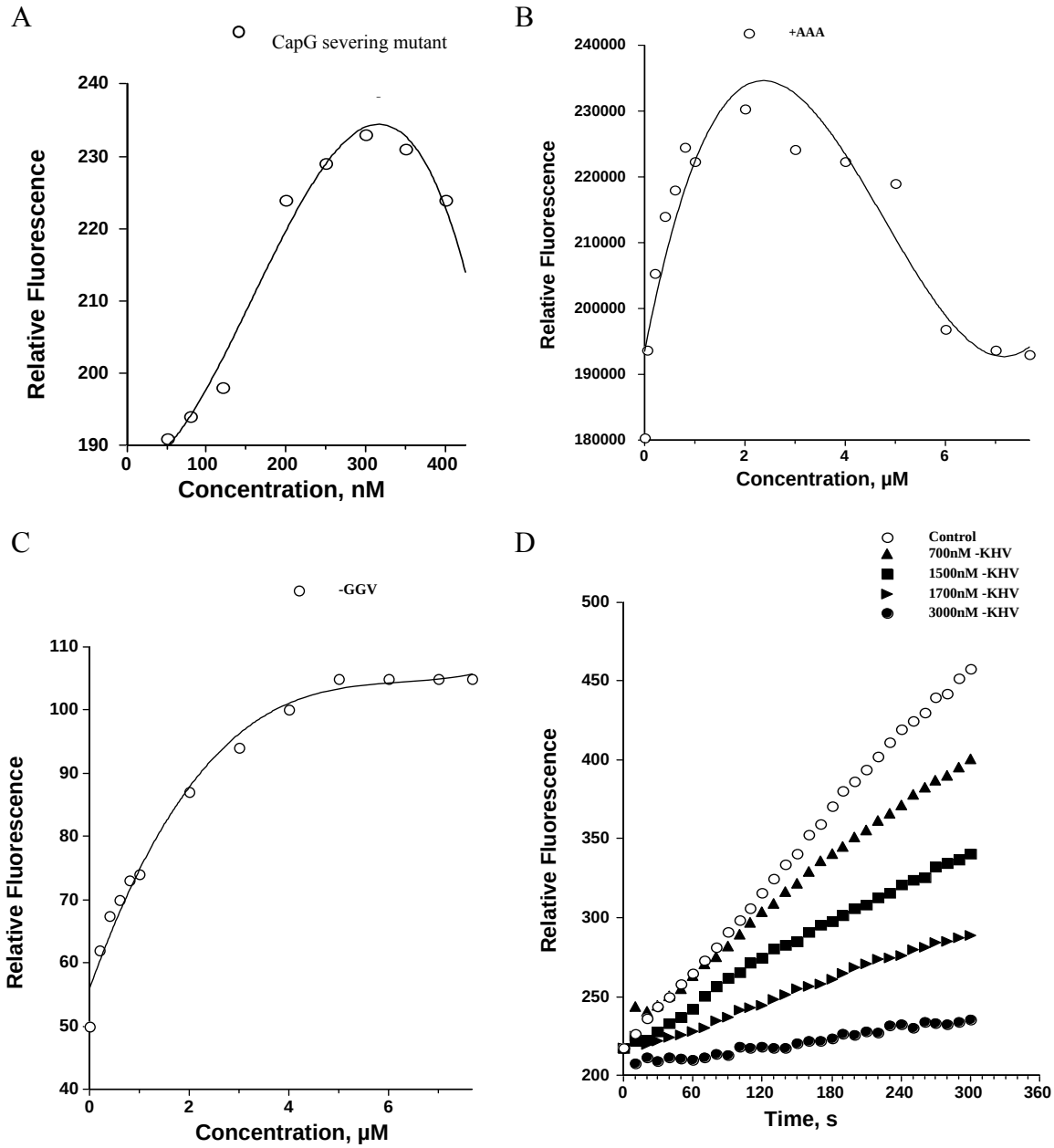


Figure 3-2. Monomer Binding of CapG Severing Mutant and its triplicate mutants. A) CapG Severing Mutant. B) +AAA. C) –GGV. Increasing amounts of capping protein were added to 300 nM G-actin in S1 buffer and mixed, and the observed fluorescence increase plotted versus the final concentrations of capping protein. D) Pointed-end growth rate inhibited by the presence of increasing amounts of CapG severing mutant –KHV. Fluorescence was monitored after the addition of 100 nM gelsolin seeded actin added to S1 buffer, capping protein, 3 µM G-actin, and 2XP buffer (refer to Materials and Methods). Open circles denotes all components mentioned above except for capping protein.

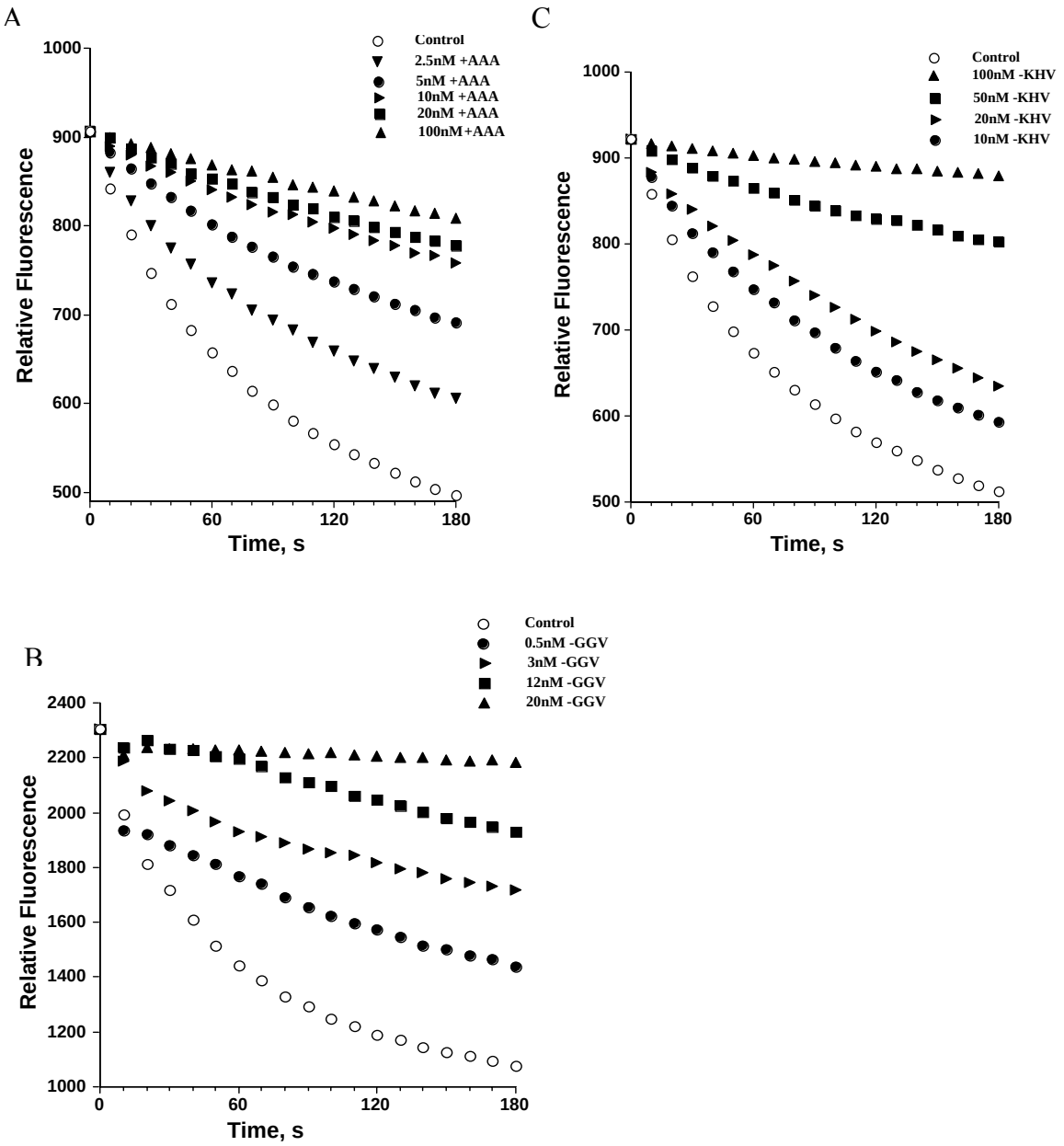


Figure 3-3. Capping activities of the CapG Severing Mutant triplicate mutants. A) +AAA. B) –GGV. C) –KHV. Depolymerization of the barbed end of 2 μM F-actin (Materials and Methods) diluted below its critical concentration to 100 nM in S1 buffer is halted by the addition of increasing amounts of capping protein (filled symbols) as evidenced by the retention of fluorescence. Note the saturation value of capping protein will never be a perfectly straight line due to the slight depolymerization occurring at the pointed end. Open circles denote the disassembly rate of 100 nM pyrene actin in S1 buffer.

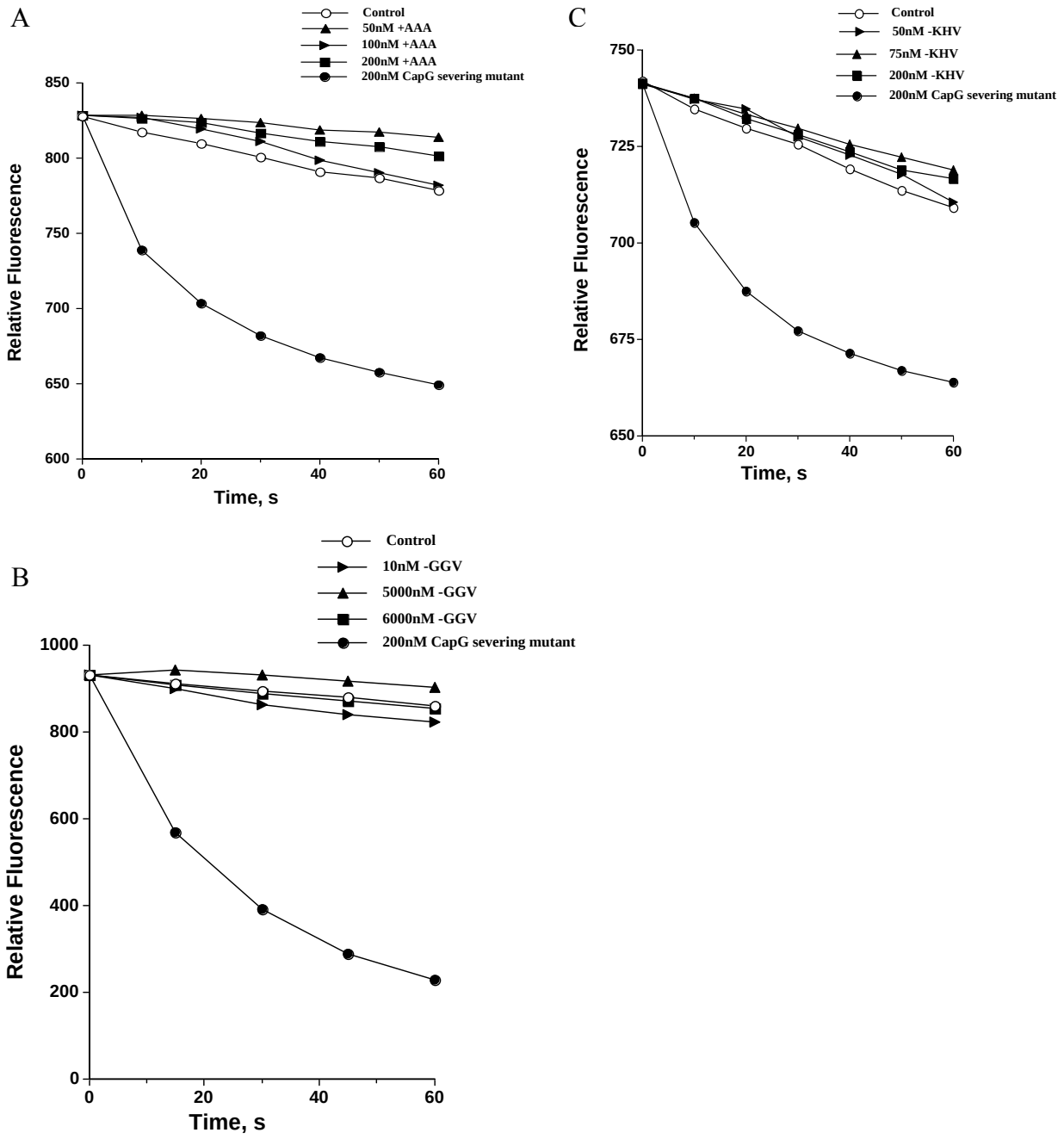


Figure 3-4. CapG Severing Mutant triplicate mutants lack severing activity. A) +AAA. B) –GGV. C) –KHV. Increasing amounts of capping protein was added to gently mixed 2 μM F-actin (refer to Materials and Methods) diluted below its critical concentration to 100 nM in S1 buffer. The depolymerization as observed in the CapG severing mutant control (filled circles) denotes severing. Open circles are the same concentration of pyrene actin in S1 buffer. No acceleration in the depolymerization rate was observed in mutants indicating a lack of severing activity.

CHAPTER 4
DISCUSSION

Role of the S1-S2 Linker Length on Function

My studies were designed to explore the effects of length change of the domain I-II linker region of the CapG severing mutant, and are based on the crystal structures of the CapG severing mutant (Figure 4-1) and gelsolin (Burtnick et al., 2004; Zhang et al., unpublished). The linker region between domains I and II in both activated CapG severing mutant and gelsolin is extended at 36Å and 30Å respectively (Burtnick et al., 2004; Zhang et al., unpublished). This linker positioning is imperative to allow the LDDYL loop steric interactions resulting in severing of the actin filament (McLaghlin et al., 1993; Zhang et al., unpublished).

Structural Determinants of Capping and Severing

The model for CapG capping is thought to be similar to gelsolin G1-G3 capping, which has been made possible through the efforts of X-ray crystallography, electron microscopy (EM), and nuclear magnetic resonance (NMR) spectroscopy (McGough et al., 2003). Figure 4-2 depicts G1 bound to subunit 1 at the very end of the actin filament and G2 bound in an area bridging subunit 1 and 3 (McGough et al., 2003). Past studies by Irobi and colleagues place G1 and G2 on adjacent monomers of the same long-pitch helix of the filament. In either case, the triplicate mutants of the CapG severing mutant did not disrupt capping activity, indicating that larger length alterations in the domain I-II linker region may be needed to misalign domain I and impair capping function.

Severing is a more complex function. In full length gelsolin, severing is initiated by Ca^{2+} which causes the protein to undergo many conformational changes (Burtnick et al., 2004). For instance Sun, et al. (1999) reports that in the presence of Ca^{2+} the extended β-sheet between G4 and G6 is broken along its interface, G6 swings out from G4 to form new contacts with G5, and actin becomes situated into the unoccupied G6 space to create an intermolecular Ca^{2+} binding site coordinated by G4 and actin. Ultimately for severing, G2-G3 attaches alongside an actin filament while the flexible G1-G2 linker extends between two adjacent protomers on the long-pitched actin strand (Burtnick et al., 2004). Simultaneously, in a separate direction the G3-G4 linker and G6 wrap over the filament's surface and direct G1 and G4 to their binding sites (Burtnick et al., 2004). A concerted pincer motion of G1 and G4 imparts a steric strain so great that enough non-covalent bonds between the actin monomers in the filament below are weakened and the filament is severed (Burtnick et al. 2004; Sun et al., 1999). Figure 4-3 illustrates the severing process in gelsolin.

CapG and other members of the gelsolin superfamily are thought to possess the same mechanism of attachment to the actin molecule as the gelsolin domain I and II linking peptide, based on sequence homology (Irobi et al., 2003). Although gelsolin severs the filament in two locations, the CapG severing mutant only severs in one location within the actin filament because it can only coordinate three subunits. We predicted that small changes in the domain I-II linker could affect the positioning of the LDDYL severing loop on the actin filament. Therefore severing would be expected to be more sensitive to alterations in the length of the linker as compared to capping.

Comparison between CapG Severing Mutant and New Mutants

To interact with actin, CapG severing mutant must first bind to one or more actin monomers within a filament. Therefore, monomer binding was first assessed. Deleting or adding amino acids in triplicate was detrimental to CapG severing mutants' monomer binding affinity as evidenced by a 7-fold reduction in +AAA, a 17-fold reduction in –GGV and a 10-fold reduction in –KHV. When compared to wild type CapG with a K_D reported to be 1000 nM (Young et al., 1990) the triplicate mutants' affinity for monomeric actin is comparable (a K_D of 1000 nM, 2,500 nM and 1,500 nM for +AAA, –GGV and –KHV mutants respectively, Table 4-1).

In Zhang et al., unpublished, it has been postulated that CapG severing mutant contains two actin binding sites. The addition mutant +AAA appears to also possess this second actin binding site as evidenced by the decrease in fluorescence at very high concentrations of the mutant protein. The deletion mutants –GGV and –KHV appear to have a stoichiometry similar to wild type CapG with only one actin binding site, since the fluorescence remains elevated at very high concentrations of protein.

The ability to cap was also hindered for the –KHV mutant who's half maximal capping constant was between 20-50 nM as compared to wild type CapG and CapG severing mutant, both with a half maximum capping at 0.5 nM. CapG severing mutant –KHV appeared to be the odd mutant out when compared to mutants +AAA and –GGV. This difference could be the consequence of electrostatic charges. The lysine (K), histidine (H), and valine (V) are associated with basic, basic, and neutral charges respectively. Whereas the other mutants addition of three neutral alanines (A) and deletion of neutral glycines (G) and valine, would have no effect on charge but simply affect the length of the linker region.

The most profound functional effect was on severing. Figure 4-1 helps illustrate how alterations in length will affect what amino acid charges get placed adjacent to each other. In mutation site II where the triplicate mutations were made, the side chains are visible. These side chains can attract or repel the actin residues that make contact with the CapG severing mutant during binding. Side binding of the actin filament is necessary for severing to occur, thus the contacts between the side-binding protein and actin need to be favorable. All triplicate mutants of the CapG severing mutant did not sever, once again stressing the importance of both length and charge of the linker region between domains I-II in order for severing to occur.

Table 4-1. Functional activities of CapG and its mutants

Protein	K_D of G-actin (nM)	½ max capping (nM)	Severing activity
CapG	1000 (Young et al., 1990)	0.5 (Southwick, 1995)	No
CapG severing mutant	150	0.5 (Zhang et al., unpublished)	Yes*
CapG severing mutant **+AAA**	1000	5	No
CapG severing mutant **-GGV**	2,500	3	No
CapG severing mutant **-KHV**	1,500	20-50	No

*Half-maximal severing reported to be 30-50 nM (Zhang et al., unpublished).

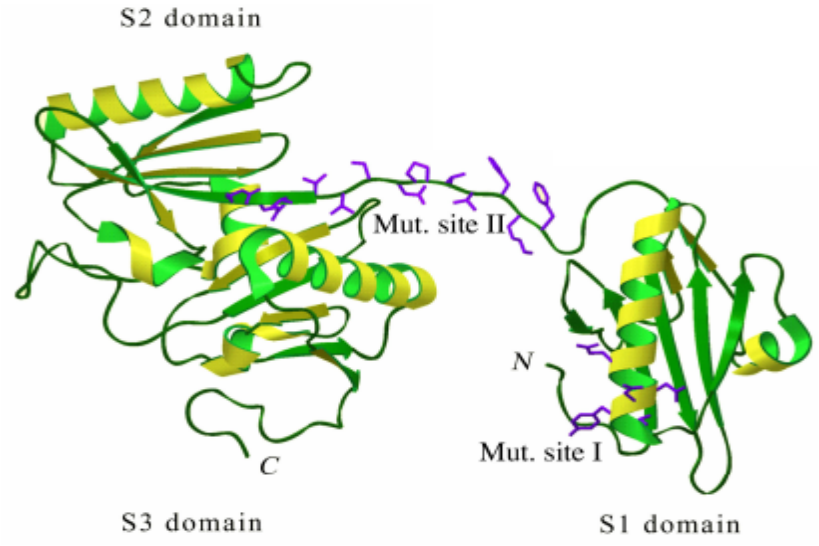
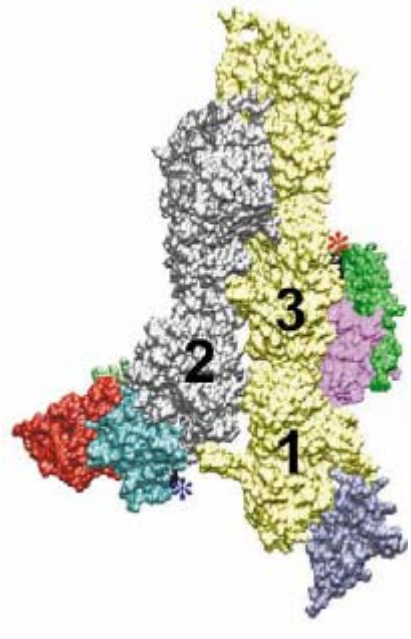


Figure 4-1. Structure of the CapG severing mutant**.** (Zhang, Y., S.M. Vorobiev, B.G. Gibson, B. Hao, G. Sidhu, V.S. Mishra, E.G. Yarmola, M.R. Bubb, S.C. Almo, and F.S. Southwick. Unpublished. A CapG gain-of-function mutant reveals critical structural and functional determinants for actin filament severing.)



G1=blue violet
G2=pink
G3=green
G4=aqua
G5=red
G6=lime green

Figure 4-2. Possible model of filament capping by gelsolin. Actin filaments are represented by space-filling models oriented with the minus or slow-growing end up. Actin subunits from one long-pitch strand are colored yellow and those from the other are colored gray. (McGough, A.M., and C.J. Staiger, J.K. Min, and K.D. Simonetti. 2003. The gelsolin family of actin regulatory proteins: modular structures, versatile functions. FEBS Lett. 552:75-81. Figure 2A, page 78.)

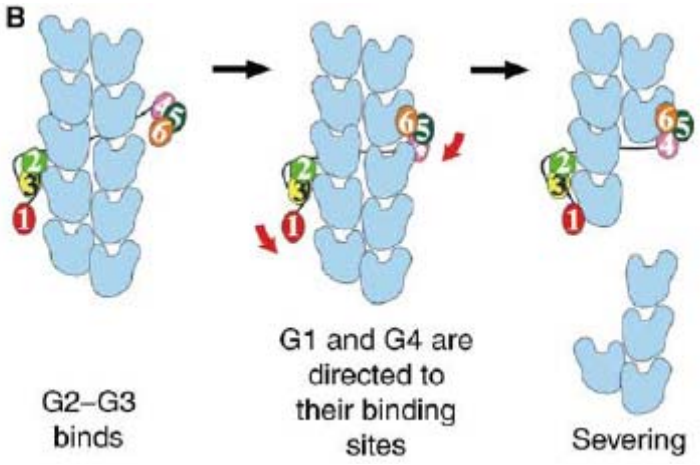


Figure 4-3. Sequence of events during severing of actin by fully activated gelsolin. Actin protomers shown in blue. Gelsolin subunits are multi-colored ovals. Reprinted by permission from Macmillan Publishers Ltd: [The EMBO Journal] (Burtnick, L.D., D. Urosev, E. Irobi, K. Narayan, and R.C. Robinson. 2004. Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. *EMBO J.* 23:2713-22. Figure 2B, page 2716.), copyright (2004)

CHAPTER 5
CONCLUSION

The reorganization of the actin network by many different proteins is essential for cell movement. The role of CapG in the cell is to cap the barbed ends of actin filaments thereby preventing further monomer growth from that end. CapG is unique among its gelsolin family members in that it does not possess the ability to sever filamentous actin. Severing is necessary for cellular plasticity; it erases old actin networks so that new ones can be formed (Ferrary et al., 1999). CapG was mutated to resemble gelsolin in two separate locations, ^{124}GFKHVVPNEVVVQR^{137} and ^{84}LDDYLGG^{90} to create the CapG severing mutant (Zhang et al., unpublished). When the length of the region linking domains I-II is altered by three amino acids, severing function is lost but capping activity is preserved. To further investigate how length translates structurally one would need to perform a crystallographic analysis to view the exact positioning of the affected domain I-II linker in the triplicate mutants compared to the CapG severing mutant.

LIST OF REFERENCES

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. The Cytoskeleton in *Molecular biology of the cell, 4th ed*. p.916, Garland Science: New York, New York, 2002.

Ayscough, K.R. *In vivo* functions of actin-binding proteins. 1998. *Curr*. *Opin*. *Cell Biol*. 10:102-11.

Bettinger, B.T., D.M. Gilbert, and D.C. Amburg. 2004. Actin up in the nucleus. *Nat*. *Rev*. *Mol*. *Cell Biol*. 5(5):410-5.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal*. *Biochem*. 72:248-54.

Burtnick, L.D., E.K. Koepf, J. Grimes, E.Y. Jones, D.I. Stuart, P.J. McLaughlin, and R.C. Robinson. 1997. The crystal structure of plasma gelsolin: implications for actin severing, capping, and nucleation. *Cell*. 90:661-70.

Burtnick, L.D., D. Urosev, E. Irobi, K. Narayan, and R.C. Robinson. 2004. Structure of the N-terminal half of gelsolin bound to actin: roles in severing, apoptosis and FAF. *EMBO J*. 23:2713-22.

Cooper, J.A., S.B. Walker, and T.D. Pollard. 1983. Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. *J*. *Muscle Res*. *Cell Motil*. 4: 253-62.

Dabiri, G.A., C.L. Young, J. Rosenbloom, and F.S. Southwick. 1992. Molecular cloning of human macrophage capping protein cDNA. A unique member of the gelsolin/villin family expressed primarily in macrophages. *J*. *Biol*. *Chem*. 267:16545-52.

Ferrary, E., M. Cohen- Tannoudji, G. Pehau-Arnaudet, A. Lapillonne, R. Athman, T. Ruiz, L. Boulouha, F. El Marjou, A. Doye, J.J. Fontaine, C. Antony, C. Babinet, D. Louvard, F. Jaisser, and S. Robine. 1999. In vivo, villin is required for Ca(2+)-dependent F-actin disruption in intestinal brush borders. *J*. *Cell Biol*. 146(4):819-30.

Irobi, E., L.D. Burtnick, D. Urosev. K. Narayan, and R.C. Robinson. 2003. From the first to the second domain of gelsolin: a common path on the surface of actin: *FEBS Lett*. 552:86-90.

Kouyama, T. and K. Mihashi. 1981. Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromysin. *Eur. J. Biochem*. 114:33-8.

Marx, J. 2003. How cells step out. *Science*. 302: 214-6.

McGough, A.M., and C.J. Staiger, J.K. Min, and K.D. Simonetti. 2003. The gelsolin family of actin regulatory proteins: modular structures, versatile functions. *FEBS Lett*. 552:75-81.

Mishra, V.S., and E.P. Henske, D.J. Kwiatkowski, and F.S. Southwick. 1994. The human actin-regulatory protein cap G: gene structure and chromosome location. *Genomics*. 23:560-5.

Onoda, K., and H.L. Yin. 1993. gCap39 is phosphorylated. Stimulation by okadaic acid and preferential association with nuclei. *J. Biol. Chem*. 268(6):4106-12.

Onoda, K., F.X. Yu, and H.L. Yin. 1993. gCap39 is a nuclear and cytoplasmic protein. *Cell Motil. Cytoskeleton*. 26(3):227-38.

Oosawa, F., and S. Asakura. 1975. Thermodynamics of the polymerization of protein. *Academic Press*. *New York*. 41-55, 90-108.

Pardee, J.D., and J.A. Spudich. 1982. Purification of muscle actin. *Methods Cell Biol*. 24:271-89.

Parikh, S.S., S.A. Litherland, M.J. Clare-Salzler, W. Li, P.A. Gulig, and F.S. Southwick. 2003. CapG $^{-/-}$ mice have specific host defense defects that render them more susceptible than CapG $^{+/+}$ mice to *Listeria monocytogenes* infection but not to *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun*. 71(11):6582-90.

Prendergast, G.C., and E.B. Ziff. 1991. Mbh1: a novel gelsolin/severin-related protein which binds actin *in vitro* and exhibits nuclear localization *in vivo*. *EMBO J*. 10:757-66.

Southwick, F.S. 1995. Gain-of-function mutations conferring actin-severing activity to human macrophage cap G. *J. Biol. Chem*. 270:45-8.

Southwick, F.S., and M.J. DiNubile. 1986. Rabbit alveolar macrophages contain a Ca^{2+}-sensitive, 41,000-dalton protein which reversibly blocks the "barbed" ends of actin filaments but does not sever them. *J. Biol. Chem*. 261:14191-5.

Spudich, J.A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem*. 246:4866-71.

Sun, H.Q., M. Yamamoto, M. Mejillano, and H.L. Yin. 1999. Gelsolin, a multifunctional actin regulatory protein. *J. Biol. Chem*. 274(47):33179-82.

Van Impe, K., V. de Corte, L. Eichinger, E. Bruyneel, M. Mareel, J. Vandekerckhove, and J. Gettemans. 2003. The nucleo-cytoplasmic actin-binding protein CapG lacks a nuclear export sequence present in structurally related proteins. *J. Biol. Chem*. 278:17945-52.

Witke, W., W. Li, D.J. Kwiatkowski, and F.S. Southwick. 2001. Comparisons of CapG and gelsolin-null macrophages: demonstration of a unique role for CapG in receptor-mediated ruffling, phagocytosis, and vesicle rocketing. *J. Cell Biol*. 154:775-84.

Yin, H.L., and T.P. Stossel. 1979. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature (London)*. 281:583-6.

Young, C.L., A. Feierstein, and F.S. Southwick. 1994. Calcium regulation of actin filament capping and monomer binding by macrophage capping protein. *J. Biol. Chem*. 269:13997-4002.

Young, C.L., F.S. Southwick, and A. Weber. 1990. Kinetics of the interaction of a 41-kilodalton macrophage capping protein with actin: promotion of nucleation during prolongation of the lag period. *Biochemistry*. 29:2232-40.

Yu, F.X., P.A. Johnston, T.C. Sudhof, and H.L. Yin. 1990. gCap39, a calcium ion- and polyphosphoinositide-regulated actin capping protein. *Science*. 250(4986):1413-5.

Yu, F.X., D.M. Zhou, and H.L. Yin. 1991. Chimeric and truncated gCap39 elucidate the requirements for actin filament severing and end capping by the gelsolin family of proteins. *J. Biol. Chem*. 266(29):19269-75.

BIOGRAPHICAL SKETCH

Andrea Roebuck was born in Michigan and moved to Florida at the age of four. Studies at the University of Florida (UF) began in June 1999, with a Bachelor of Science awarded in microbiology and cell science in May 2003. Andrea continued her graduate studies at UF to obtain a Master of Science in molecular genetics and microbiology in May 2006.