The *S. cerevisiae* calponin homologue Scp1 regulates stability and organization of the actin cytoskeleton

by

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ABSTRACT

Calponins and transgelins are members of a conserved family of actin-associated proteins widely expressed from yeast to humans. While a role for calponin in muscle cells has been described, the biochemical activities and in vivo functions of non-muscle calponins and transgelins are largely unknown. I have used genetic and biochemical analyses to characterize the budding yeast member of this family, Scp1, which most closely resembles transgelin and contains one calponin homology (CH) domain.

I showed that Scp1 is a novel component of yeast cortical actin patches and shares in vivo functions and biochemical activities with Sac6/fimbrin, the one other actin patch component that contains CH domains. Similar to Sac6, purified Scp1 binds directly to actin, cross-links actin filaments, and stabilizes filaments against disassembly. Furthermore, Scp1 competes with Sac6 for binding to actin filaments and may share an overlapping binding site on actin. Overexpression of *SCP1* suppresses *sac6Δ* defects and deletion of *SCP1* enhances *sac6Δ* defects. Together, these data show that Scp1 and Sac6/fimbrin function together to stabilize and organize the yeast actin cytoskeleton.

I used the genetic interactions between *SCP1* and *SAC6* to develop the first in vivo assay for function of any transgelin-like protein and established that actin binding is important for at least some Scp1 functions. Sequences necessary and sufficient for actin cross-linking were identified in the carboxyl terminus of Scp1, outside the CH domain.

Scp1 may regulate actin cytoskeleton not only via direct binding to actin filaments, but also via its interaction with another actin binding protein, Abp1. Scp1 and Abp1 physically interact in a yeast two hybrid and co-immunoprecipitation assays. In vivo patch localization of Scp1 mutant defective for binding to actin filaments requires src-homology 3 (SH3) domain of Abp1. In vitro, Scp1 specifically modulates Abp1-dependent activation of the Arp2/3 complex. In summary, Scp1 may function in complex with Abp1 to regulate actin nucleation by the Arp2/3 complex.

Thesis supervisor: Gerald R. Fink

Title: American Cancer Society Professor of Genetics

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CHAPTER 1. CALPONIN FAMILY OF THE ACTIN-BINDING PROTEINS.

INTRODUCTION

This thesis focuses on the regulation of the yeast actin cytoskeleton by an actin-binding protein Scp1. Scp1 belongs to the calponin protein family, which has been conserved between fungi and animals. The cellular functions of calponins appear to be involved in the regulation of the actin cytoskeleton, but they have not been well characterized. The actin cytoskeleton plays a key role in many aspects of cellular physiology. Research focused on actin and actin-binding proteins not only provides insights into fundamental questions of cell biology, but also could have a profound impact on understanding mechanisms of disease and developing new therapies. The complexity of mammalian actin cytoskeleton poses a challenge to the investigation of diverse actin functions and makes use of the model organisms especially attractive. Discoveries made in model organisms usually apply to the more complex organisms because of the evolutionary conservation of protein structure and function. The two main questions addressed in this thesis are:

1. What are the in vivo functions of Scp1?

2. How does Scp1 interact with actin on the molecular level?

In the first chapter, I provide an overview of the actin cytoskeleton and the calponin protein family. In the second chapter, I present biochemical and genetic analysis of Scp1 functions that partially overlap with fimbrin functions. In chapter three, I focus on the intriguing association of Scp1 with another actin binding protein Abp1. In chapter four, I discuss the future directions for investigation of Scp1 functions in yeast and for extending our knowledge of Scp1 functions to other calponin family members.

FUNCTIONS AND ORGANIZATION OF THE ACTIN CYTOSKELETON

Diverse roles of actin in vivo

Actin is, arguably, the most versatile protein in the cell. It is difficult to find a cellular process that has not been linked to actin. Actin monomers assemble into filaments and form the microfilament cytoskeleton of the cell. The roles of the actin cytoskeleton in cell morphology and motility have been well documented. Organization of the actin filaments into higher order structures provides a framework for establishing polarity and shape of all eukaryotic cells. In animal cells, highly cross-linked sub-cortical actin filament networks provide dynamic rigidity responsible for changes in cell shape during motility. Organization of actin filaments into tight parallel bundles is critical for formation of specialized structures in animal cells (e.g. hair cell stereocilia in the inner ear and microvilli in intestinal brush border; stereocilia (Shibayama et al., 1987; Drenckhahn et al., 1991). Even in plant and fungal cells, where the cell wall provides rigid "exoskeleton," the actin cytoskeleton directs the cell wall synthesis, thereby influencing cell shape and/or movements. For instance, plant actin is involved in establishment of the plane of cell division, pollen tube growth, and guard cell movements (reviewed in McCurdy et al., 2001).

Other well-established functions of the actin cytoskeleton include endocytosis and exocytosis, organelle inheritance and nuclear segregation, intra- and extra-cellular signaling. In all eukaryotic cells, actin filaments provide tracks for transport of molecules, vesicles, and organelles by the myosin protein motors. Interaction of the actin filaments with the myosin motors also plays a very specialized role in generating a contractile force during cell motility, cytokinesis, and muscle contraction in animal cells. The actin cytoskeleton serves not only as a scaffold for various signaling pathways, but also actively participates in signaling events via interactions with numerous signaling molecules (Calderwood et al., 2000; Machesky and Insall, 1999; Volkmann and Baluska, 1999). Dynamic reorganization of the cortical actin cytoskeleton leads to receptor clustering and

changes in intracellular and/or extracellular signaling in yeast and animal cells (Ayscough and Drubin, 1998; Wojciak-Stothard et al., 1999; Matsuda and Hirai, 1999). In plant cells, actin has been implicated in intercellular communications through the regulation of the structure of the intercellular junctions (plasmodesmata) and the active protein transport across these junctions (McCurdy et al., 2001).

In other cellular processes, such as translation, transcription, and metabolism, the functions of actin are not as well understood. Actin is clearly involved in compartmentalization of protein synthesis, and both mRNA and protein components of polyribosomes have been found in association with actin filaments in vitro and in vivo (Hesketh, 1994). Further, gene expression is spatially and temporally regulated by an actin-dependent transport and localization of specific mRNAs in the cytoplasm (reviewed in Stebbings, 2001). Actin has also been found in the nucleus associated with the chromatin remodeling complex, where it may affect chromatin structure and the processes dependent on chromatin structure, like transcription and DNA replication (Boyer and Peterson, 2000; Rando et al., 2000). Actin's role in metabolism has been proposed based on the observation that several metabolic enzymes (GAPDH, kinase phosphorylase, and lactate dehydrogenase) interact with actin in vitro and co-localize with actin structures in vivo (Minaschek et al., 1992; Schmitz and Bereiter-Hahn, 2002; Cao et al., 1999). The actin cytoskeleton may coordinate metabolic processes via spatial organization of the metabolic enzymes in the cytoplasm and may regulate metabolism in response to signaling events (Poglazov, 1983; Schmitz and Bereiter-Hahn, 2002).

Several aspects of actin function are particularly important in medicine. Bacterial and viral pathogens (e.g. *Listeria*, *Shigella*, and vaccinia virus) use the force generated by actin polymerization to propel themselves within a cell and/or for infection of new cells (Cossart, 2000; Ploubidou and Way, 2001). The prominent role of actin filaments in cell motility makes the actin cytoskeleton an important factor in the function of immune system (e.g. macrophage and

neutrophil motility), as well as in cancer metastases (recently reviewed in Feldner and Brandt, 2002; Frame and Brunton, 2002; Pawlak and Helfman, 2001). Signaling links between extracellular matrix and actin cytoskeleton have also been implicated in loss of contact inhibition and cellular transformation.

Yeast actin cytoskeleton

Many of the insights into the cellular roles of actin came from studies of the budding yeast *S. cerevisiae.* Yeast continues to serve as an excellent reference organism because it has a relatively simple actin organization and because it is amenable to both the traditional biochemical and genetic approaches, as well as to the novel genomic and proteomic tools. Yeast filamentous actin is found in three sub-cellular structures: an actomyosin contractile ring, sub-cortical cables, and motile cortical patches. Actomyosin contractile ring assembles at the bud neck and facilitates cytokinesis (Epp and Chant, 1997; Lippincott and.Li, 1998). Actin cables are likely comprised of actin bundles, because actin bundling proteins Sac6/fimbrin and Abp140 co-localize with cables and are required for normal actin cable morphology (Adams et al., 1989; Adams et al., 1991; Asakura et al., 1998). Actin cables are oriented along the mother-bud cell axis and are thought to function as tracks for myosin V-driven transport of vesicles, organelles and mRNA to the bud (reviewed in Pruyne and Bretscher, 2000).

The functions of actin patches have not been as clearly defined as those of the bud-neck ring and cables. Actin patches are motile foci of filamentous actin that localize to the sites of new growth. The distribution of actin patches changes throughout the mitotic cell cycle (Kilmartin and Adams 1984; Figure 1.1). At the start of a new cell cycle, patches are polarized to the site of the future bud. During bud growth, patches are concentrated at the bud tip, then briefly become depolarized during isotropic growth, and re-polarize to the bud neck during cytokinesis. This localization of actin patches to sites of new growth is consistent with a function of actin patches in directing the cell wall

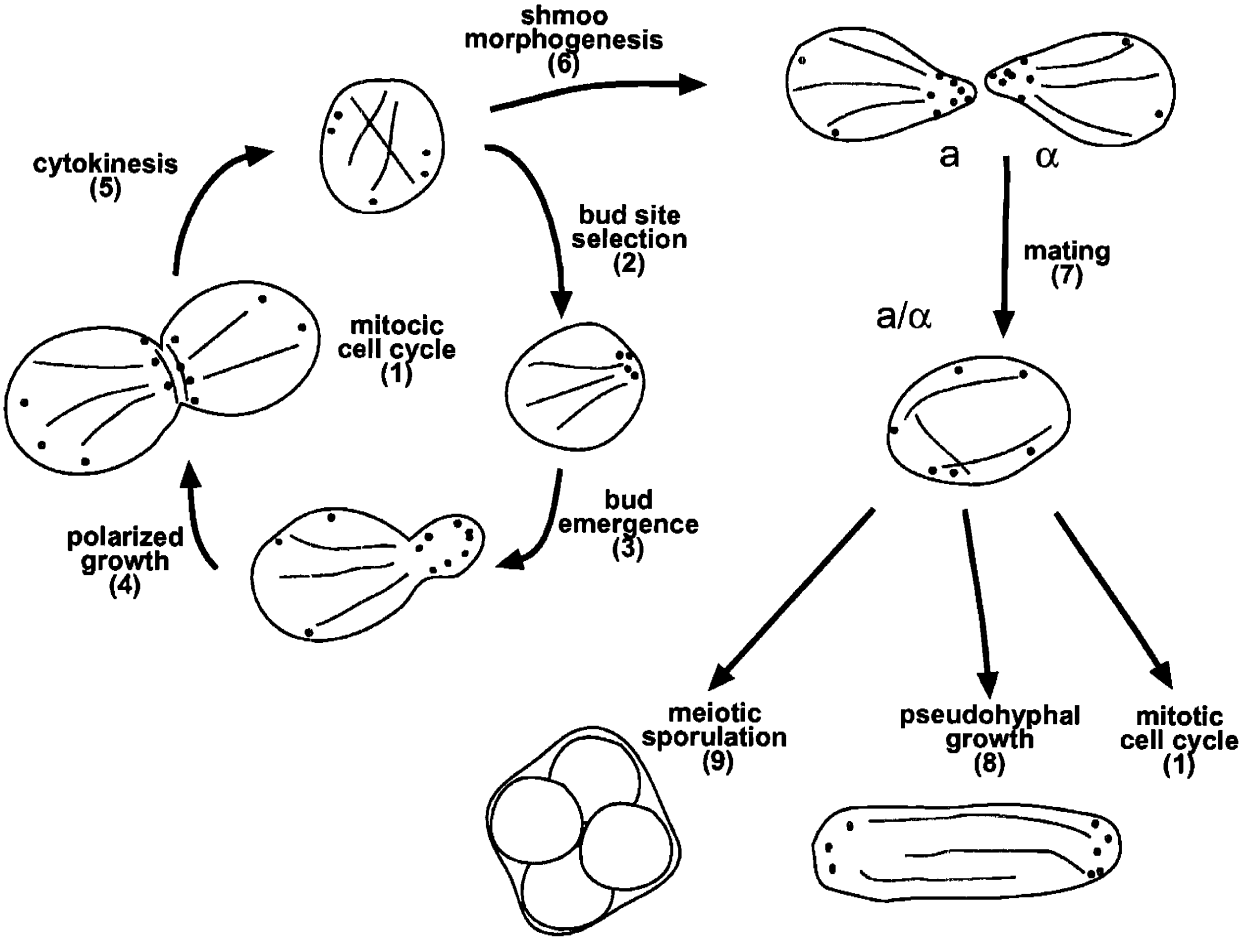


Figure 1.1 Actin plays an important role in yeast cell polarity and morphogenesis. The diagram shows the actin structures (red) at the different stages of the yeast life cycle. Processes that require an intact actin cytoskeleton are labeled. (1) McMillan et al., 1998; (2) Zahner et al., 1996; Yang et al., 1997; (3) Bender and Pringle 1991;(4) Adams and Pringle, 1984; (5) Lippincott and Li, 1998; Osman and Cerione 1998; (6,7) Yorihuzi and Ohsumi, 1994; Valtz and Hersowitz, 1996; Dorer et al., 1997; (8) Cali et al., 1998; (9) Haarer et al., 1994; Whitecare et al., 2001.

biosynthesis. Mutations in actin patch components result in abnormal cell wall composition and morphology (Mulholland et al., 1994; Gabriel and Kopecka 1995; Mulholland et al., 1997; Utsugi et al., 2002, Li et al., 2002).

Another strong functional link has been identified between actin patches and endocytosis. Mutations in actin and actin-associated proteins lead to defects in endocytosis, but the exact role of actin is unclear. Several lines of evidence suggest that cortical actin patches may provide mechanical forces that drive vesicle fission, internalization or transport. Immunoelectron microscopy of yeast cells revealed coils of actin filaments surrounding finger-like invaginations of the plasma membrane (Mulholland et al., 1994). This observation lead to a speculation about actomyosin-dependent fission of endocytic vesicles. Another possibility is that actin serves as a scaffold for assembly of endocytic machinery. Discovery that Pan1, yeast homologue of endocytic adapter Eps15, directly activates Arp2/3-dependent actin nucleation provided the first direct link between endocytic apparatus and actin assembly (Duncan et al., 2001). Multiple interactions of Pan1 with actin associated and endocytic proteins (Sla1, Sla2, End3, Ent1-4) may regulate membrane remodeling during endocytosis (reviewed in Goode and Rodal, 2001).

Other phenotypes associated with mutations in actin patch components include defects in shmoo morphogenesis and mating (Yorihuzi and Ohsumi 1994; Valtz and Herskowitz 1996; Dorer et al., 1997), pseudohyphal growth (Cali et al., 1998), sporulation (Haarer et al., 1994; Whitecare et al., 2001), bud site selection (Zahner et al., 1996; Yang et al., 1997), bud emergence (Bender and Pringle 1991), polarized growth (Adams and Pringle, 1984), and cytokinesis (Lippincott and Li, 1998; Osman and Cerione 1998). Moreover, depolymerization of actin filaments in the presence of Latrunculin A, triggers a morphogenesis checkpoint in the cell cycle (McMillan et al., 1998). These data suggest that organization of the actin cytoskeleton is critical for progression of the cell cycle. Clearly, actin is required for many cellular functions in yeast (Figure 1.1). Great progress has been made in the last twenty years in identifying the genetic

functions and the molecular components of the yeast actin cytoskeleton. The next challenge is to understand how all these proteins work together to regulate the structure, dynamics and functions of the yeast actin cytoskeleton.

Regulation of actin filament dynamics and organization

The discussion above highlights the diverse roles of actin in almost every aspect of cell physiology. How can actin be involved in so many different processes? The key to actin's versatility lies in its interactions with the numerous proteins that regulate actin filament dynamics and organization. Actin filaments exhibit intrinsic polarity and dynamic behavior (Figure 1.2). ATP-bound actin subunits are found at the barbed (+) ends of the filaments, ADP-bound subunits are at the pointed (-) ends, and both ends can add or lose subunits. However, at steady state, when no net assembly or disassembly is occurring, actin filaments undergo turnover/treadmilling: addition of the ATP-bound actin monomers at the barbed (+) ends and loss of ADP-bound actin subunits at the pointed (-) ends of the filaments.

A convenient way for studying the dynamics of actin assembly and disassembly in vitro utilizes fluorescently labeled actin monomers. When pyrene-labeled actin monomer is incorporated into an actin filament, the change in molecular environment of the fluorophore leads to increase in fluorescent signal, which can be monitored in a fluorimeter over time (Cooper and Pollard, 1982). Conversely, when polymers that contain fluorescently labeled actin subunits depolymerize, the signal decreases. When actin polymerization is monitored in this fashion, the fluorescence curve has three regions: a lag phase, an exponential phase, and a plateau (Figure 1.3). Lag phase reflects the slow formation of actin dimers and trimers that serve as nuclei for rapid elongation of the actin filaments during the exponential phase. The slope of the curve during exponential phase is proportional to the rate of actin filament assembly. The fluorescence plateau indicates that reaction has reached a steady state, in which polymerization is balanced by depolymerization (treadmilling). If small amount of

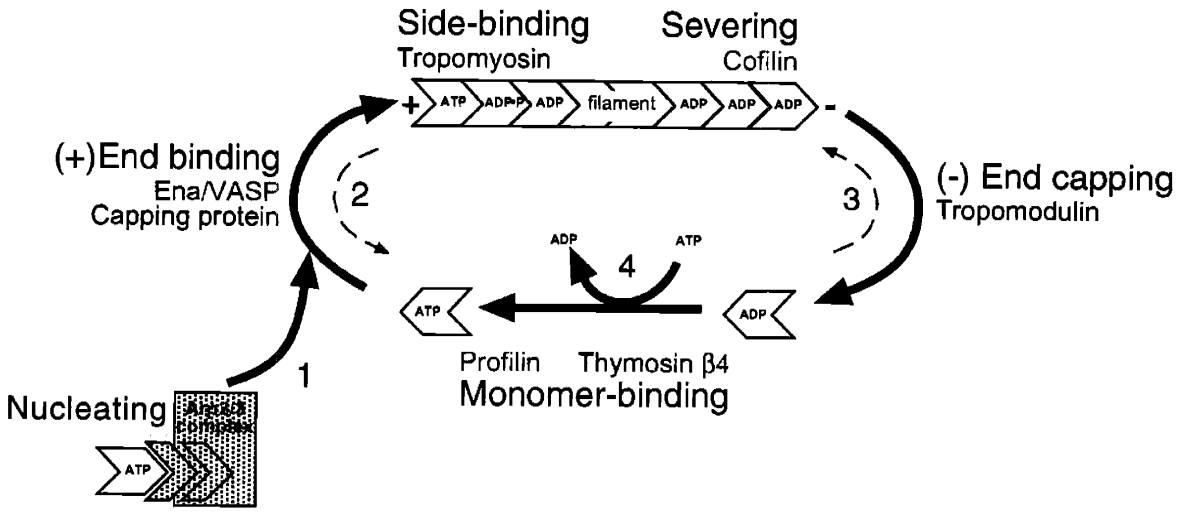


Figure 1.2 Regulation of actin filament dynamics. Diagram represents nucleation and turnover of the actin filaments. The major steps contributing to the dynamic properties of actin filaments (indicated by arrows) include (1) de novo nucleation of actin filaments, (2) addition of the actin monomers at the barbed (+) ends of the filaments, (3) loss of the actin monomers at the pointed ends, and (4) nucleotide exchange on the actin monomers. Biochemical activities of different regulatory proteins for each step are indicated, with examples of specific proteins colored red if they increase actin filament dynamics (e.i. increase rates of assembly or disassemby) and colored blue if they decrease actin dynamics. See text for references.

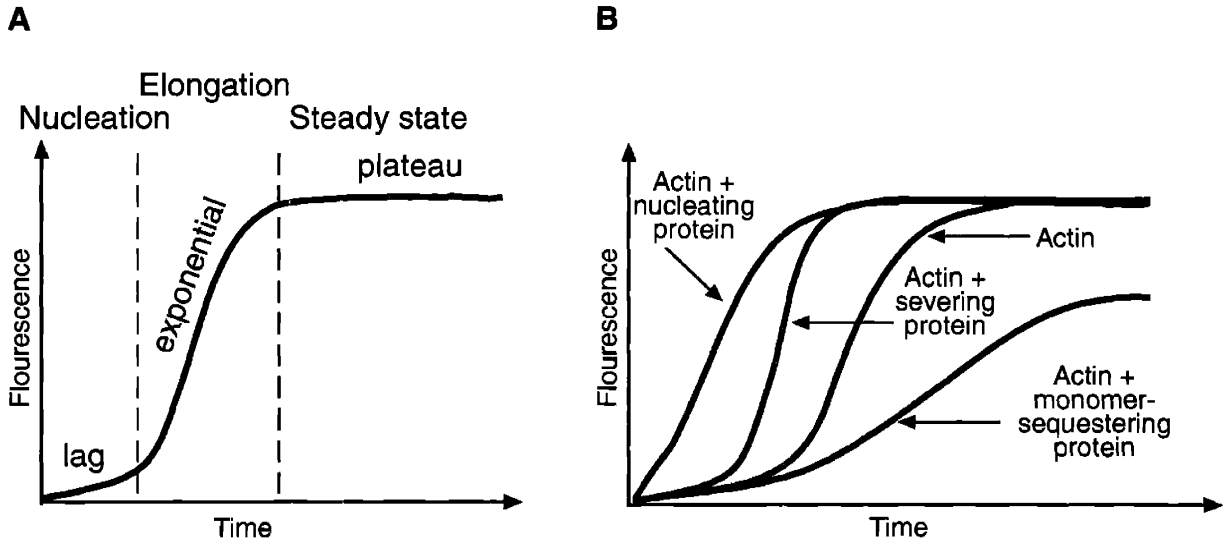


Figure 1.3 Fluorimetry assay for monitoring actin polymerization. A. Hypothetical curve of pyrene-actin flourescence over time during actin polymerization from monomers. B. Effects of actin-binding proteins on the time course of actin polymerization. Hypothetical curves show the effects of addition of a nucleating nucleating, such as CapZ (Cooper and Pollard, 1985), a monomer sequestering protein, such as profilin (Pollard and Cooper, 1984); and a severing protein, such as cofilin/actophorin (Cooper et al., 1986).

pre-formed actin filaments is added to the reaction, polymerization proceeds without the lag phase. The shape of the actin assembly and disassembly curves can change dramatically in the presence of actin-binding proteins that regulate actin dynamics in vitro (Figure 1.3). This method has been used extensively to elucidate the distinct mechanisms of actin dynamics regulation.

Spontaneous de novo assembly of actin filaments is a slow process that can be catalyzed by actin nucleating factors, such as the Arp2/3 complex and formins (Machesky et al., 1994; Pruyne et al., 2002; Sagot et al., 2002). The Arp2/3 complex consists of seven proteins, including two that are closely related to actin (Arp2 and Arp3). Binding of an actin monomer to the Arp2/3 complex produces a free barbed end favorable for filament elongation. Arp2/3 mediated actin nucleation in vitro is slow in the absence of activators, which include the WASP/SCAR protein family, Abp1, type I myosin, Pan1/Eps15, and others. Regulation of the pathways for activation of the Arp2/3 complex is a very active area of research (Higgs, 2001; Higgs and Pollard, 2001). In addition to the Arp2/3 complex, formins also nucleate actin filaments, although their mechanism of action is not well understood. (Pruyne et al., 2002; Sagot et al., 2002).

Once nucleation has occurred, actin filament assembly and disassembly is regulated by three broad classes of proteins: filament capping, filament side binding, and monomer-binding proteins (Figure 1.2). Binding of the capping proteins to the ends of actin filaments can either prevent filament disassembly at the pointed (-) ends (e.g. tropomodulin; Weber et al., 1994; Weber et al., 1999), or limit elongation at the barbed (+) ends of the filament (e.g. capping protein; Schafer et al., 1996). In addition, a novel "end-protection" activity has been recently identified in the proteins of the ena/VASP family. These proteins bind barbed ends of the filaments, protecting them from the capping proteins, while allowing filament elongation to proceed (Bear et al., 2002)

Side-binding proteins can regulate filament disassembly by binding along the filament and providing additional contacts stabilizing interactions between actin subunits in the filament (e.g. tropomyosin; Wegner, 1982). A special sub-

class of side binding proteins has a severing (e.g. cofilin; Thorstensson et al., 1982) or severing and capping activities (e.g. gelsolin; Kwiatkowski et al., 1989). Their effects on actin dynamics can be complicated since severing can lead to either increased polymerization (more barbed ends for elongation) or depolymerization (more pointed ends to disassemble). For example, severing and barbed-end capping by gelsolin initially inhibits assembly at the barbed ends, but upon uncapping, leads to the net increase in polymerization.

Monomer-binding proteins also can promote either stabilization or destabilization of the actin filaments. Profilin promotes actin assembly by facilitating nucleotide exchange and addition of the monomer to the barbed end of the filaments (Goldschmidt-Clermont et al., 1992; Pantaloni and Carlier, 1993). In contrast, thymosin β4 binds and sequesters actin monomers, preventing nucleotide exchange and, therefore, inhibiting actin polymerization (Carlier et al., 1993). In summary, multiple actin binding proteins utilize different strategies to regulate dynamic behavior of the actin filaments.

Actin binding proteins also regulate organization of the actin filaments either by nucleating new filaments from the sides of existing filaments (branching) or by cross-linking filaments into higher order structures (bundling and gelation; Figure 1.4). Cross-linking requires either more than one actin binding site on a single protein or multimerization of an actin binding protein. Bundling proteins bring actin filaments into tight bundles (e.g. fimbrin and fascin; de Arruda et al., 1990; Otto and Schroeder, 1984), while gelation factors cross link actin filaments into loose networks (e.g. spectrin; Fishkind et al., 1985). The distinction between the two types of cross-linkers may be artificial, since several proteins (e.g. alpha-actinin, filamin) induce formation of either actin bundles or gels depending on the assay conditions (Wachsstock, 1993; Cortese and Frieden, 1990; Hou et al., 1990). Branching and cross-linking of actin filaments in vitro is clearly also relevant in vivo for generation of different cellular structures. Branching of actin filaments by the Arp2/3 complex is critical in lamelapodial ruffling and extension (Bailly et al., 2001), whereas tight bundles

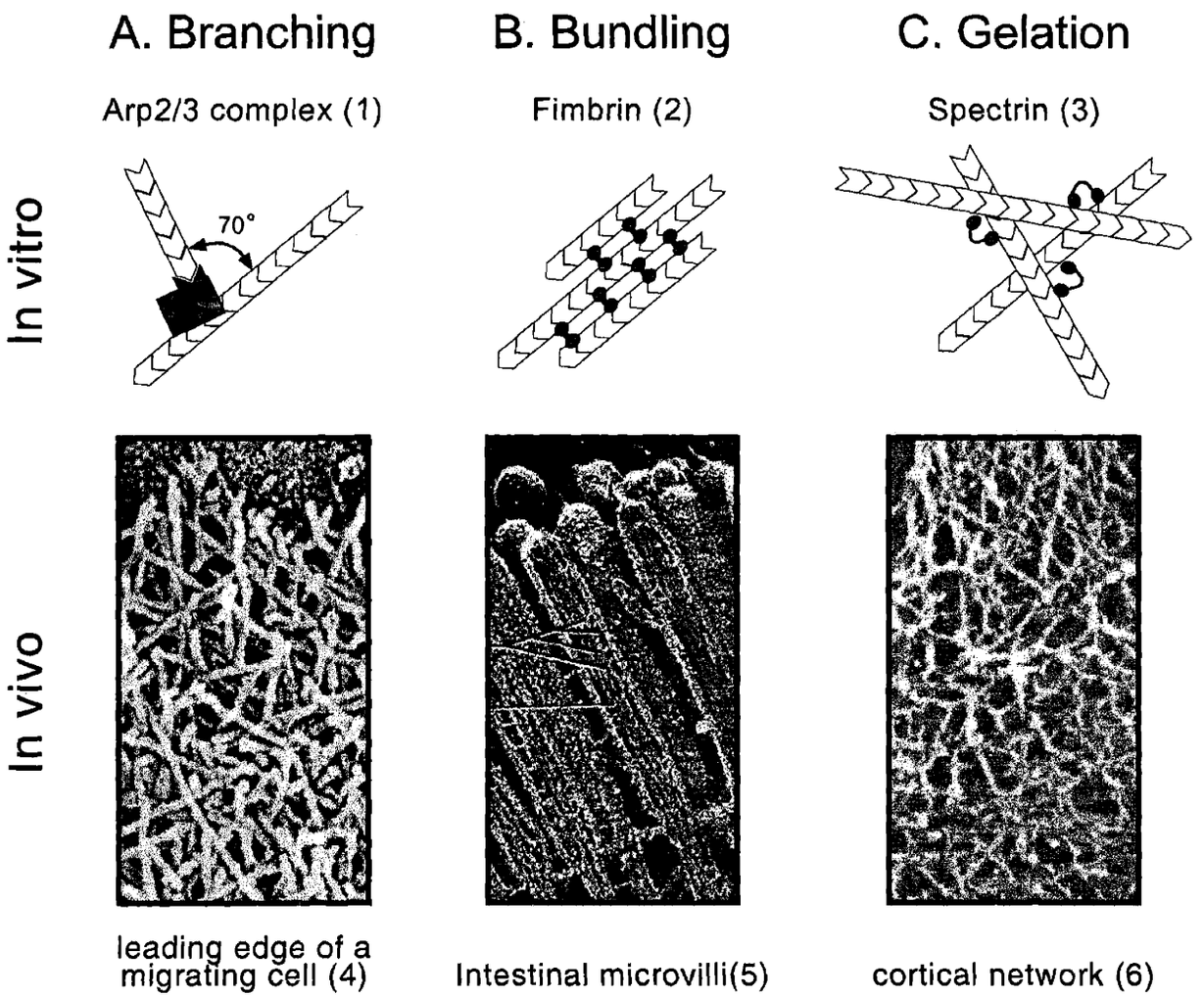


Figure 1.4 Mechanisms regulating actin filament organization. A. Branching occurs via assembly of the new filaments from the sides of pre-existing filaments. B. Bundling occurs by cross-linking actin filaments into parallel arrays. C. Gelation occurs by cross-linking actin filaments into orthogonal networks. The diagram shows the arrangement of the actin filaments and the proteins regulating filament organization (red) in vitro; 1-3 are specific examples. Below the diagram, electron micrographs show examples (4-6) of the corresponding in vivo structures. (1) Mullins et al., 1998; (2) de Arruda et al., 1990; (3) Fishkind et al., 1985; (4) Svitkina et al., 1997; (5) Hirokawa et al., 1982; (6) Hartwig, 1992.

of actin filaments are critical for maintaining specialized structures of intestinal microvilli and hair stereocilia (Shibayama et al., 1987; Drenckhahn et al., 1991).

Actin filament dynamics and organization are regulated by a large number of actin-associated proteins that belong to conserved protein families. It is remarkable that these protein families use only a limited set of structural modules to achieve great diversity of biochemical activities (Matsudaira, 1991; Pollard et al., 1994; Puius et al., 1998). One example of such module is the calponin homology (CH) domain, found in many proteins, including members of the calponin family.

DOMAIN ORGANIZATION OF THE CALPONIN PROTEIN FAMILY

Evolution of calponin protein family

The calponin protein family is characterized by a single calponin homology (CH) domain at the amino-terminus and either one or more calponin-like repeats (CLR) at the carboxyl terminus (Prinjha et al., 1994). Based on domain organization, proteins in this family can be divided into two subfamilies: calponins, containing three or more CLR sequences, and transgelins, containing a single CLR (Figure 1.5A). Phylogenetic analysis based on protein sequence, however, suggests that invertebrate calponin- and transgelin-like proteins may belong to subfamilies distinct from their vertebrate counterparts (Figure1.5B). The evolutionary history of the calponin protein family sets up the context in which the functions of the family members can be understood.

Members of the calponin family are found in fungi and animals, although none have been identified in plants. While sequencing of *Arabidopsis taliana* genome has been completed, sequencing additional plant genomes will reveal whether calponin family members are truly absent from the plant kingdom. Fungal genomes *(S. cerevisiae, S.pombe,* and *N. crassa*) contain a single transgelin-like gene, whereas higher eukaryotic genomes have multiple

Figure 1.5 Calponin family. A. Domain organization of calponin and transgelin members of calponin protein family. The calponin homology (CH) domain is labeled and calponin–like repeats are represented by black rectangles. B. Phylogram of calponins and transgelins. Species are indicated in parentheses as following: C.e., *C. elegans*; D.m., *Drosophila melanogaster*; E.g., *Echinococcus granulosus*; H.s., *Homo sapiens*, N.c., *Neurospora crassa*; S.c., *S. cerevisiae*; S.p., *S. pombe*. Sequences were obtained from the GeneBank (NCBI) and labeled following nomenclature of either GeneBank (NCBI) or BioKnowledge® Library (Proteome Inc.) nomenclature. For *N. crassa*, and *S. pombe*, predicted proteins were identified as transgelin (TGN) based on domain organization. The phylogram was generated using TreeVew software based on the ungapped multiple alignment generated using ClustalX.

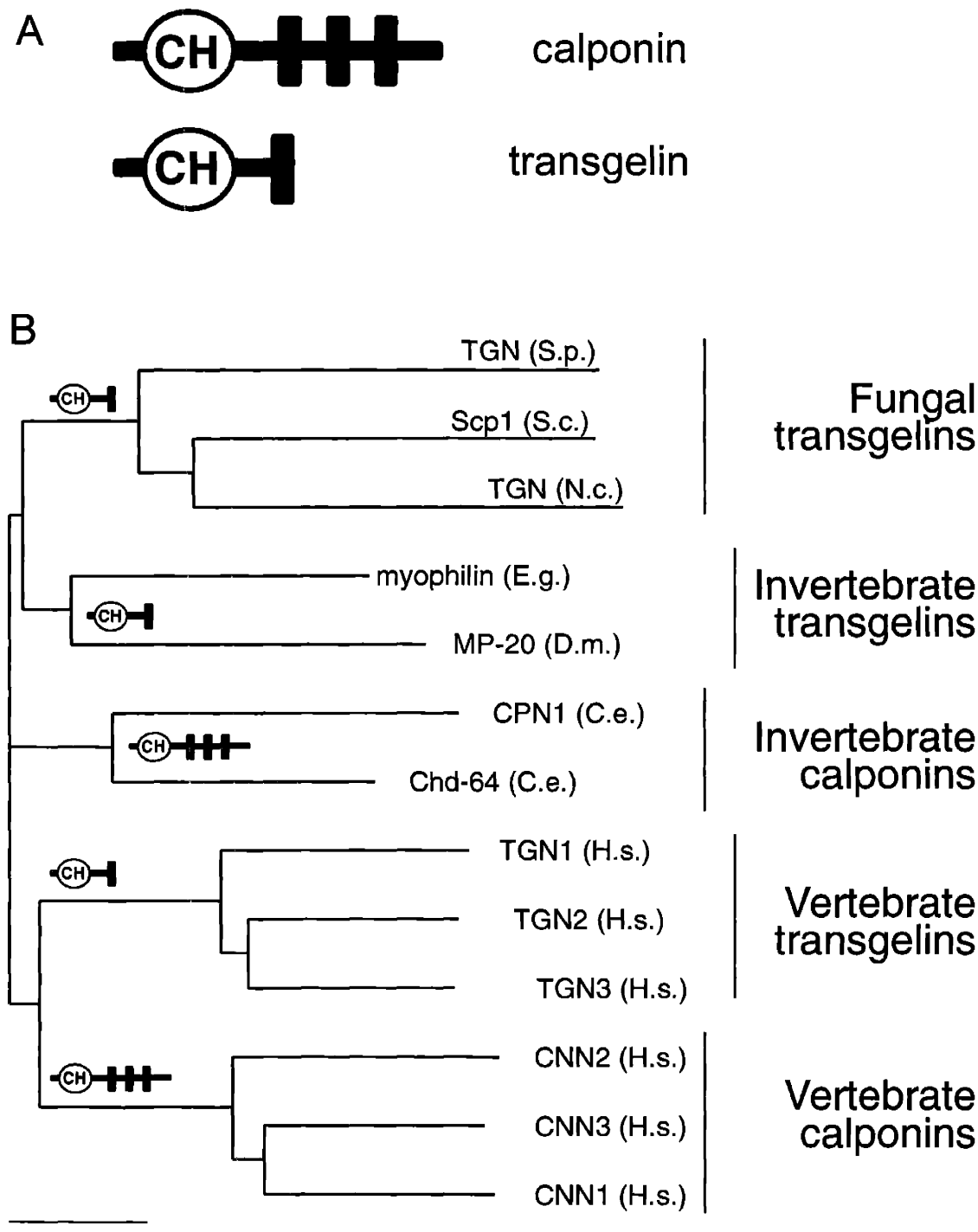

A
CH
calponin
CH
transgelin
B
TGN (S.p.)
Scp1 (S.c.)
TGN (N.c.)
Fungal transgelins
myophilin (E.g.)
MP-20 (D.m.)
Invertebrate transgelins
CPN1 (C.e.)
Chd-64 (C.e.)
Invertebrate calponins
TGN1 (H.s.)
TGN2 (H.s.)
TGN3 (H.s.)
Vertebrate transgelins
CNN2 (H.s.)
CNN3 (H.s.)
CNN1 (H.s.)
Vertebrate calponins

Figure 1.5

transgelins and calponins. Several interesting observations can be made based on the phylogenetic analysis of calponin family members (Figure 1.5B).

First, invertebrate transgelins are more closely related to fungal transgelins than they are to vertebrate transgelins. Therefore, fungal and invertebrate transgelins may resemble the ancestral member of this family, whereas vertebrate transgelins represent more specialized proteins that evolved later. Second, vertebrate calponins are more closely related to vertebrate transgelins than to other calponins. This comparison suggests that calponins may have evolved independently in vertebrate and invertebrate animals. The carboxy terminal sequence of transgelin-like gene was probably expanded by duplication and unequal crossing-over to give rise to the multiple calponin-like repeats of calponins.

Third, it is interesting to consider the relationships between tissue specific calponin family members. Among vertebrate members, both transgelins and calponins have three isoforms: smooth muscle specific, ubiquitous, and neuronal. The neuronal calponin Cnn3 is more closely related to other calponins than to neuronal transgelin isoform Tgn3. Similarly, muscle-specific calponin Cnn1 is more closely related to other calponins than to muscle specific transgelin Tgn1. These observations suggest that tissue specific functions evolved independently in each subfamily after the split between vertebrate calponins and transgelins. In addition to vertebrate Cnn1 and Tgn1, at least two invertebrate muscle-specific proteins have been characterized: MP-20 in *Drosophila* and myophilin in the tapeworm. The fact that transgelins and calponins that perform muscle-specific functions are not clustered together in the phylogenetic tree, suggests that muscle-specific functions of calponin family members evolved independently by convergent evolution, rather than from a single ancestral muscle specific gene that gave rise to multiple orthologs.

What structural elements make calponin family members particularly suitable to function in muscle, what function do they perform in muscle, and what are the functions of calponin family members outside the muscle? The

evolutionary conservation of the proteins in the calponin family suggests that these proteins may have conserved functions in vivo, yet our understanding of these functions is limited. Below, I review the roles assigned to each of the calponin structural domains and, in later sections, discuss the unsolved mysteries and open questions regarding each of the protein subfamilies.

Calponin homology (CH) domain

CH domains are found in many actin-associated proteins that perform diverse functions (Figure 1.6A). Proteins containing CH domains cross-link actin filaments (e.g. spectrin, filamin, fimbrin), link actin to other cytoskeletal systems (e.g. fimbrin, plectin), and form signaling scaffolds (e.g. IQGAP, ARHGEF6) and others (reviewed in Gimona et al., 2002; Korenbaum and Rivero, 2002). Based on the primary sequence, five types of CH domains have been identified (Gimona et al., 2002). Type 1 and type 2 CH domains generally occur as a pair and comprise an actin binding domain (ABD) characteristic of the spectrin protein superfamily (Figure 1.6A). Fimbrin, which is often included in the spectrin superfamily, contains two classical ABDs in tandem, comprised of four CH domains (Matsudaira, 1991; Hartwig, 1995). The sequence of the fimbrin CH is sufficiently divergent from the CH1 and CH2 to warrant designation CH1.1, CH2.1, CH1.2 and CH2.2 (Gimona et al., 2002, Figure 1.6). Type 3 CH domains are found at the amino-termini of calponins and of the signaling proteins like Vav and IQGAP. Type 4 and 5 CH domains are found in a recently identified parvin/actopaxin protein family (Nikolopoulos and Turner, 2000; Olski et al., 2000). Since little is known about the type 4 and 5 CH domains, they will not be discussed further.

The three-dimensional structures of several CH domains have been published (Goldsmith et al., 1997; Norwood et al., 2000; Bramham et al., 2002). These domains share a unique fold characterized by a pair of parallel alpha-helices sandwiched between another pair of alpha-helices and connected by flexible loops (Figure 1.6B). The arrangements of the helices in the type 1, 2 and

3 CH domains are very similar, and variations occur mainly in the loops connecting the alpha helices. These divergent loops may be responsible for the functional differences among the different types of CH domains.

It is well established that a pair of type 1 and type 2 CH domains forms an actin-binding domain (e.g. alpha-actinin and utrophin; Way et al., 1992; Winder et al., 1995). The two CH domains in this pair are not equivalent with respect to actin binding because isolated type 1 CH domains of alpha-actinin and utrophin bind actin filaments, while truncated proteins containing only CH2 bind actin filaments either weakly (utrophin, spectrin; Winder et al., 1995; Djinovic-Carugo et al., 1997) or not at all (alpha-actinin; Way et al., 1992). Because the isolated CH1 domains have reduced affinity for actin filaments compared to the ABD, CH2 must contribute to the binding affinity of ABD for actin, perhaps by helping to position the CH1 domain on the actin filament (Way et al., 1992; Gimona et al., 2002)

Whether the single type 3 CH domain can bind actin filaments has been a subject of controversy (Gimona and Winder, 1998; Morgan and Gangopadhyay, 2001). On the one hand, 3D-helical reconstructions of decorated actin filaments suggest that calponin, which contains a single CH3 domain, and proteins containing a tandem pair of CH domains (fimbrin and utrophin) interact similarly with actin filaments (Bramham et al., 2002; Hanein et al., 1997; Hanein et al., 1998; Hodgkinson et al., 1997; Figure 1.7). On the other hand, the single CH domain of calponin (or transgelin) is not sufficient for binding to actin filaments. Carboxy-terminal truncations of calponin or transgelin that leave the CH domain intact, result in failure of the truncated protein to co-pellet with actin filaments in vitro and co-localize with actin filaments in vivo (Gimona and Mital 1998, Fu et al., 2000). As will be discussed in chapter 2, it remains possible that the single CH domain of calponins represents a weak actin-binding module that serves as a 'locator' for the strong actin-binding site.

Whether CH3 domains of other proteins can bind to actin filaments is also not clear. CH domain of the Rho family GTPase activating protein IQGAP is

Figure 1.6 Calponin homology (CH) domain. A. Schematic representation of the molecular structure of proteins containing CH domains. Figure was generated using The Simple Modular Architecture Research Tool (SMART). B. Diagram of the three-dimensional structure of the calponin CH domain solved by NMR (PDB: 1h67) and of the first CH domain of fimbrin (PDB: 1aoa; amino acids 121-230). Coordinates were retreived from PDB and visualized using RasMol software.

A

Type1/2 superfamily

b-spectrin

interaptin

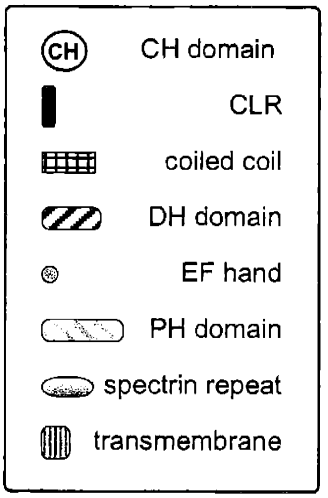
a-actinin

dystonin

utrophin

calmin

smoothelin



Type 3 superfamily

calponin

transgelin

ARHGEF6

Vav

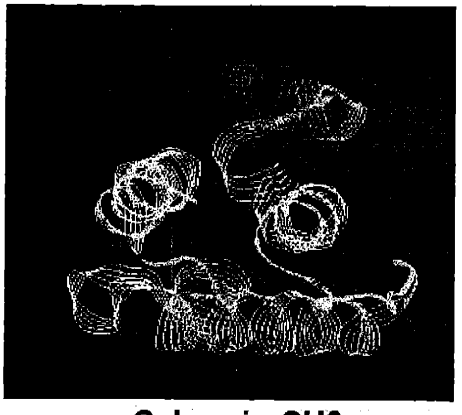
IQGAP

Fimbrin family

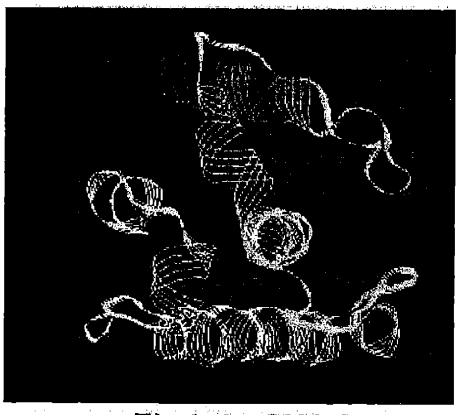
Type 1.1 2.1 1.2 2.2

fimbrin

B



Calponin CH3



Fimbrin CH1.1

Figure 1.6

perhaps the strongest candidate for such function. It was previously thought that IQGAP bound actin via a pair of CH3 domains, held together by dimerization via a coiled-coil domain located carboxy-terminal to the CH domain. Recent actin-binding experiments with the IQGAP truncated at the carboxyl-terminus suggest that the single CH3 domain of IQGAP may bind actin filaments (Morris et al., 2002). However, the contributions to the actin binding affinity by the amino-terminal sequences outside the CH domain have not yet been examined.

The CH3 domain of another actin-associated and signaling protein RhoGEF Vav may to perform a regulatory, rather than actin binding function (Abe et al., 1999). Deletion of the Vav CH domain activates Vav-dependent signaling pathways in vivo, while co-expression of the activated Vav (missing CH domain) with the amino-terminus containing CH domain inhibits Vav-dependent signaling (Abe et al., 1999). The CH domain is not likely to be required for the interactions with actin because deletion of the CH domain increases association of Vav with actin in vivo (Kranewitter and Gimona 1999). The CH domain may regulate Vav signaling by sterically blocking a docking site for Rho family GTPases or by providing binding sites for additional regulatory molecules (Gimona et al., 2002). In support of the latter model, partial deletion of the CH domain abolishes interaction of Vav with the nucleotide dissociation inhibitor RhoGDI in immunoprecipitation and GST-pull down experiments (Groysman et al., 2000).

In addition to regulatory and actin binding roles, CH domains may also function in coupling the actin cytoskeleton to signaling pathways and to other cytoskeletal systems. A signaling function has been proposed for the CH3 domain of calponin based on its association with the extracellular regulatory protein kinase ERK1 in blot overlay assays (Leinweber et al., 1999). The same study also described binding of CH domains of alpha-actinin to ERK1 and competition of the calponin CH domain with alpha-actinin for ERK1. These results support the interaction of CH domains with signaling molecules in vitro. However, convincing evidence supporting the signaling role of CH domain in vivo is lacking (see discussion below).

Figure 1.7. Calponin, fimbrin, and utrophin in complex with F-actin. (A) Reconstruction of the single calponin CH domain bound to F-actin (Bramham et al., 2002). (B) Reconstruction of fimbrin ABD, comprising two CH domains, bound to F-actin (Hanein et al., 1998). (C) Reconstruction of utrophin ABD bound to F-actin at a density of 1 utrophin per actin (Moores et al., 2000). (D) Reconstruction of utrophin ABD bound to F-actin `half-decorated' – one utrophin per two actins (Galkin et al., 2002). (E) Reconstruction of utrophin ABD bound to F-actin `single-decorated' – one utrophin per actin (Galkin et al., 2002). In (A-E), the two actin monomers from one strand of the F-actin filament are shown in spacefilling representation colored red and blue, from pointed to barbed end, respectively. The CH-domain-containing ABD is shown as a yellow and gold ribbon representation with the amino-terminal CH domain (or single CH domain in the case of calponin) in yellow in each case. (modified from Winder, 2003)

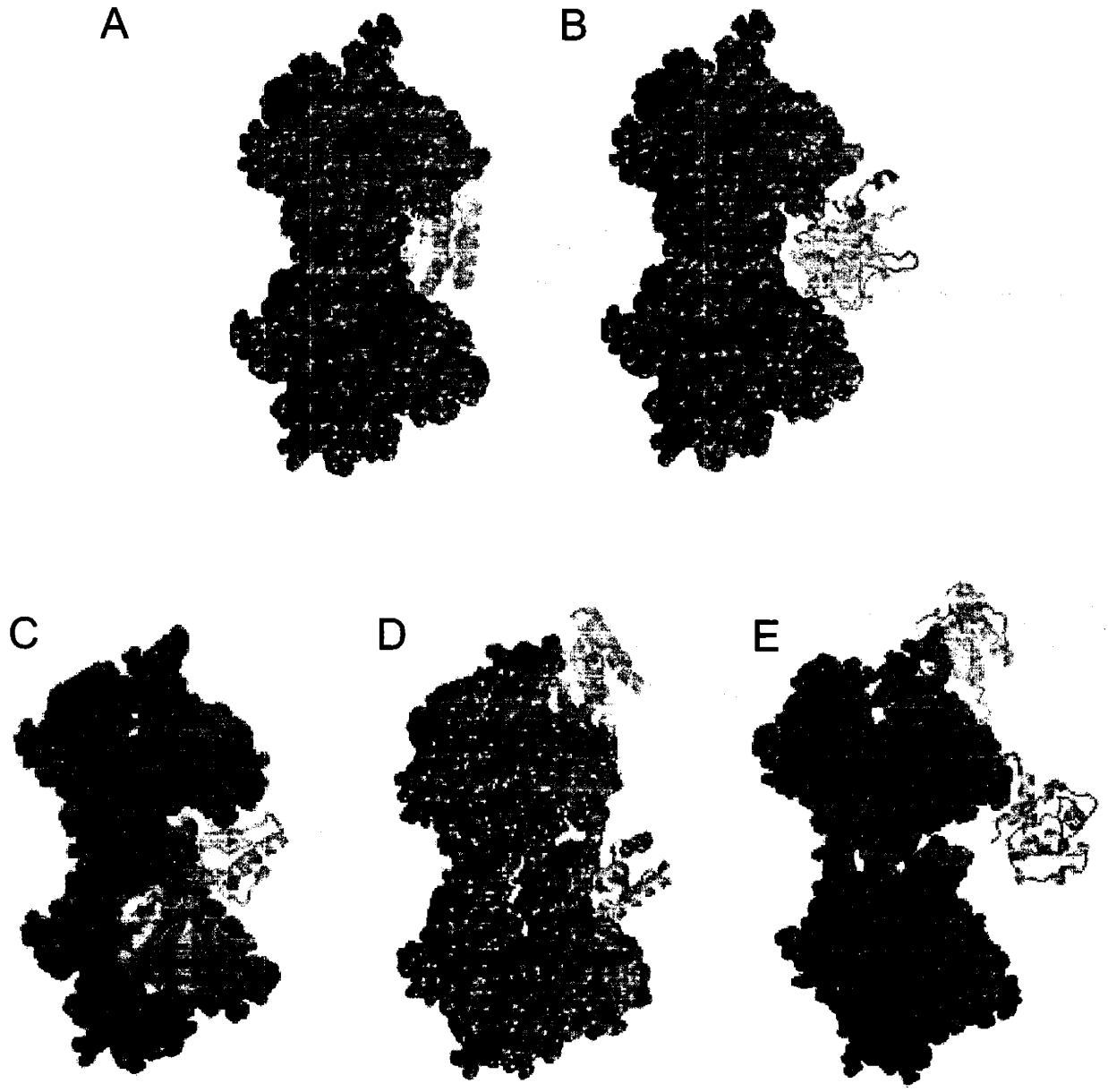


Figure 1.7

CH domains may also function in binding to intermediate filaments. The CH3 domain of calponin binds to desmin in vitro and CH1.1 domain of fimbrin binds vimentin (Mabuchi et al., 1997; Correia et al., 1999; Fujii et al., 2000). Mapping of the vimentin-binding site on CH1.1 domain of fimbrin using synthetic peptides demonstrated it to be distinct from the actin-binding site, which also resides on the CH1.1 domain (Correia et al., 1999). In the case of fimbrin, the interaction is clearly relevant in vivo, since both fimbrin and vimentin co-localize in cultured fibroblasts, and micro-injection of specific peptides disrupting the fimbrin-vimentin interaction also disrupts cell motility. Therefore, CH domains serve as integrators of the different cytoskeletal systems in the cell.

Recently, the cast of CH domain containing proteins has been expanded to include a broad family of acetyltransferases (CAT and CPT), kinesin-like proteins (KLP), abnormal spindle proteins (Asp) and others that were not previously suspected to contain CH domains (Korenbaum and Rivero, 2002). The sequences of the CH domains in these proteins are less conserved compared to the type1-5 CH domains and many of these proteins are not known to associate with actin. It will be interesting to learn whether these proteins associate with actin via their CH domains or whether in these proteins, CH domains have evolved to carry out an entirely different function.

Calponin-like repeats

The calponin-like repeat is a conserved sequence of 26 amino acids, which is found almost exclusively in the calponin protein family. No structural data is available for this motif and the protein sequence analysis predicts that the triple CLR sequence of calponin is primarily a random coil (Kranewitter et al., 2001). CLRs are found in nature exclusively in odd numbers: one in transgelin-like proteins, three in calponins, five repeats have recently been noted in *Shistosoma mansoni* calponin, and seven repeats are present in unc-87-like proteins discussed below.

In calponin, the three tandem repeats of the CLR sequences are sufficient for co-localization with actin in vivo, although their ability to bind to actin filaments in vitro has not been demonstrated (Gimona and Mital, 1998). A peptide corresponding to the first CLR of calponin binds to actin filaments in vitro (Mino et al., 1998). Additional evidence supporting the actin binding activity of the multiple CLRs comes from studies of an actin bundling protein which appears to be unique to nematodes (unc87 in *C. elegans*) and contains seven tandem CLRs with no other sequence similarity to known proteins (Kranewitter et al., 2001). The three amino-terminal CLRs of unc87 bind actin filaments in vitro and co-localize with actin in vivo when overexpressed in mammalian cell culture. Therefore, the triple calponin-like repeat functions as an actin binding module.

Whether the single CLR also binds to actin filaments has been controversial. Truncation of one or two CLRs of calponin abolishes calponin actin filament binding (Gimona and Mital, 1998). In addition, some have argued that transgelins, which contain a single CLR, do not bind to actin filaments (Gimona and Mital, 1998). Others have demonstrated transgelin binding to actin filaments under conditions of low ionic strength (Fu et al., 2000). In vitro binding experiments demonstrated that transgelin CLR is required for binding of transgelin to actin filaments, but whether it is also sufficient for actin binding has not been demonstrated (Fu et al., 2000). Thus, the role of a single calponin-like repeat is unclear.

CALPONINS

Calponin is characterized by a single calponin homology domain at the amino-terminus and multiple calponin-like repeats at the carboxyl terminus. Most calponins contain three repeats, although an unusual five-repeat calponin has been recently discovered in *Schistosoma mansoni* (Stradal et al., 1998). Since the initial identification of calponin (Takahashi et al., 1988), substantial progress has been made toward understanding the biochemical activities and the role of calponin in smooth muscle. Calponin is an actin bundling protein that regulates

smooth muscle contraction. Two non-smooth muscle isoforms of calponin have also been characterized, although not to the same extent as the muscle-specific calponin. Below, I discuss three open questions that will need to be answered to provide a more complete picture of calponin's role in the cell.

What is the mechanism of actin bundling by calponin?

Actin filament binding and bundling of calponin have been well documented. The bundling activity and stochiometry of binding is sensitive to the ionic strength of solution, and weak ionic interactions appear to make a significant contribution toward the binding affinity (Tang et al., 1997). The mechanism by which calponin cross-links actin filaments remains unresolved. Two actin-binding sites have been proposed for the muscle specific calponin isoform CNN1 (Figure 1.8). The first site (S1), comprised of residues 145-163, is a strong actin binding site (el-Mezgueldi et al., 1996; Mezgueldi et al., 1995). This sequence is not conserved among calponins and, therefore, is not likely to be used by other calponin family members for actin filament binding (Burgstaller et al., 2002). The second actin binding site (S2; amino acids 172-187) resides in the first CLR and is conserved in both calponins and transgelins (Mino et al., 1998; see Figure 1.8). Although peptide binding studies suggested that both S1 and S2 can bind actin filaments, only S1 inhibits actomyosin ATPase in vitro (el-Mezgueldi et al., 1996). It is not clear whether the two sites comprise a single actin filament binding site or function as two autonomous sites. An additional actin-binding site may reside in the CH domain of calponin (see above). If calponins contain only one actin-binding site, then bundling of actin filaments may occur via dimerization of calponin molecules. This hypothesis was previously rejected based on the inability to detect dimerization of purified calponin in sedimentation velocity, sedimentation equilibrium, and gel filtration experiments (Stafford et al., 1995). However, the possibility that dimerization occurs specifically after binding to actin filaments cannot yet be ruled out. One

Figure 1.8 Sequence alignment of calponin family members. Protein sequences of the yeast Scp1 and mouse Cnn1, Cnn2 and Tgn1 were retreived from the GeneBank; alignment was generated using Clustal X software and visualized in SeqView. Conserved amino acids are highlighted in green. Proposed actin binding sites of calponin are underlined (site 1, single line; site 2, double line) and residues shown by mutagenesis to be involved in actin binding are in red boxes .

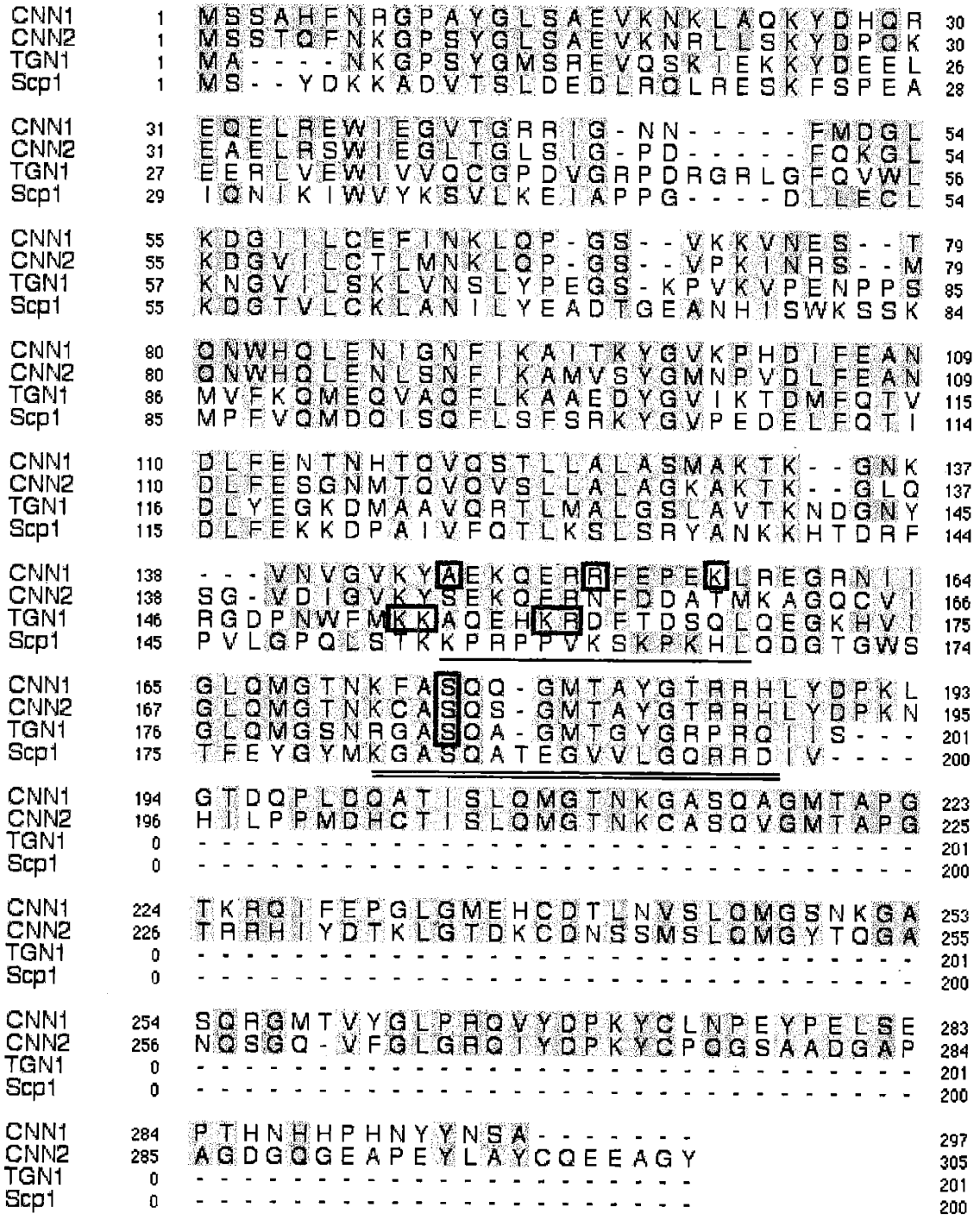

CNN1 1 MSSAHFNRGPAYGLSAEVKNKLAQKYDHQR 30
CNN2 1 MSSTQFNKGPSYGLSAEVKNRLLSKYDPQK 30
TGN1 1 MA----NKGPSYGMSREVQSKIEKKYDEEL 26
Scp1 1 MS--YDKKADVTSLDEDLRQLRESKFSPEA 28
CNN1 31 EQELREWIEGVTGRRIG-NN-----FMDGL 54
CNN2 31 EAELRSWIEGLTGLSIG-PD-----FQKGL 54
TGN1 27 EERLVEWIVVQCGPDVGRPDRGRLGFQVWL 56
Scp1 29 IQNIKIWVYKSVLKEIAPPG----DLLECL 54
CNN1 55 KDGIILCEFINKLQP-GS--VKKVNES--T 79
CNN2 55 KDGVILCTLMNKLQP-GS--VPKINRS--M 79
TGN1 57 KNGVILSKLVNSLYPEGS-KPVKVPENPPS 85
Scp1 55 KDGTVLCKLANILYEADTGEANHISWKSSK 84
CNN1 80 QNWHQLENIGNFIKAITKYGVKPHDIFEAN 109
CNN2 80 QNWHQLENLSNFIKAMVSYGMNPVDLFEAN 109
TGN1 86 MVFKQMEQVAQFLKAAEDYGVIKTDMFQTV 115
Scp1 85 MPFVQMDQISQFLSFSRKYGVPEDELFQTI 114
CNN1 110 DLFENTNHTQVQSTLLALASMAKTK--GNK 137
CNN2 110 DLFESGNMTQVQVSLLALAGKAKTK--GLQ 137
TGN1 116 DLYEGKDMAAVQRTLMALGSLAVTKNDGNY 145
Scp1 115 DLFEKKDPAIVFQTLKSLSRYANKKHTDRF 144
CNN1 138 ---VNVGVKYAEKQERREPEKLREGRNII 164
CNN2 138 SG-VDIGVKYSEKQERNFDDATMKAGQCVI 166
TGN1 146 RGDPNWFMKKAQEHKRDFTDSQLQEGKHVI 175
Scp1 145 PVLGPQLSTKKPRPPVKSKPKHLQDGTGWS 174
CNN1 165 GLQMGTNKFASQQ-GMTAYGTRRHLYDPKL 193
CNN2 167 GLQMGTNKCASQS-GMTAYGTRRHLYDPKN 195
TGN1 176 GLQMGSNRGASQA-GMTGYGRPRQIIS--- 201
Scp1 175 TFEYGYMKGASQATEGVVLGQRRDIV---- 200
CNN1 194 GTDQPLDQATISLQMGTNKGASQAGMTAPG 223
CNN2 196 HILPPMDHCTISLQMGTNKCASQVGMTAPG 225
TGN1 0 ---------- 201
Scp1 0 ---------- 200
CNN1 224 TKRQIFEPGLGMEHCDTLNVSLQMGSNKGA 253
CNN2 226 TRRHIYDTKLGTDKCDNSSMSLQMGYTQGA 255
TGN1 0 ---------- 201
Scp1 0 ---------- 200
CNN1 254 SQRGMTVYGLPRQVYDPKYCLNPEYPELSE 283
CNN2 256 NQSGQ-VFGLGRQIYDPKYCPQGSAADGAP 284
TGN1 0 ---------- 201
Scp1 0 ---------- 200
CNN1 284 PTHNHHPHNYYNSA------- 297
CNN2 285 AGDGQGEAPEYLAYCQEEAGY 305
TGN1 0 ---------- 201
Scp1 0 ---------- 200

Figure 1.8

way to test this model would be to compare chemical cross-linking of calponin to itself in the presence and absence of the actin filaments.

By what mechanism does calponin regulate smooth muscle contraction?

Calponin was first isolated from chicken gizzard smooth muscle as a protein recognized by antibodies to troponin I, a regulator of the skeletal muscle contraction (Takahashi et al., 1986). Mounting in vitro and in vivo evidence supports a regulatory role for calponin in smooth muscle; however, the mechanism of regulation remains controversial (reviewed in Winder et al., 1998; Morgan and Gangopadhyay, 2001). Two competing models have been proposed. According to one, binding of calponin to actin directly inhibits the actomyosin Mg-ATPase (Abe et al., 1990; Winder and Walsh, 1990a; Winder and Walsh, 1990b). This model proposes that during muscle contraction, phosphorylation of calponin and/or binding of calcium-binding proteins (caltropin/S100 or calmodulin) inhibits actin binding of calponin and relieves the inhibition of the actomyosin ATPase. Analysis of the in vitro 'contractile' system consisting of purified proteins, as well as studies of calponin's inhibition of actin filament motility over immobilized myosin are quite convincing (Winder and Walsh 1990; Miki et al., 1992; Shirinsky et al., 1992; Haeberle 1994; Borovikov et al., 1996). Moreover, in vitro analysis of calponin regulation by phosphorylation (Winder et al., 1993; Tang et al., 1996) and by calcium-binding proteins (Takahashi et al., 1986; Wills et al., 1993; Fujii et al., 1994) also supports this model. However, an in vivo test of this mechanism has relied largely on the use of permeabilized or de-membranated muscles and produced contradictory data (Itoh et al., 1994; Horowitz et al., 1996; Obara et al., 1996; Malmqvist et al., 1997; Morgan and Gangopadhyay, 2001). Some investigators observed changes in force, shortening velocity and/or muscle tone upon addition of exogenous calponin to the muscles stripped of endogenous calponin, while others have not. Furthermore, the in vivo relevance of these systems has been

questioned because potential regulators of contraction are lost during tissue preparation (Morgan and Gangopadhyay, 2001).

An alternative mechanism of calponin-mediated regulation of the smooth muscle contraction proposes a signaling rather than structural role for calponin (Menice et al., 1997). Such a role appears plausible given that other proteins containing type 3 CH domain function in signal transduction cascades (see discussion above for IQGAP, Vav). Compelling in vitro evidence supports the idea that calponin regulates muscle contraction via its interactions with the signaling proteins in the MAP kinase and PKC pathways. Calponin binds PKC and ERK kinase in vitro and can be phosphorylated by the PKC and calmodulin kinases (Leinweber et al., 1999). Moreover, calponin binding activates PKC in vitro, consistent with the signaling role in the PKC pathway (Leinweber et al., 2000). Whether these biochemical interactions are relevant in vivo has been difficult to demonstrate. Probably the strongest evidence in support of the signaling role of calponin, comes from recent studies using an antisense RNA approach. Chemical loading of calponin antisense oligonucleotides into ferret aorta strips resulted in ~50% reduction of calponin levels, but not of other smooth muscle proteins (Je et al., 2001). Calponin antisense RNA decreased muscle contractions induced specifically by the activators of the PKC pathway and also reduced ERK phosphorylation in response to PKC activators (Je et al., 2001). Since the basal intrinsic muscle tone was not affected by calponin antisense RNA, calponin is not likely to directly regulate actomyosin interactions, at least in the ferret aorta.

At this point, it is not clear whether one or both of these mechanisms operate in mammalian tissues. The main obstacle to understanding the mechanisms of smooth muscle regulation by calponin has been the absence of a good in vivo model system. Different studies describe contractility of permeablilized muscles or cells harvested from different species and different organs, which makes it difficult to compare different studies and interpret contradictory results. Initial characterization of a knock out mouse has not

provided the insight into the mechanism of calponin function because the changes in expression of other smooth muscle proteins, including actin, desmin, and caldesmon, make interpretation difficult (Fujishige et al., 2002; Morgan and Gangopadhyay, 2001). Ultimately, the understanding of muscle-specific role of calponin may require the use of more genetically tractable model organisms, like *D. melanogaster* or *C. elegans* (discussed below).

What are the functions of calponin outside the smooth muscle?

Several lines of evidence suggest that calponins may perform important functions outside the smooth muscle. First, the most obvious phenotype resulting from deletion of the muscle specific calponin CNN1 in mice was increased bone formation, suggesting that calponin may function as a negative regulator of bone formation (Yoshikawa et al., 1998). This result lead to discovery of CNN1 expression in osteoblasts. Second, two non-smooth muscle calponin isoforms have been identified in mammals. Rat acidic calponin (CNN3) localizes with actin in the growth cones of neuronal cells and may regulate neuronal remodeling and migration (Ferhat et al., 1996; Plantier et al., 1999). Neutral calponin CNN2, a ubiquitously expressed isoform, localizes to the ends of actin stress fibers and lamelapodial protrusions in the fibroblasts (Danninger and Gimona, 2000; Masuda et al., 1996). Based on localization, CNN2 is thought to function in the organization of the actin cytoskeleton, although its precise role in vivo is unknown. Understanding in vivo functions of calponins, especially outside smooth muscle, may emerge from an analysis of calponins' interactions with other proteins.

Several calponin-binding proteins have been identified in recent years. The next challenge will be to understand which of these interactions is biologically relevant. Calponin is reported to bind calmodulin, caltropin, myosin, tropomyosin, desmin, microtubules, PKC and ERK kinase (Reviewed in Hodgkinson, 2000; Small and Gimona, 1998). It is possible that calponin functions as a scaffold for other regulatory molecules; however, the in vivo

significance of the multiple protein-protein interactions have not yet been established. Particularly interesting will be to understand whether the reported interaction of calponin with microtubules and intermediate filaments has any in vivo relevance and whether calponin functions to integrate the three cytoskeletal systems in the cell.

TRANSGELINS

Transgelins are proteins of unknown function sharing a high degree of sequence similarity with calponins. Transgelins are characterized by a single CH domain at the amino-terminus and a single calponin like repeat at the carboxyl terminus (Figure 1.5). Given the absence of the functional assays for transgelin, previous studies have focused largely on in vitro activities and in vivo expression of transgelin. The two challenges for the future are characterization of biochemical activities and in vivo functions of transgelins.

Does transgelin bind and cross-link actin filaments in vitro and by what mechanism?

Transgelin was named for its in vitro gelation activity on actin filaments (Shapland et al., 1993), but this activity has been controversial due to the sensitivity of gelation to ionic strength (reviewed in Morgan 2001). Actin gelation has only been demonstrated in the absence or presence of very minimal salt concentrations and some groups have not been able to detect any actin biding of transgelin (Gimona and Mital, 1998; Kobayashi et al., 1994; Shapland et al., 1993). Therefore, careful characterization of biochemical activities of transgelin on actin will be required to resolve this controversy. Once actin filament binding and cross-linking by transgelin is established, the mechanism of actin cross-linking will need to be elucidated. One actin-binding site of transgelin may reside in the CLR, because deletion or mutation of this region inhibits actin filament binding in vitro (Fu et al., 2000). The second actin-binding site is unknown. The

sequences of the calponin actin-binding site 1 (S1; Figure 1.8) are not conserved in transgelin, but the overall positive charge of the region immediately adjacent to the CLR is conserved. Moreover, the importance of the positively charged dipeptides in this region has been demonstrated by mutational analysis (Fu et al., 2000). Therefore, the second actin-binding site may reside in a positively charged region adjacent to the CLR, analogous to the S1 of calponin. Alternatively, the CH domain or dimerization may be involved in actin filament cross-linking and will need to be investigated further (see discussion for calponins).

What are the functions of transgelin and how are they regulated?

Perhaps a more important, yet more difficult question to address, is the in vivo role of transgelins. Like calponins, mammalian transgelins are represented by three isoforms: smooth muscle specific (TGN1), neuronal (NP25/TGN3) and a ubiquitously expressed (TGN2). No in vivo function has been characterized for any transgelin isoform. A transgelin (TGN1) knock out mouse was generated, but it exhibited no mutant phenotype (Zhang et al., 2001). The absence of the phenotype may be due to the functional redundancy among calponin family members. It would be interesting to determine protein levels of other transgelins and calponins in TGN1-deficient mice to test whether loss of TGN1 leads to compensatory increase in expression of other calponin family members. Alternatively, transgelin function may not be required for homeostatic functions; instead transgelin may be involved in cellular senescence (see below) or in a stress response (e.g. wound healing). How transgelin is regulated is also unknown. Regulation by phosphorylation analogous to that of calponin has been proposed based on the in vitro phosphorylation of a conserved serine residue (ser-184) of transgelin by protein kinase C (Fu et al., 2000). However, in vivo phosphorylation of transgelin has not been detected (Gimona et al.,1992; Fu et. al., 2000).

What role does transgelin play in senescence and regulation of cell proliferation?

Several groups have focused on characterization of the mammalian transgelin expression and, based on the expression patterns, suggested a role for transgelin in cellular senescence and inhibition of cell proliferation. Transgelin was initially purified as a smooth muscle specific protein (SM22α; Lees-Miller et al., 1987a; Lees-Miller et al., 1987b) and is still used as one of the earliest markers for smooth muscle differentiation (Zhang et al., 2001). An independent group identified transgelin/WS3-10 as a cDNA overexpressed in cells from patients with premature aging/Werner's syndrome and in senescent fibroblasts (Thweatt et al., 1992). Several groups found that transgelin is up regulated in differentiated and senescent cells (Grigoriev et al., 1996; Thweatt et al., 1992), and down regulated in transformed (e.g. rhabdomyosarcoma) and proliferating cells (Genini et al., 1996; Lawson et al., 1997; Shapland et al., 1988). However, this correlation between expression level and cell proliferation does not constitute functional data or prove causality. One group was able to demonstrate that transgelin overexpression in cultured fibroblasts caused increased potassium currents at the plasma membrane (Liu et al., 1994). These changes were consistent with cellular senescence, but the effects could have been indirect. Therefore, what role, if any, transgelin plays in senescence and cell proliferation remains uncertain.

INVERTEBRATE CALPONIN FAMILY MEMBERS

The data discussed above were generated from studies of vertebrate calponins and transgelins. Virtually nothing is known about the functions of invertebrate calponin family members. Most of the invertebrate calponin genes were identified by homology in the sequenced genomes of model organisms and have not been characterized further. The two exceptions are MP-20 and myophilin. MP-20 was identified in a *D. melanogaster* screen for muscle specific genes and localized specifically to synchronous muscles (Ayme-Southgate et al.,

1989). Myophilin was identified in *Echinococcus granulosus* as a muscle specific antigen and localized to the contractile myofibrils of smooth muscle cells by immunoelectron microscopy (Martin et al., 1995). The muscle specific expression is a common theme shared by both vertebrate and invertebrate calponin family members. It is plausible that the muscle-specific functions of these proteins have been conserved in evolution and invertebrate model organisms may provide good tools for understanding the functions of mammalian calponin family members in smooth muscle.

The existence of non-muscle calponins and transgelins, as well as the presence of transgelin-like genes in fungi, suggest that these proteins may also perform a less specialized cellular function. Understanding this function may come from characterizing non-muscle isoforms of calponin family members in less complex eukaryotes, amenable to advanced genetic and biochemical approaches.

CHAPTER 2. SCP1 FUNCTIONS WITH Sac6/FIMBRIN TO REGULATE STABILITY AND ORGANIZATION OF THE ACTIN CYTOSKELETON.

BACKGROUND

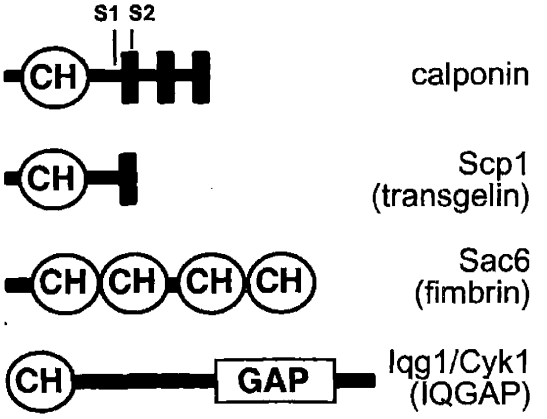
Actin filament assembly and organization is regulated by a large number of actin-associated proteins that use a limited set of structural modules to achieve great diversity of activities (Matsudaira, 1991). One such protein module, the calponin homology (CH) domain, is found in actin-associated proteins that cross-link actin filaments (e.g. spectrin, filamin, fimbrin), link actin to other cytoskeletal systems (e.g. fimbrin, plectin), and form signalling scaffolds (e.g. IQGAP, Vav; reviewed in Gimona et al., 2002 and Chapter 1). It is well established that a pair of CH domains forms a classic actin binding domain (e.g. α-actinin and fimbrin; reviewed in Matsudaira, 1991). In contrast, calponin family members contain only a single CH domain, and it remains controversial whether this domain can bind to actin filaments (Gimona and Winder, 1998; Fu et al., 2000; Winder, 2003).

The calponin protein family, which includes calponins and transgelins, is characterized by a single CH domain located at the amino-terminus and either one or more calponin-like repeats (CLR) located at the carboxyl-terminus (Prinjha et al., 1994). Both mammalian transgelin and *S. cerevisiae* calponin (Scp1) have a single CLR, whereas mammalian calponin contains three CLRs (Figure 2.1A). The calponin family is highly conserved from yeast to humans. Fungal genomes (*S. cerevisiae, S.pombe,* and *N. crassa*) contain a single transgelin-like gene, whereas higher eukaryotic genomes have multiple transgelins and calponins. This evolutionary conservation of calponin family proteins suggests that they may have highly conserved functions *in vivo*, yet our understanding of these functions is limited. Calponin is a regulator of smooth muscle contraction, but the functions of non-muscle calponins are not as well understood (reviewed in Morgan and Gangopadhyay, 2001).

Transgelin (also called SM22 and WS3-10) was named for its *in vitro* gelation activity on actin filaments (Shapland et al., 1993), but this activity has

Figure 2.1 Domain organization of Scp1 and related proteins. (A) Domain organization of calponin and the three calponin homology (CH) domain-containing proteins in budding yeast: Scp1, Sac6, and Iqg1/Cyk1. CH domains are circled, calponin-like repeats (CLR) appear as solid rectangles, the two putative actin binding sequences in calponin are labeled (S1 and S2), and the GTPase activating protein (GAP) domain in Iqg1/Cyk1 is boxed. (B) Primary sequence of Scp1. The CH domain is shaded, the CLR is underlined, and the sites of mutations in *scp1* are indicated by arrows. (C) Diagram of the mutant constructs generated in this study and summary of their activities and in vivo functions.

A



B

```
  1 MSYDKKADVT SLDEDLRQLR ESKFSPEAIQ NIKIWVYKSV  40
 41 LKEIAPPGDL LECLKDGTVL CKLANILYEA DTGEANHISW  80
 81 KSSKMPFVQM DQISQFLSFS RKYGVPEDEL FQTIDLFEKK 120
121 DPAIVFQTLK SLSRYANKKH TDRFPVLGPQ LSTKKPRPPV 160
161 KSKPKHLQDG TGWSTFEYGY MKGASQATEG VVLGQRRDIV 200
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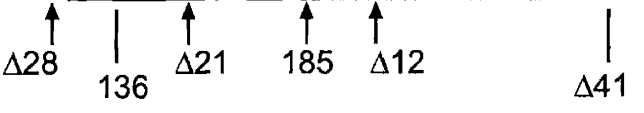


C

		Actin filament binding	Actin filament cross-linking	In vivo function shared with Sac6
WT	CH	+	+	+
S185A	CH A	–	–	–
S185D	CH D	+/–	+	+
N(1-136)	CH	–	–	n.d.
C(136-200)		+	+	n.d.
Δ12	CH	n.d.	n.d.	–/+
Δ28	CH	n.d.	n.d.	–/+
Δ21	CH	–/+	n.d.	n.d.
Δ41	CH	–	n.d.	n.d.

Figure 2.1

been questioned because it does not occur at physiological salt concentrations (see Discussion). In vivo, transgelin localizes to actin structures such as stress fibers (Fu et al., 2000), yet the ability of transgelin to bind directly to actin filaments in vitro also has been disputed (Morgan and Gangopadhyay, 2001). Increased levels of transgelin expression have been correlated with cell differentiation and senescence, but the function, if any, of transgelins in these processes has not been demonstrated (Thweatt et al., 1992; Grigoriev et al., 1996). Thus, little is known about the in vitro or in vivo functions of transgelins.

The *S. cerevisiae* genome contains a single open reading frame with homology to the calponin protein family, and this gene was annotated as *S. cerevisiae* calponin homologue, *SCP1* (Epp and Chant, 1997). However, the domain organization of Scp1 more closely resembles transgelin than calponin. As shown in Figure 2.1B, Scp1 contains a single CH domain (residues 28-139; shaded) and one calponin-like repeat (residues 174-200; underlined). The yeast genome encodes two other proteins with readily apparent CH domains, Sac6 (fimbrin), and Iqg1/Cyk1 (IQGAP), shown schematically in Figure 2.1A. IQGAP has a single CH domain, localizes to the bud neck, and functions in cytokinesis, but little is known about its interactions with actin (Epp and Chant, 1997; Lippincott and Li, 1998). Sac6/fimbrin binds to and bundles actin filaments through a tandem pair of actin binding domains, each comprised of two CH domains. Sac6 localizes to cortical actin patches and actin cables and is important for actin organization, endocytosis, and cell polarity in vivo (Drubin et al., 1988; Adams et al., 1991; Kubler and Riezman, 1993).

Here, I show that the *S. cerevisiae* transgelin homologue Scp1 is a novel component of the cortical actin cytoskeleton and a *bona fide* actin filament binding and cross-linking protein. The sequences in Scp1 critical for actin filament binding and cross-linking reside outside of the CH domain. Genetic interactions between *SCP1* and *SAC6*/fimbrin and similar biochemical activities suggest that these two CH domain-containing proteins cooperate in vivo to regulate the stability and organization of the cortical actin cytoskeleton.

RESULTS

Scp1 localizes to cortical actin patches in vivo

To investigate the in vivo function of Scp1, I first examined localization of Scp1 in yeast cells. I was unable to localize endogenous Scp1 using anti-Scp1 antibodies or HA-tagged Scp1 using anti-HA antibodies, likely because Scp1 is expressed at low levels (see below). Therefore, I examined the localization of GFP-Scp1 expressed under the control of the *SCP1* promoter from a low copy plasmid. As shown in Figure 2.2A, GFP-Scp1 localized to motile cortical patches, largely polarized in the bud, but also present in the mother cell. The GFP-Scp1 patches disappeared rapidly after cells were treated briefly with 200μM latrunculin A, an actin monomer sequestering agent (Figure 2.2A). Thus, filamentous actin is required for GFP-Scp1 localization. In fixed cells, GFP-Scp1 patches co-localized with rhodamine phalloidin stained actin patches, demonstrating that GFP-Scp1 localizes to actin patches (Figure 2.2B). Whereas all GFP-Scp1 patches overlapped with actin patches, about 16% of actin patches (n=89) did not have a corresponding GFP signal. This leaves open the possibility that some actin patches do not contain Scp1.

Deletion of *SCP1* enhances *sac6*Δ phenotypes

To further study *SCP1* in vivo function, I generated a complete deletion of the *SCP1* gene. This mutation alone had no salient phenotype in haploid or diploid cells, but did show specific genetic interactions with *sac6*Δ. Among the many phenotypes tested for the *scp1*Δ single mutants were growth at a full range of temperatures, growth under various stresses (e.g. NaCl, caffeine, benomyl), cell morphology, bipolar budding pattern, actin cytoskeleton organization, and endocytosis (assayed by lucifer yellow, FM4-64 uptake and Ste6 internalization). The only detectable phenotype of *scp1*Δ was a modest but reproducible sensitivity to latrunculin A (Table 2.1). In a desperate attempt to find a molecular phenotype of *scp1*Δ mutant strains, I have taken two steps further. First, I have compared genome-wide gene expression in wild type and *scp1*Δ strains using Affimetrix microarrays and detected no significant differences (data

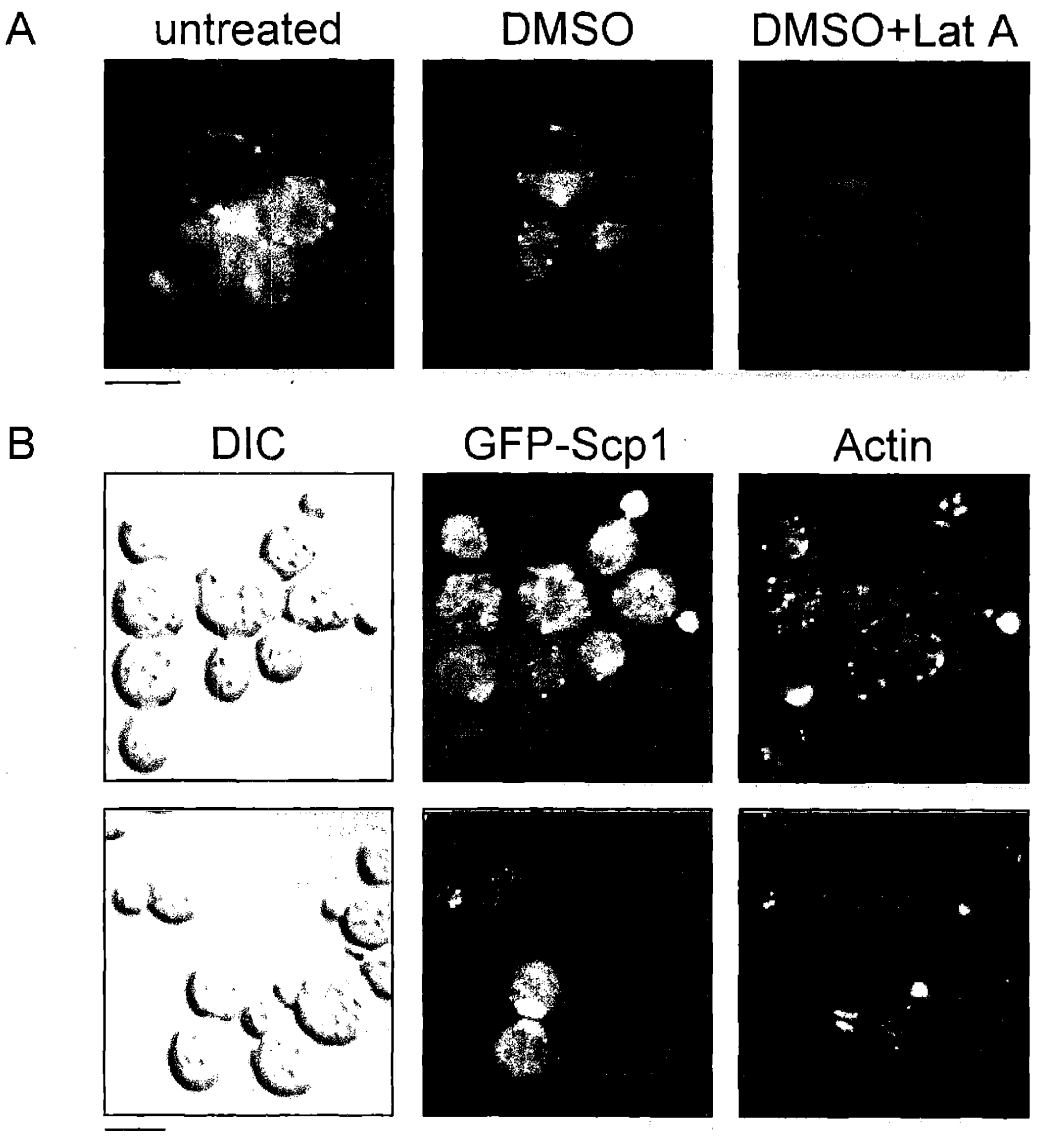


Figure 2.2 Localization of GFP-Scp1 to cortical actin patches. (A) Localization of GFP-Scp1 in live yeast cells untreated, treated with DMSO, or treated with 200μM latrunculin A in DMSO. (B) Co-localization of GFP-Scp1 and rhodamine phalloidin actin staining in fixed cells. Bar, 5μm.

Figure 2.2

Table 2.1 Latrunculin A sensitivity of *scp1*Δ and *sac6*Δ mutant strains.

Relevant genotype	Relative sensitivity to latrunculin A
wild type	1.0
*sac6*Δ	2.2
*scp1*Δ	1.4
*sac6*Δ *scp1*Δ	3.5
*sac6*Δ *scp1*Δ, *pSCP1/CEN*	2.0

The latrunculin A sensitivities of cells were measured by halo assays as previously described (Ayscough et al., 1997). Relative sensitivity was defined as the ratio of latrunculin A concentrations required to produce halos of the same diameter for wild type and mutant strains.

not shown). Second, I compared total protein extracts on the two-dimensional gels and found only minor differences between wild type and scp1Δ strains (Figure 2.3).

Given the high degree of functional redundancy among components of cortical actin patches (reviewed in Goode and Rodal, 2001; Pruyne and Bretscher, 2000), I tested for synthetic genetic interactions between *SCP1* and other genes that regulate actin function. The *scp1Δ* mutants showed synthetic defects only with *sac6Δ*, but not with *abp1Δ*, *aip1Δ*, *arp2-1*, *cap2Δ*, *cof1-22*, *crn1Δ*, *end3Δ*, *las17Δ*, *pan1-4*, *rvs167Δ*, *sla1Δ*, *sla2Δ*, *srv2Δ*, *tpm1Δ*, or *tpm2Δ*. Deletion of *SCP1* enhanced many phenotypes of *sac6Δ*, including temperature and caffeine sensitivity (Figure 2.4A), salt sensitivity (unpublished observation), and latrunculin A sensitivity (Table 2.1) and others (Appendix 1). Deletion of *SCP1* did not further enhance the actin organization or endocytosis phenotypes of *sac6Δ* cells, which already have depolarized actin cytoskeleton and fail to accumulate Lucifer Yellow dye in the vacuole (unpublished observations).

The *scp1Δ sac6Δ* double mutant cells provided a genetic background that permits direct testing of the Scp1 function in vivo. Mutation of a conserved serine residue in the CLR of mammalian calponin (Ser175) and transgelin (Ser184) disrupts actin binding in vitro (Tang et al., 1996; Fu et al., 2000). To test whether the analogous residue in Scp1 (S185; Figure 2.1B) is important for in vivo function, I generated two substitutions (S185A and S185D) and deletions of 12 and 28 carboxyl terminal residues of Scp1 (Δ12 and Δ28). *scp1Δ sac6Δ* cells transformed with wild type or mutant *SCP1* constructs were analyzed for growth phenotypes. Both wild type *SCP1* and *scp1S185D* suppressed the growth defects of *scp1Δ sac6Δ* cells, indicating that these constructs restore *SCP1* function. In contrast, cells expressing *scp1S185A* grew only slightly better than control cells carrying an empty vector (Figure 2.4A). The *scp1Δ12* and *scp1Δ28* truncation mutants showed a level of suppression intermediate between wild type *SCP1* and *scp1S185A* (Figure 2.4B). Stable expression of the mutant proteins was verified by immunoblotting (Figure 2.6B and unpublished observations).

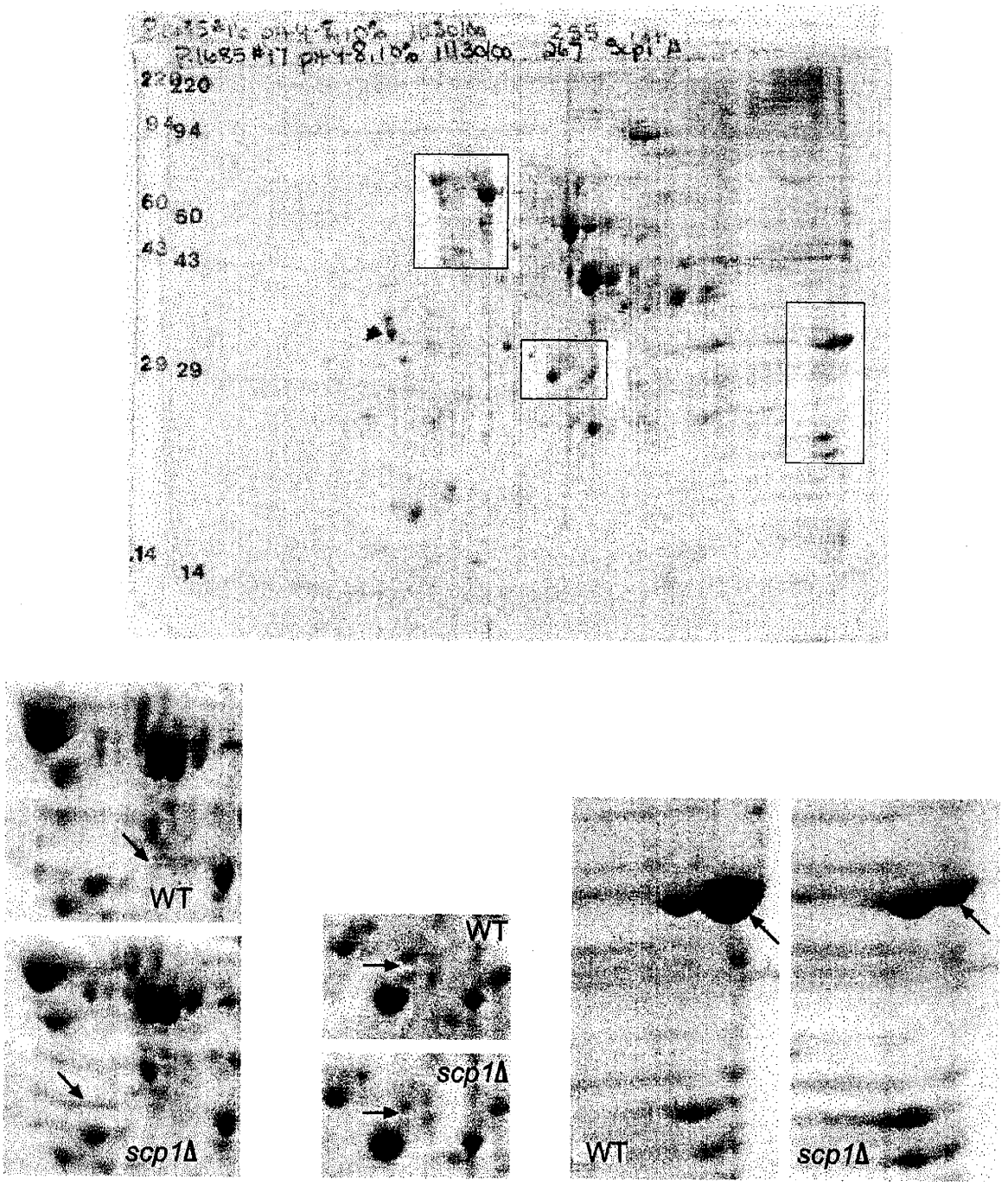


Figure 2.3 Comparison of protein extracts from wild type and *scp1Δ* cells. A. Protein extracts from AGY255 *(SCP1/SCP1)* and AGY267 *(scp1Δ/scp1Δ)* were separated by the two dimensional gel electrophoresis and stained by Coomassie. Scanned gels were superimposed (green- wild type, red - *scp1Δ)* and cpompared. Regions where differences were detected are boxed and enlarged. Arrows indicate spots that are different between the two gels.

These data indicate that the conserved serine residue (located in the CLR) is critical for Scp1 function in vivo.

Additional copies of *SCP1* partially suppress the *sac6Δ* growth phenotype

SCP1 expressed from a low copy plasmid suppressed the temperature sensitivity of *sac6Δscp1Δ* double mutant cells (Figure 2.4). To investigate the basis of this effect, I quantified the expression levels of actin, Sac6, and Scp1 in cells, comparing cell extracts to standard curves of purified proteins (actin, Sac6, and Scp1) by immunoblotting (Figure 2.5). The level of Scp1 (~0.01 ng/μg of total cellular protein) was considerably lower than that of Sac6 (~0.15 ng/μg) and actin (~1ng/μg). From these values, I calculated that the molar ratio of actin, Sac6, and Scp1 in cells is approximately 65:6:1. The expression level of Scp1 in *sac6Δscp1Δ* cells carrying a low-copy *SCP1* plasmid (Figure 2.5 lane E) was 2-4 fold higher than endogenous Scp1 levels in wild type cells (Figure 2.5 lane B). In addition, expression of *SCP1* from a low copy plasmid in wild type cells also resulted in 2-4 fold increase in Scp1 levels (Figure 2.6A). These observations suggested that extra copies of *SCP1* can suppress *sac6Δ* cell growth defects. To test this hypothesis directly, I transformed *sac6Δ* cells with low copy plasmids expressing wild type and mutant Scp1 proteins. As shown in Figure 2.6C, a wild type *SCP1* plasmid partially suppressed the temperature sensitivity of the *sac6Δ* mutant. *scp1S185D* also partially suppressed the *sac6Δ* phenotype, whereas *scp1S185A* showed no suppression. Thus, low-level overexpression of *SCP1* partially suppresses the temperature sensitive growth phenotype of the *sac6Δ* mutant, and a specific mutation in *SCP1* abolishes this suppression.

High level overexpression of *SCP1* disrupts the actin cytoskeleton

To study the functional role of Scp1 in cortical actin patches, I examined the effects of overproducing Scp1 at high levels on cell growth and morphology. Overexpression of *SCP1* from a galactose-inducible promoter inhibited cell growth (Figure 2.7A) and increased the percentage of large, round, multi-budded and multinucleated cells (see Table 2.2 and Figure 2.7B). Rhodamine-phalliodin

Figure 2.4 Synthetic genetic interactions between *SCP1* and *SAC6*. (A) Genetic interactions of scp1Δ, *scp1S185A* and *scp1S185D* with a *sac6Δ* null mutation. Homozyogous diploid yeast (wild type, *scp1Δ*, sac6Δ, or *sac6Δ scp1Δ*) carrying vector alone or *SCP1* plasmids were serially diluted and grown on YPD at different temperatures in the presence or absence of 5mM caffeine. (B) Genetic interactions of *scp1Δ*12 and *scp1Δ*28 with a *sac6Δ* null mutation. Wild type, *sac6Δ*, or *sac6Δ scp1Δ* haploid yeast carrying vector alone or *SCP1* plasmids were serially diluted and grown on YPD at different temperatures.

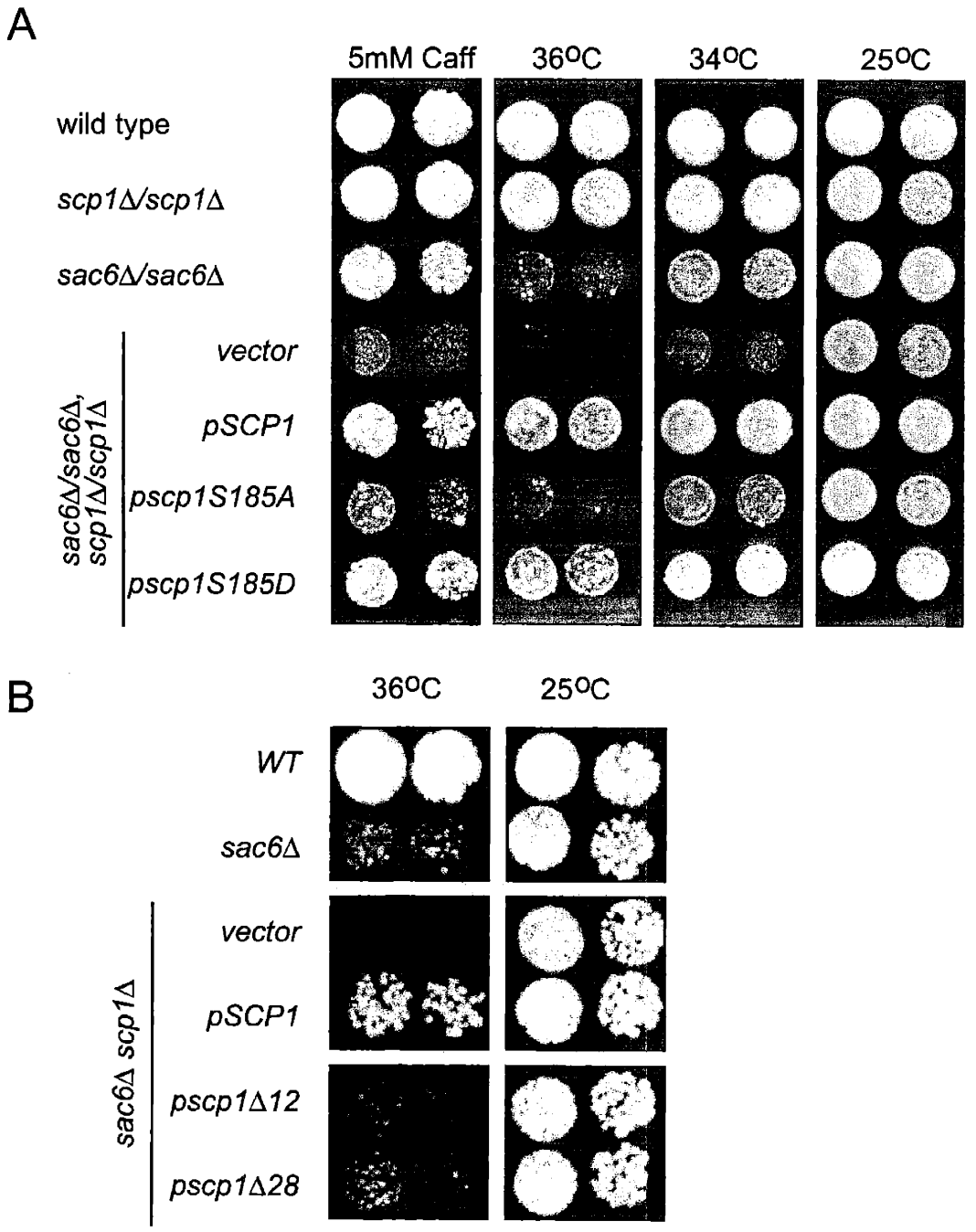

A
5mM Caff
36°C
34°C
25°C
wild type
scp1Δ/scp1Δ
sac6Δ/sac6Δ
sac6Δ/sac6Δ, scp1Δ/scp1Δ
vector
pSCP1
pscp1S185A
pscp1S185D
B
36°C
25°C
WT
sac6Δ
sac6Δ scp1Δ
vector
pSCP1
pscp1Δ12
pscp1Δ28

Figure 2.4

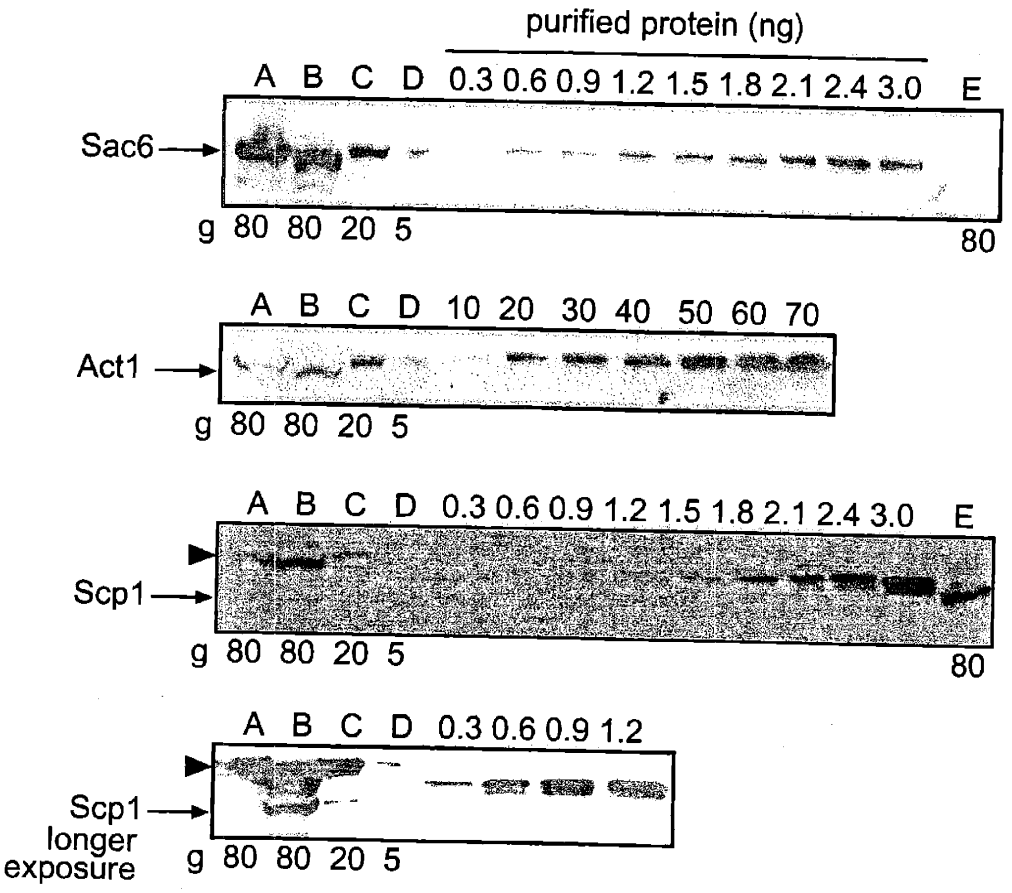


Figure 2.5 Levels of Sac6, actin and Scp1 expression in wild type cells. Immunoblots comparing the amounts of protein in total cell extracts with the standard amounts of purified protein. 80 g of total protein from *scp1Δ/scp1Δ* (lane A), 80 g (laneB),20 g (laneC) and 5 g (lane D) of total protein from *SCP1/SCP1* cells, and 80 g of total protein from *scp1Δ/scp1Δ,sac6Δ/sac6Δ pSCP1/CEN* (lane E) were separated by PAGE along side the purified proteins (amounts in ng are indicated above the lanes). Protein levels were determined by immunoblotting with anit-Sac6, anti-actin and anti-Scp1N antibodies.

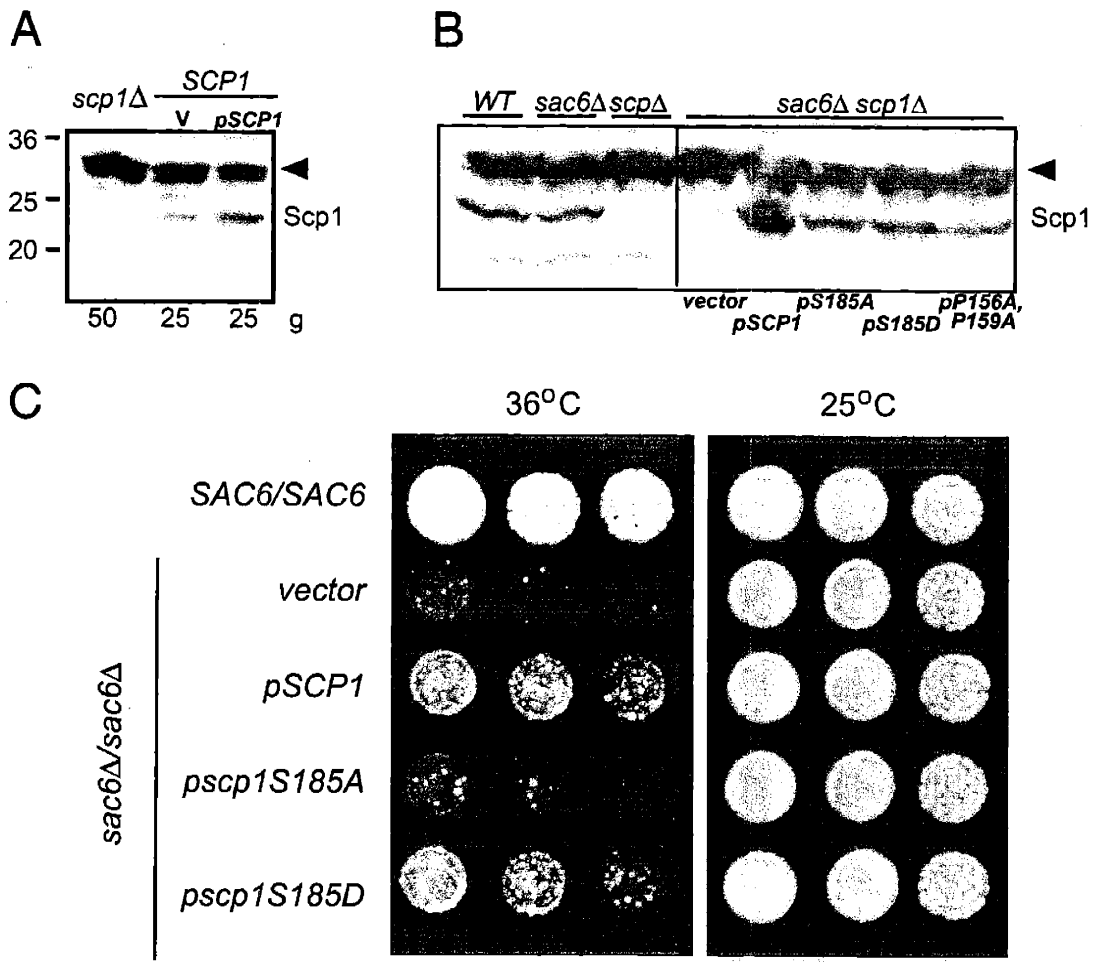


Figure 2.6 Suppression of *sac6*Δ null mutant phenotype by additional copies of *SCP1*. (A) Additional copy of Scp1 on a low copy plasmid increases Scp1 expression 2-4 fold. Protein extracts from AGY197 transformed with pRS 316 vector, AGY195 transformed with pRS316 vector, and AGY 195 transformed with pAG20 were analysed by immunoblotting with anti-Scp1N antibody. (B) Expression of wild type and mutant Scp1. AGY 195, 196, 197, and 198 were transformed with either empty vectors or plasmids carrying wild type or mutant Scp1 (pAG16, 17, 18, and 20). Protein extracts from these strains were analysed by immunoblotting with anti-Scp1N antibody. (C) Serial dilutions of wild type and sac6Δ/sac6Δ homozygous diploid yeast cells carrying vector or low copy *SCP1* plasmids grown at different temperatures.

Table 2.2 Effect of Scp1 overexpression on cell morphology and actin organization.

Phenotype	Vector	*Gal1-SCP1*
Multi-budded cells	1%	20%
Large cells	0.5%	12.7%
Multinucleated cells	0.7%	14.4%
Aggregated actin	6%	34%

Greater than 200 cells were scored to determine the percentage of cells with the mutant phenotypes listed. Examples of phenotypes are shown in Figure 2.7B.

Figure 2.7 Effects of Scp1 overexpression on cell growth and morphology. (A) Growth of serially diluted L4852 strains carrying vector (pRS423Gal1) or *SCP1* overexpression plasmids (pAG49, pAG48, pAG39) on glucose or galactose plates. (B) Galactose-induced L4852 cells carrying vector (pRS423Gal1) or wild type *SCP1* overexpression plasmid (pAG49) stained with rhodamine-phalloidin and DAPI. For *Gal1-SCP1*, only cells with abnormal actin organization are shown. The phenotypes are quantified in table 2.2. (C) Expression levels of wild type and mutant Scp1 after galactose induction. 80 or 2 μg of protein extracts from the yeast strains in (A) grown in glucolse or galactose were analyzed along side purified His6-Scp1 by immunoblotting with anti-Scp1N antibody. Arrowhead points to a non-specific band recognized by the antibody in protein extracts from glucose-grown cells.

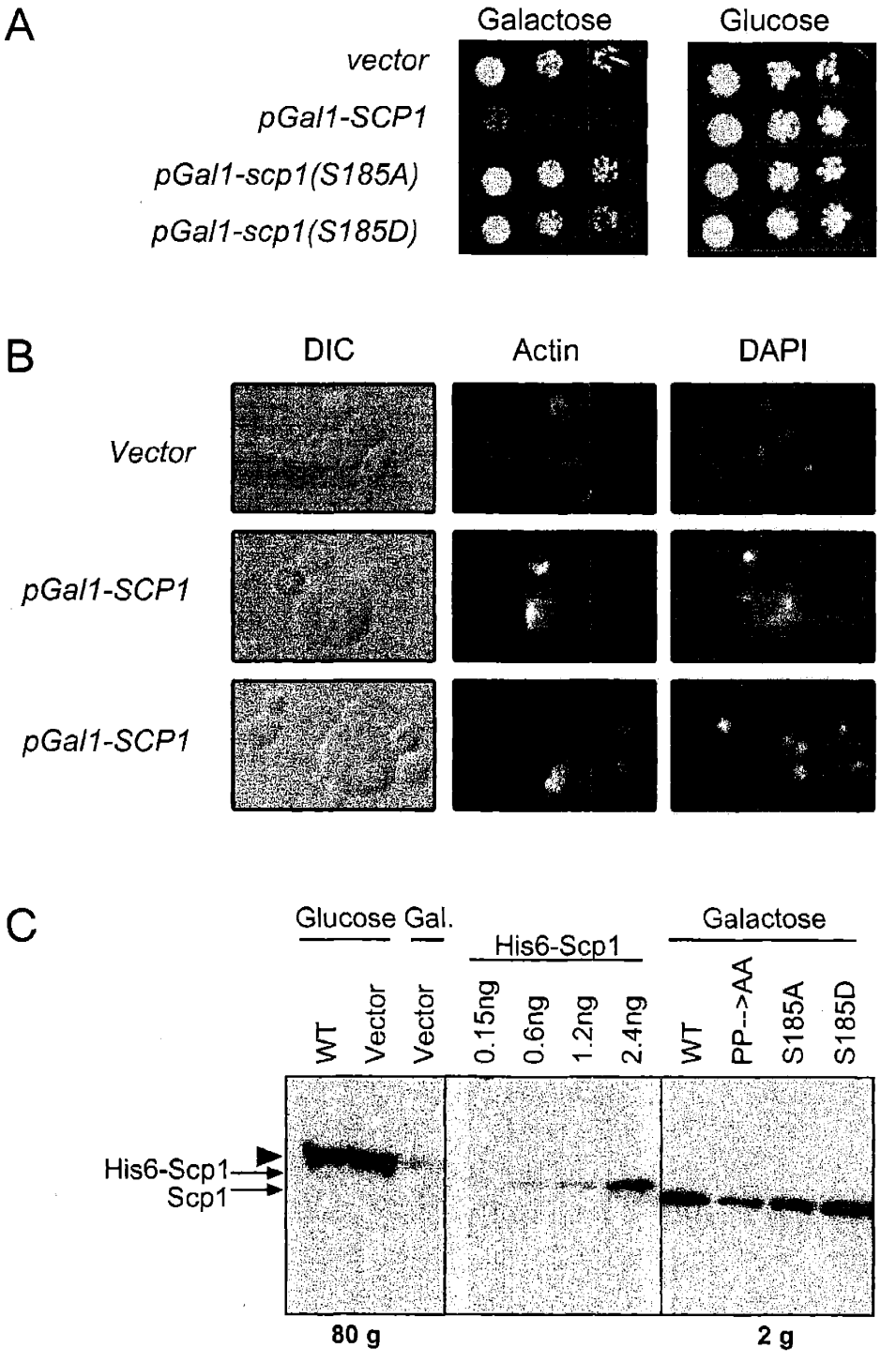

A
Galactose
Glucose
vector
pGal1-SCP1
pGal1-scp1(S185A)
pGal1-scp1(S185D)
B
DIC
Actin
DAPI
Vector
pGal1-SCP1
pGal1-SCP1
C
Glucose
Gal.
His6-Scp1
Galactose
WT
Vector
Vector
0.15ng
0.6ng
1.2ng
2.4ng
WT
PP-->AA
S185A
S185D
His6-Scp1
Scp1
80 g
2 g

Figure 2.7

staining revealed that *SCP1* overexpression caused the formation of actin clumps. To test whether the *SCP1* overexpression phenotype requires normal interactions between Scp1 and actin, I over expressed *scp1S185A* and *scp1S185D* mutants. High level expression of all Scp1 proteins was confirmed by immunoblotting (Figure 2.7C). As shown in figure 2.7A, overexpression of either Scp1*S185A* or Scp1*S185D* had no detectable effect on cell growth. These data suggest that the *SCP1* overexpression phenotype is sensitive to mutation in the carboxyl terminus of Scp1.

Scp1 binds to and cross-links actin filaments *in vitro*

To test whether Scp1 interacts directly with actin filaments in vitro, I over expressed Scp1 in yeast using a galactose inducible promoter, purified the protein, and measured its ability to bind actin filaments in a high-speed co-sedimentation assay. As shown in Figure 2.8 A and B, Scp1 bound to yeast actin filaments in a concentration-dependent manner with micromolar binding affinity (Kd = 0.7μM) and a molar saturation stochiometry of 1:2 Scp1 to actin. Hexahistidine (His6) tagged Scp1 expressed and purified from *E. coli* bound to actin filaments with a similar affinity (Figure 2.8C).

Mammalian calponin family members have been shown to cross-link actin filaments (Shapland et al., 1993; Kolakowski et al., 1995; Tang et al., 1997). Using several complementary approaches, I found that Scp1 has a similar activity. First, His6-Scp1 increased light scattering of yeast actin filaments in a concentration dependent manner (Figure 2.9A), suggesting that Scp1 organized filaments into larger structures (e.g. bundles or networks). Second, I analyzed the reactions from the light scattering experiment in a low speed pelleting assay. In the absence of Scp1, most actin remained in the supernatant as expected, but with increasing amounts of Scp1, actin shifted to the pellet (Figure 2.9B). This concentration-dependent increase in actin pelleting correlated with the observed increase in light scattering (Figure 2.9A). To ensure that actin cross-linking by His6-Scp1 was not due to the tag, I tested untagged Scp1 in the low-speed actin-pelleting assay. Figure 2.9C shows that the effects of 1μM untagged Scp1 are

Figure 2.8. Binding of Scp1 to actin filaments. (A) Co-sedimentation assay using 2.5 μM yeast actin filaments and varying concentrations of Scp1. The samples are labeled below the pellet (P) and supernatant (S) lanes: A, 0.5 μM; B, 1 μM; C, 2 μM; D, 3 μM; E, 4 μM. Half of the supernatant was loaded in each lane compared to pellet. (B) Resulting binding curve. The amount of Scp1 bound (μM) in each reaction was calculated from densitometry measurements of the gel in (A) and plotted versus the total concentration of Scp1 in the reaction. (C) Co-sedimentation assay using 5 μM yeast actin filaments and/or 1 μM of His6-Scp1 or untagged Scp1. Equivalent amounts of pellet and supernatant were loaded in each lane. The samples are labeled below the pellet and supernatant lanes: 1, actin alone; 2, Scp1; 3, actin and Scp1; 4, His6-Scp1; 5, actin and His6-Scp1. Note: in (A), a contaminant that does not pellet with actin is marked with an asterisk, and in (C), a proteolytic fragment of Scp1 is visible below the full-length protein.

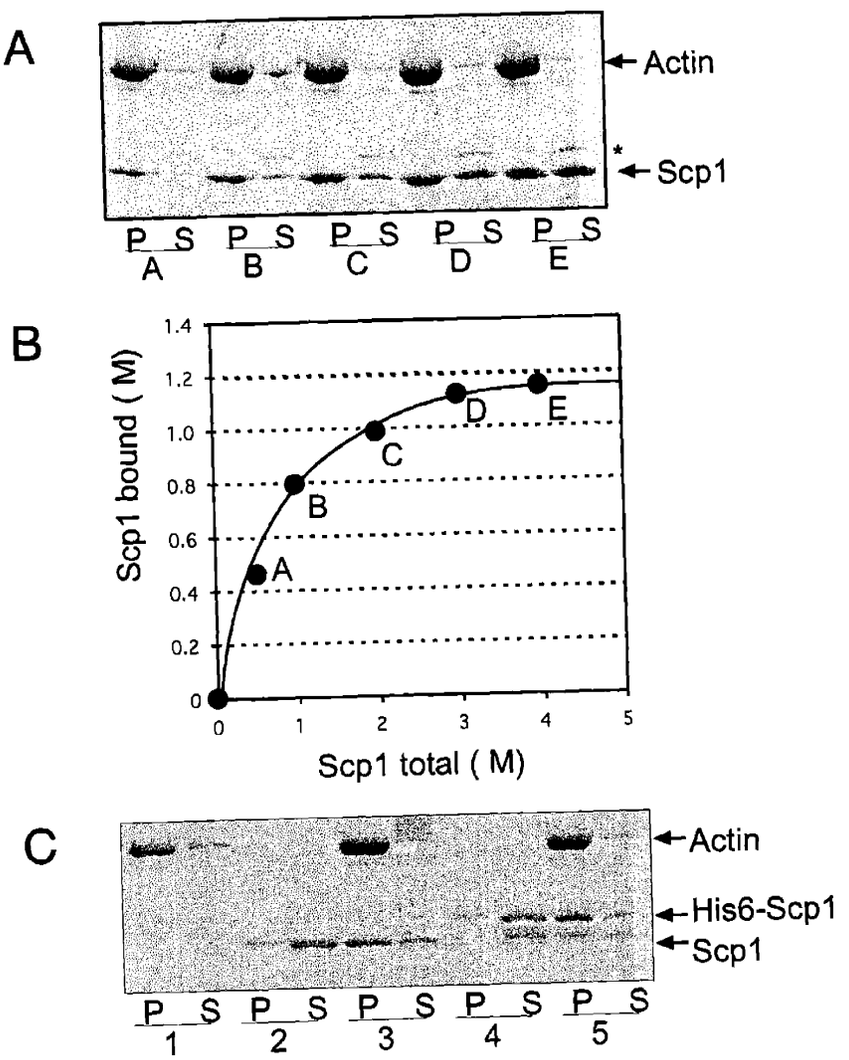

A
Actin
*
Scp1
P S P S P S P S P S
A B C D E
B
Scp1 bound (M)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B
C
D
E
0 1 2 3 4 5
Scp1 total (M)
C
Actin
His6-Scp1
Scp1
P S P S P S P S P S
1 2 3 4 5

Figure 2.8

nearly identical to the effects of 1 μM His6-Scp1. These results demonstrate that Scp1 cross-links actin filaments in vitro.

To characterize the actin cross-linking activity of Scp1 further, I used the falling ball assay (Pollard and Cooper 1982) to measure the apparent viscosity of the actin filament solution in the presence and in the absence of His6-Scp1. The apparent viscosity of a 4μM actin filament solution increased markedly when Scp1 concentration exceeded 1μM (Figure 2.9D). I also examined by electron microscopy negatively stained actin filaments in the presence and in the absence of His6-Scp1. In the absence of Scp1, actin filaments were distributed evenly throughout the grid (Figure 2.10A). When actin was polymerized in the presence of Scp1, actin filaments formed loose bundles tangled into networks (Figure 2.10 B and C). Scp1 cross-linked bundles appeared wavy and the spacing between filaments in a bundle was non-uniform. In contrast, Sac6/fimbrin bundles were straight with uniform spacing between filaments (Figure 2.10D).

Truncation of the carboxyl terminus of Scp1 reduces its actin binding affinity.

Purification of His6-tagged Scp1 from E. coli produced not only the full length protein, but also two truncated products (Figure 2.11C, marked with asterisks). These truncated forms of Scp1 did not co-pellet with actin in the high speed pelleting assays, suggesting that compared to the full length Scp1, truncated proteins had reduced affinity for actin filaments (Figure 2.11 A and C). I used antibodies raised against the amino terminal (anti-Scp1N) and carboxyl terminal (anti-Scp1C) peptides of Scp1 to characterize these truncations. The full length His6-Scp1 was recognized by both antibodies, while the truncated proteins were recognized only by the anti-Scp1N antibody (not shown). This suggested that truncated proteins were missing carboxy-terminal residues. Using mass spectrometry, I determined the mass of the full length and truncated proteins to be 26426, 24165 and 21914 kDa respectively. These data indicated that all bacterially expressed constructs were missing the amino-terminal methionine Met-1. Using the amino acid sequence of Scp1 in conjunction with

Figure 2.9. Actin filament cross-linking by Scp1. (A) Actin filament assembly and organization monitored by light scattering. Monomeric yeast actin (4 μM) was polymerized in the absence or presence of His6-Scp1 (1 or 2 μM) and monitored for change in light scattering (360 nm) over time. (B) Low speed pelleting assay for actin filament cross-linking. The reactions shown in (A) were centrifuged at low speed (10,000 x *g*) to precipitate cross-linked actin filament structures. Pellets and supernatants were analyzed by SDS-PAGE and Coomassie staining. (C) Low-speed pelleting assay of 2.5 μM actin filaments incubated with and without untagged Scp1 (middle two lanes) or His6-Scp1 (last two lanes). Note: a proteolytic fragment of actin is marked by a single asterisk. (D) Effect of His6-Scp1 on viscosity of actin filaments in solution. 4 μM actin was polymerized in capillary tubes in the presence of varying concentrations of His6-Scp1 (0, 0.23, 0.46, 0.93, 1.4, 1.8, 3.7 μM). Viscosity was measured after 1 hour incubation. Viscosity is inversely proportional to the velocity of the ball moving through the sample (Pollard and Cooper, 1982). Gelation (as indicated by the ball-bearing remaining stationary beneath the meniscus) was observed at Scp1 concentrations above 1.4μM.

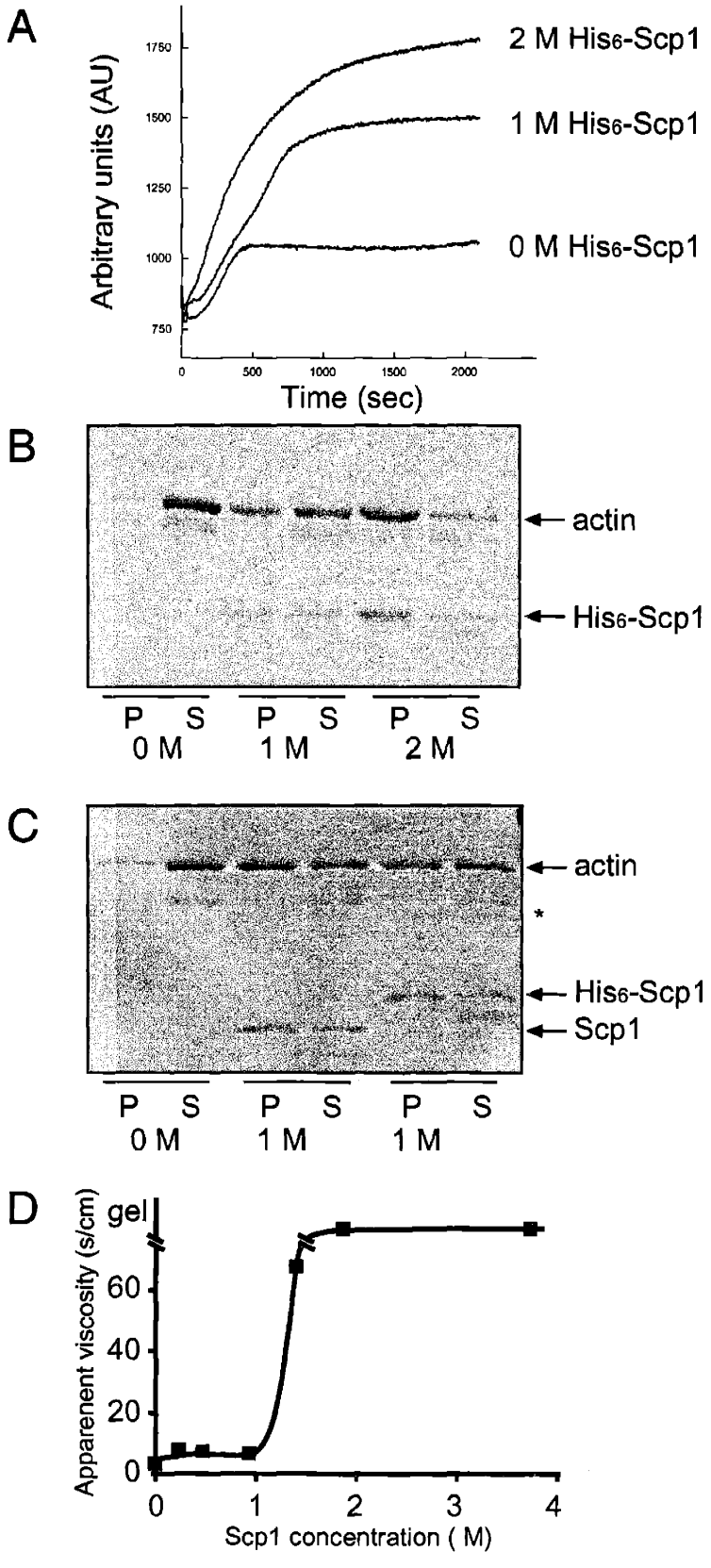

A
Arbitrary units (AU)
1750
1500
1250
1000
750
0
500
1000
1500
2000
Time (sec)
2 M His6-Scp1
1 M His6-Scp1
0 M His6-Scp1
B
actin
His6-Scp1
P S P S P S
0 M 1 M 2 M
C
actin
*
His6-Scp1
Scp1
P S P S P S
0 M 1 M 1 M
D
Apparenent viscosity (s/cm)
gel
60
40
20
0
0 1 2 3 4
Scp1 concentration (M)

Figure 2.9

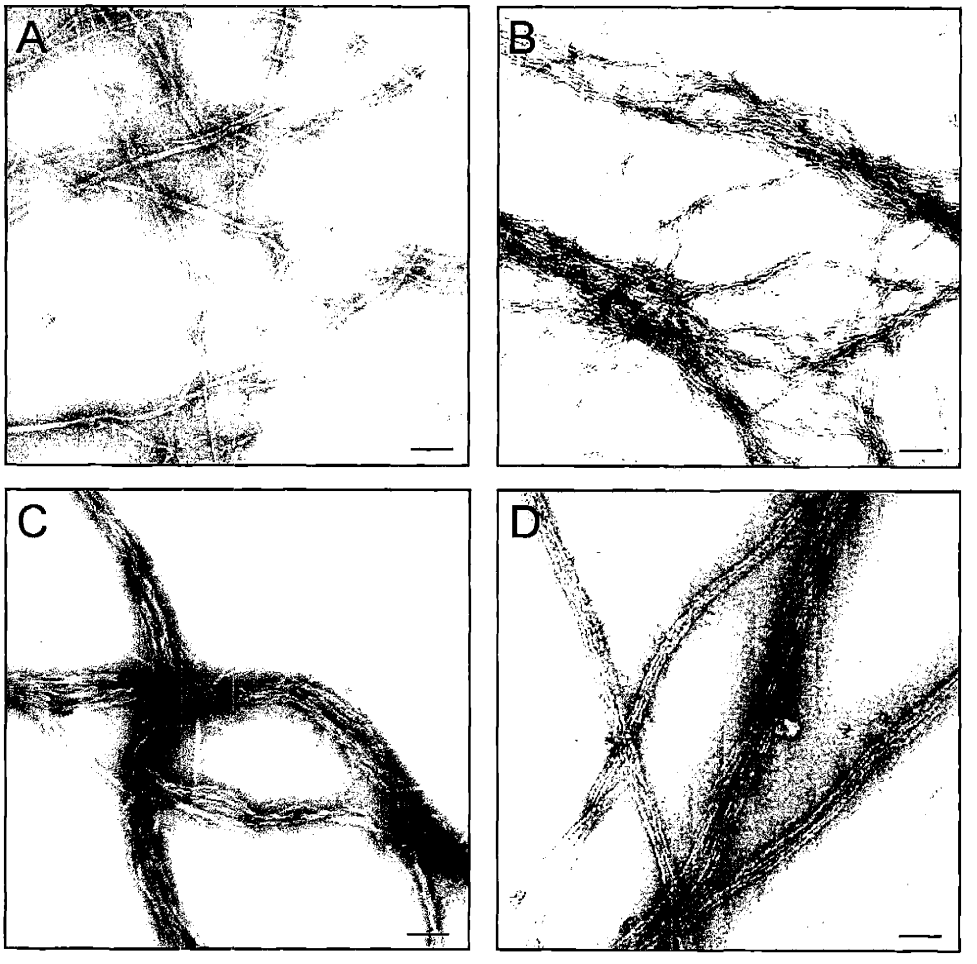


Figure 2.10. Electron micrographs of negatively stained actin filaments cross-linked by Scp1 or Sac6. Actin filaments (15 μM) were polymerized in the presence of control buffer (A), 7.5μM His6-Scp1 (B, C), or 7.5μM Sac6 (D), negatively stained with uranyl acetate, and photographed at 21,000x magnification. Bar, 100nm.

the immunoblotting and mass spectrometry data, I determined that truncated proteins were missing 21 and 41 amino acid residues at the carboxyl terminus. Therefore, loss of carboxy-terminal residues reduces in vitro actin binding affinity of Scp1.

The carboxyl terminus of Scp1 alone can cross-link actin filaments.

To better understand the molecular mechanism of actin binding and cross-linking by Scp1, I expressed and purified amino-terminal N(1-136) and carboxyl-terminal C(136-200) fragments of Scp1 (Figure 2.1C). While I attempted to generate both His6-tagged and untagged constructs in *E.coli*, I was able to isolate only the His6-tagged N(1-136) and untagged C(136-200). I tested the purified proteins for actin binding in the high speed pelleting assays. In contrast to the full length Scp1, the N(1-136) fragment did not co-pellet with actin filaments (Figure 2.11A). The C(136-200) fragment, on the other hand, co-pelleted with actin filaments, indicating that at least one actin-binding site resides in the carboxyl terminus of Scp1. I also tested the ability of the truncated proteins to cross-link actin filaments. In a low-speed pelleting assay, actin remained in the supernatant in the absence of Scp1 and in the presence of N(1-136) (Figure 2.11B). However, actin was found mostly in the pellet in the presence of the carboxy-terminal fragment or the full length Scp1. Therefore, untagged carboxyl terminus of Scp1 is sufficient to cross-link actin filaments.

In addition, I addressed whether a specific residue in the carboxyl terminus (S185) critical for in vivo function of Scp1 (see above), was also important for actin filament binding. I purified the His6-tagged S185A and S185D mutant Scp1 proteins and compared their ability to bind and cross-link actin filaments with the wild type Scp1. Scp1S185D bound to actin filaments in a high-speed actin pelleting assay (Figure 2.11C), cross-linked actin filaments in the low speed actin pelleting assay (unpublished observations), and increased light scattering of the actin filaments similar to wild type Scp1 (Figure 2.11D). In contrast, Scp1*S185A* had greatly diminished actin binding and cross-linking activities (Figure 2.11 C and D and unpublished observations). These results

Figure 2.11. Effects of Scp1 mutations on actin filament binding and cross-linking. (A) High speed actin filament co-sedimentation assay. Reactions contained 0 or 5μM yeast actin filaments and 5 μM Scp1: full-length His6-Scp1, amino terminus His6-Scp1N(1-136), or carboxyl terminus Scp1C(136-200) (designated WT, N, and C, respectively). Actin filaments were pelleted by high speed centrifugation and equivalent amounts of the pellet and supernatant were analysed by SDS-PAGE and Coomassie staining. (B) Low speed pelleting assay for actin filament cross-linking. 5μM actin filaments was mixed with 5μM Scp1 (His6-Scp1, His6-Scp1N(1-136), Scp1C(136-200)) or control buffer and centrifuged for 10 minutes at 10,000 x *g*. The pellets and supernatants were analyzed as above. (C) High speed actin filament co-sedimentation assay comparing wild type and mutant Scp1 proteins. 10 μM yeast actin filaments was mixed with 6 μM His6-Scp1 (wild type, S185A, or S185D mutant) or buffer alone. Actin filaments were pelleted by high speed centrifugation and pellets and supernatants were analyzed by SDS-PAGE and Coomassie staining. Lanes: 1, actin and Scp1; 2, actin and Scp1 *S185A*; 3, actin and Scp1 *S185D*; 4: actin alone. Note: proteolytic fragments of Scp1 are marked by asterisks. (D) Actin filament assembly and organization monitored by light scattering. Monomeric yeast actin (10 μM) was polymerized in the absence or presence of 5 μM wild type or mutant His6-Scp1 and monitored for change in light scattering (360 nm) over time.

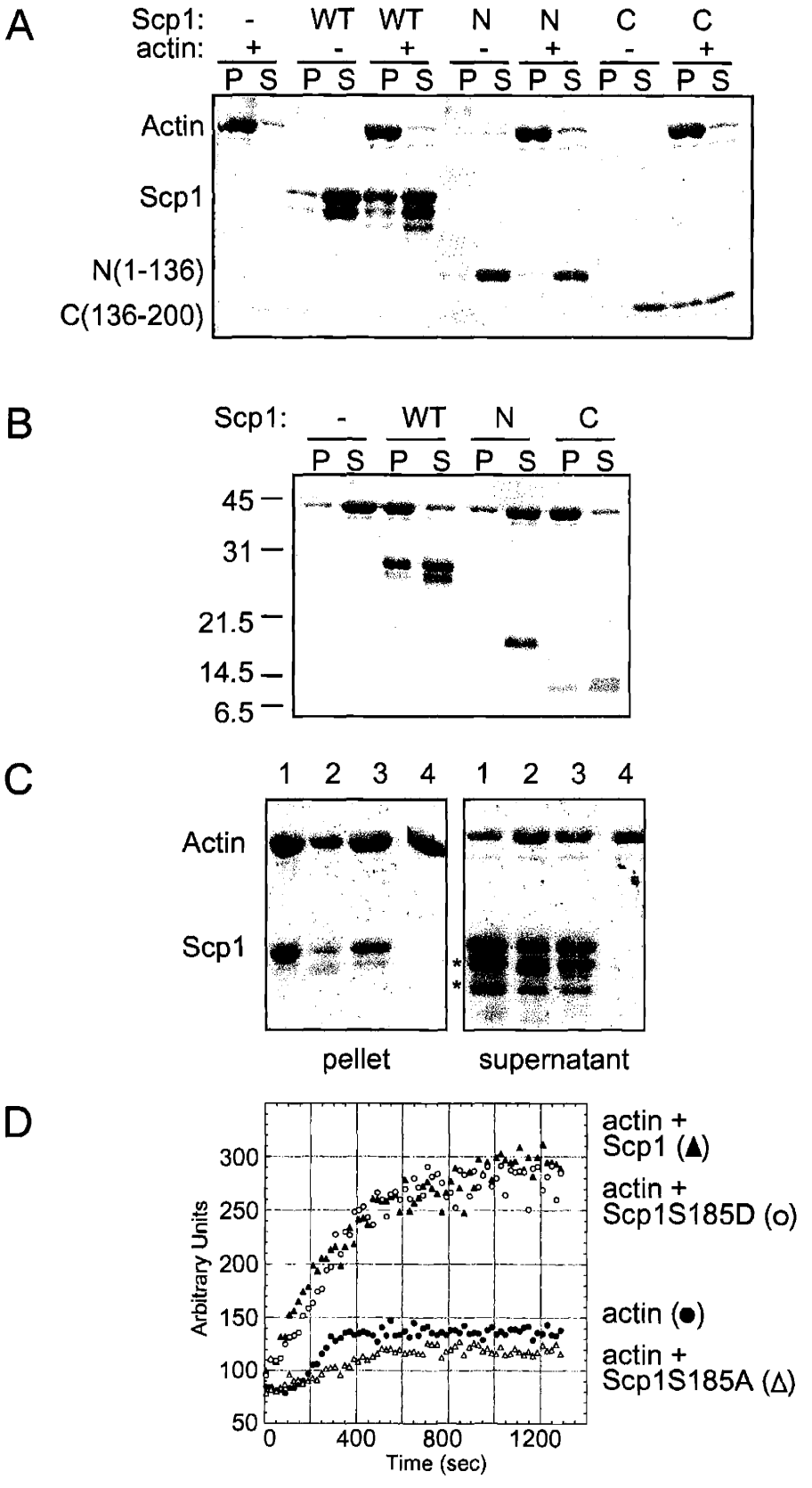

A
Scp1: - WT WT N N C C
actin: + - + - + - +
P S P S P S P S P S P S P S
Actin
Scp1
N(1-136)
C(136-200)
B
Scp1: - WT N C
P S P S P S P S
45
31
21.5
14.5
6.5
C
1 2 3 4 1 2 3 4
Actin
Scp1
pellet
supernatant
D
Arbitrary Units
300
250
200
150
100
50
0
400
800
1200
Time (sec)
actin + Scp1 (▲)
actin + Scp1S185D (o)
actin (●)
actin + Scp1S185A (Δ)

Figure 2.11

suggest that Scp1S185D retains much of the wild type Scp1 interaction with actin. However, Scp1S185D showed reduced actin binding affinity compared to wild type Scp1 at higher salt concentrations (150mM KCl; unpublished observations). Therefore, the Scp1S185D interaction with actin may be weakened, but not nearly to the extent of Scp1S185A.

Scp1, like fimbrin, decreases the rate of actin filament disassembly

Both Sac6/fimbrin (Bretscher, 1981; Adams et al., 1991) and Scp1 (this work) bind to and cross-link actin filaments; in addition, Sac6 stabilizes actin filaments against disassembly in vitro(see Figure 5 in Goode et al., 1999). To address whether Scp1 similarly can stabilize actin filaments, I compared pyrene-actin filament disassembly kinetics (see Chapter 1) in the presence and absence of His6-Scp1. In the absence of Scp1, actin filaments disassembled rapidly, and pyrene fluorescence reached steady state by 600 seconds (Figure 2.12A). His6-Scp1 reduced the rate of actin filament disassembly in a concentration dependent manner. Similar effects were observed using untagged Scp1 (not shown). Thus, Scp1, like Sac6/fimbrin, stabilizes actin filaments.

Scp1 and Sac6 compete for actin filament binding in vitro

Given that Scp1 and Sac6/fimbrin show genetic interactions and have similar biochemical activities on actin, I investigated whether they also have overlapping binding sites on actin filaments. I tested the ability of Scp1 to compete with Sac6 for binding to actin filaments in a high speed co-sedimentation assay, using constant concentrations of Sac6 (0.5μM) and actin filaments (2 μM) and variable concentrations of His6-Scp1 (0 to 7μM). In the absence of Scp1, nearly 90% of the Sac6 bound to actin filaments (Figure 2.12 B and C). The percentage of Sac6 bound to actin filaments decreased proportionally with increasing concentrations of Scp1. To test the specificity of the competition, I assayed Sac6 binding to actin in the presence of another actin binding protein, tropomyosin (Tpm1). A range of Tpm1 concentrations (1-10 μM)

Figure 2.12. Activities of Scp1 on actin filaments similar to Sac6/fimbrin. (A) Effects of His6-Scp1 on the rate of actin filament depolymerization. Actin filament disassembly was initiated by the addition of 20 μM latrunculin A to 2 μM preformed actin filaments (1% pyrene labeled) in the presence of 0, 67, 200, 670, 2000 nM His6-Scp1, and change in pyrene-actin fluorescence was monitored over time. (B) Competition of Scp1 with Sac6 for binding to actin filaments. Co-sedimentation assay using 2 μM yeast actin filaments and variable concentrations of His6-Scp1 (0, 0.35, 0.7, 1.75, and 7 μM). The pellets and supernatants were analyzed by SDS-PAGE and Coomassie staining. (C) The concentrations of Sac6 (✿) and Scp1 (✿) bound to actin filaments were calculated from gel densitometry and plotted versus the total concentration of His6-Scp1 in the reactions.

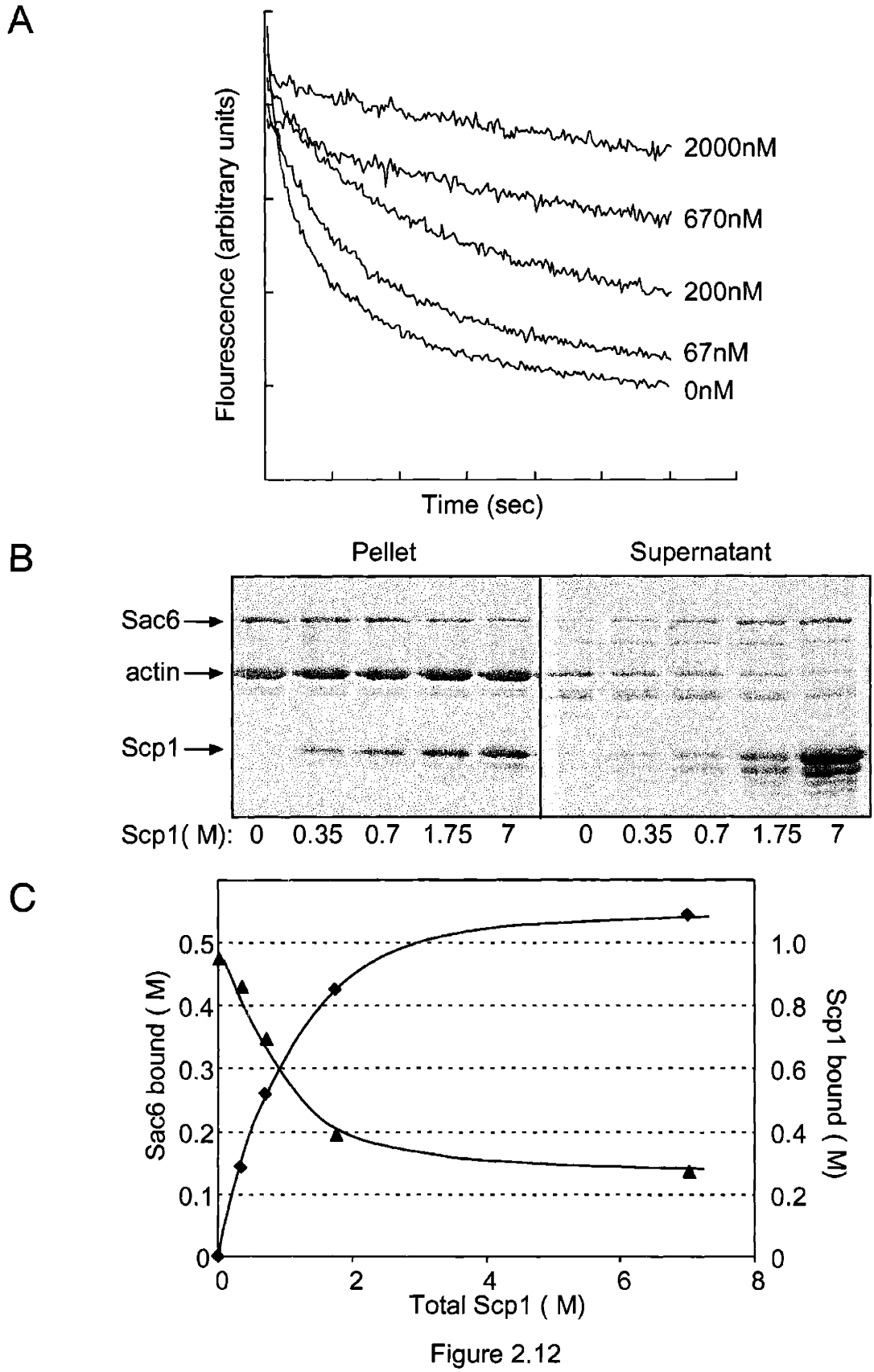

A
Flourescence (arbitrary units)
2000nM
670nM
200nM
67nM
0nM
Time (sec)
B
Pellet
Supernatant
Sac6
actin
Scp1
Scp1(M): 0 0.35 0.7 1.75 7 0 0.35 0.7 1.75 7
C
Sac6 bound (M)
Scp1 bound (M)
Total Scp1 (M)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.8
1.0
2
4
6
8

Figure 2.12

had no effect on Sac6 binding to actin filaments (not shown). Thus, Scp1 specifically competes with Sac6 for binding to actin.

DISCUSSION

Calponins and transgelins comprise one of the most widely conserved actin associated protein families, yet their in vivo function in non-muscle cells has remained elusive. Furthermore, controversy has surrounded the issue of whether transgelins even bind to actin filaments and/or localize to actin structures in vivo (reviewed in Small and Gimona, 1998; Morgan and Gangopadhyay, 2001). Here, I have shown unambiguously that the yeast transgelin homologue Scp1 binds directly to actin filaments, cross-links and stabilizes actin filaments in vitro, and localizes to actin filament structures in vivo. Further, I established that the sites on Scp1 necessary and sufficient for actin cross-linking reside in the carboxyl terminus, outside the CH domain. I also provided the first *in vivo* evidence for transgelin cellular function, showing that Scp1 cooperates with Sac6 (fimbrin) to organize and stabilize the actin cytoskeleton. Finally, mutant analysis revealed a correlation between in vivo phenotypes and in vitro activities on actin, demonstrating that actin binding is required for at least some in vivo functions of Scp1.

Activities of Scp1 on actin filaments *in vitro*

Scp1 binds directly to actin filaments with an affinity (Kd ~ 0.7μM) similar to that reported for other members of the calponin family: 1μM for calponin (Lu et al., 1995; Tang et al., 1996) and 1.3 and 1.4μM for transgelin (Shapland et al., 1993; Kobayashi et al., 1994). Scp1 also cross-links actin filaments. Previous studies have reported actin filament bundling for calponin (Kolakowski et al., 1995) and actin filament gelation for transgelin (Shapland et al., 1993). However, the ability of transgelin to cross-link actin filaments has been questioned (Morgan and Gangopadhyay, 2001), in part because the gelation activity occurs specifically in low ionic strength buffer and is blocked by the addition of 10mM KCl (Shapland et al., 1993). Using four independent assays, I showed that His6-

Scp1 and untagged Scp1 each cross-link actin filaments in buffer containing 50mM KCl. One possible explanation for the discrepancy between previous results and ours is that previous experiments tested transgelin and actin from different species, whereas our experiments used transgelin (Scp1) and actin from the same organism. This raises the possibility that other transgelins besides Scp1 also cross-link actin filaments.

By what mechanism does Scp1 cross-link actin filaments? Cross-linking requires either the presence of two actin-binding sites within a single polypeptide chain or dimerization of an actin binding protein. All of the data available for calponin family members suggest that they do not dimerize, because they behave as monomers in sedimentation velocity, sedimentation equilibrium, and gel filtration experiments (Lees-Miller et al., 1987b; Stafford et al., 1995). Similarly, I found no evidence for Scp1 dimerization using several methods: gel filtration, yeast two-hybrid assay, and co-immunoprecipitation of HA-tagged Scp1 with untagged Scp1 (unpublished observations). I can not rule out the possibility that Scp1 dimerizes (e.g. it may dimerize specifically when bound to actin). However, based on the available data, I speculate that Scp1 (and possibly other calponins) cross-link actin filaments via two distinct actin binding sites.

The location of the two sites required for actin filament cross-linking was revealed by the analysis of the mutant proteins. One actin binding site probably resides in the CLR (Figure 2.1B), because specific mutation of a single residue in the CLR (S185A) abolished actin filament cross-linking and greatly reduced actin binding affinity of Scp1. These results are in agreement with the previous studies that identified the analogous serine residue to be critical for actin binding of other calponin family members (Winder et al., 1993; Tang et al., 1996; Fu et al., 2000). These data also suggest that the mechanism of actin binding by calponins is highly conserved.

Analysis of the truncated Scp1 constructs revealed the location of a second site required for actin filament cross-linking. The amino-terminal Scp1 fragment His6-N(1-136) containing CH domain did not bind to actin filaments, whereas the carboxy-terminal fragment C(136-200) not only bound to actin

filaments, but also cross-linked them. It is formally possible that the hexa-histidine tag interfered with the actin binding of the amino-terminal fragment, yet it seems unlikely, given that the full length his-tagged Scp1 bound to actin filaments. In addition, single CH domains of mammalian calponin and transgelin are also not sufficient for in vitro binding to actin filaments (Gimona and Mital, 1998; Fu et al., 2000). The second actin binding site or dimerization site of Scp1 must reside in the sequences between the CH domain and the CLR. This site may be analogous to the actin binding site S1 of calponin (Figure 2.1A). While the sequence of S1 is not conserved among calponin isoforms or in transgelins (Gimona and Mital, 1998), this region is enriched in positively charged amino acids in all calponin family members. Mutations of the positively charged amino acids in this region decrease actin binding affinity of calponin and transgelin (Gong et al., 1993; Fu et al., 2000). Further mutational analysis of Scp1 will be required for precise identification of the residues required for cross-linking of the actin filaments.

Does the CH domain of Scp1 contribute to actin binding? The data in Figure 2.12 show clearly that the CH domain is neither sufficient nor necessary for actin filament binding by Scp1, yet Scp1 competes for actin binding with Sac6/fimbrin, which binds actin via two tandem pairs of CH domains. This apparent discrepancy may be explained by a recently proposed model. Based on comparing cryo-EM reconstructions of calponin and fimbrin decorated actin filaments, it was suggested that the CH domain of calponin may serve as a 'locator' domain, helping to position the true actin binding motifs in calponin (reviewed in Winder, 2003). The CH domain of Scp1 may act similarly.

Scp1 functions with Sac6 to regulate the actin cytoskeleton in vivo

Genetic and biochemical data, as well as sub-cellular localization, reveal a functional relationship between Scp1 and Sac6/fimbrin. Both GFP-Scp1 (this work) and Sac6 (Drubin et al., 1988) localize to cortical actin patches. Sac6 was also reported to co-localize faintly with actin cables by immunofluorescence; however, this was not observed with GFP-Sac6 (Doyle and Botstein, 1996).

Therefore, it is possible that Scp1 localizes in vivo to both actin patches and cables, but that I have only been able to detect patch localization with GFP-Scp1. Biochemical analyses show that Scp1 and Sac6 have similar activities on actin. Like Sac6/fimbrin, Scp1 cross-links actin filaments in vitro. Sac6 cross-links actin filaments into tight bundles, and Scp1 cross-links actin into loose bundles and networks. Scp1, like Sac6/fimbrin, not only organizes actin filaments, but also decreases the rate of actin filament disassembly (filament stabilization). The shared role of *SAC6* and *SCP1* in stabilizing the actin cytoskeleton is supported further by the latrunculin A sensitivities of *sac6Δ* and *scp1Δ* mutant cells. Taken together, these in vitro and in vivo observations suggest that Scp1 and Sac6 cooperate in organizing and stabilizing the actin cytoskeleton.

The overlapping genetic functions of *SCP1* and *SAC6* may be related to their relative abundance in cells and their ability to compete for actin binding. Using quantitative immunoblotting, I defined the in vivo molar ratios of actin, Sac6, and Scp1 to be approximately 65:6:1 (actin to Sac6 to Scp1). The higher levels of Sac6 compared to Scp1 suggest that Sac6 may provide the more 'dominant' activity on actin. This idea is supported by the relative strengths of their respective null phenotypes. Further, this could explain why as little as 2-4 fold higher expression of Scp1 partially suppresses defects in *sac6Δ* cells.

To demonstrate the importance of the Scp1-actin interaction for in vivo functions, I have used a mutant of Scp1 that has a weak affinity for actin filaments in vitro. *scp1S185A* failed to suppress loss of *SAC6* or loss of *SCP1* function in a *sac6Δ* background. On the other hand, *scp1S185D* mutant, which retained actin filament binding in vitro, suppressed the phenotypes associated with the loss of *SCP1* and *SAC6* in vivo. These results provide strong evidence that Scp1-actin interactions are required for in vivo functions of Scp1 shared with Sac6.

A functional relationship between calponins and fimbrin may be conserved in other organisms. Both protein families are widely expressed in different vertebrate tissues and have overlapping sub-cellular locations. In fibroblasts, fimbrin and calponin are both found on stress fibers (Babb et al., 1997; Jiang et

al., 1997; Messier et al., 1993; Shapland et al., 1988), where they might function together to regulate actin cross-linking and stabilization. In addition, fimbrin and calponin may play a role in adhesive actin structures, linking the actin cytoskeleton to the cell membrane. Fimbrin is found at focal adhesions and podosomes (Lin et al., 1993; Messier et al., 1993; Babb et al., 1997), and calponin is found in dense plaques, a type of adherence junction similar to podosomes and focal adhesions (North et al., 1994). Finally, it has been proposed that fimbrin may link the actin cytoskeleton to the vimentin intermediate filament cytoskeleton, and a vimentin-binding site has been mapped to the first CH domain of fimbrin (Correia et al., 1999). A similar function for calponin has been suggested by in vitro binding studies and overlapping in vivo localization of desmin and calponin (Mabuchi et al., 1996; North et al., 1994; Wang and Gusev, 1996). Thus, calponin and fimbrin may have shared in vivo functions that are conserved across a wide range of organisms.

MATERIALS AND METHODS

Strains and growth conditions

The yeast strains used in this study are listed in table 2.3. Standard methods were used for growing and manipulating yeast (Guthrie and Fink, 1991). To generate AGY189, the coding regions of *SAC6* and *SCP1* in AGY20 were replaced with *LEU2* and *HIS3*, respectively. AGY189 was sporulated, and resulting haploid strains (AGY 195, 196, 197, and 198) were crossed to the congenic strains of opposite mating type to generate AGY490, AGY491, AGY492 and AGY493. For growth assays, homozygous diploid strains carrying vectors (pRS313, pRS314, pRS315, pRS316) and/or plasmids (Table 2.4) were grown to log phase, then serially diluted 5 fold, spotted on plates, and grown for an additional 2-3 days. Latrunculin A sensitivity of cells was measured by halo assays as previously described (Ayscough et al., 1997).

Plasmid construction

The coding region of the *SCP1* gene (*YOR367w*), plus 397 bases of sequence upstream of the translation start site, was amplified by PCR from wild type yeast genomic DNA. The PCR product was cloned into the *ClaI* and *SmaI* sites of pRS316 (Sikorski and Hieter, 1989), generating pAG20. For additional *SCP1* constructs, I introduced by site directed mutagenesis (Quick Change™ Kit; Stratagene Inc.; La Jolla, CA) a *BglII* site at the start codon of *SCP1* in pAG20, generating pAG3. To construct an amino terminal GFP-*SCP1* fusion plasmid (pAG9), I cloned GFP as a *BamHI* fragment from plasmid B3355 (Fink lab collection) into the *BglII* site of pAG3. To generate an *E. coli* expression amino terminal hexahistidine-*SCP1* fusion construct (pAG22), *SCP1* was excised from pAG3 as a *BglII-XhoI* fragment and cloned into the *BamHI* and *XhoI* sites of pTrcHisA (Invitrogen; Carlsbad, CA). To generate *SCP1* Gal-overexpression plasmids (pAG179), the *BglII-XhoI SCP1* fragment was cloned into the *BamHI* and *XhoI* sites of pRS426Gal1 (Christianson et al., 1992). Point mutations in *SCP1* (S185A and S185D) were generated by site-directed mutagenesis as above. To generate N136 and 136C constructs, sequences coding for the

Table 2.3 Strains used in chapter 2.

Name	Genotype	Source
AAY1918	*MATa, ura3, trp1, leu2, his3, prb1, can1, sac6::LEU2, pep4::HIS3, pGal10-SAC6/CEN/URA*	Sandrock et al., 1997
AGY20	*MATa/MATα, his3Δ200/his3Δ200, leu2-3,112/ leu2-3,112, ura3-52/ura3-52, trp1::HisG/trp1::HisG*	Fink lab
AGY189	*MATa/MATα, his3Δ200/his3Δ200, leu2-3,112/ leu2-3,112, ura3-52/ura3-52, trp1::HisG/trp1::HisG, SAC6/sac6Δ::LEU2, SCP1/scp1Δ::HIS3*	This study
AGY195	*MATa, his3Δ200, leu2-3,112, ura3-52, trp1::HisG*	This study
AGY196	*MATa, his3Δ200, leu2-3,112, ura3-52, trp1::HisG, sac6ΔLEU2*	This study
AGY197	*MATa, his3Δ200, leu2-3,112, ura3-52, trp1::HisG, scp1Δ::HIS3*	This study
AGY198	*MATa, his3Δ200, leu2-3,112, ura3-52, trp1::HisG, sac6Δ::LEU2, scp1Δ::HIS3*	This study
AGY490*	*MATa/MATα, SAC6/SAC6, SCP1/SCP1*	This study
AGY491*	*MATa/MATα, sac6Δ::LEU2/sac6Δ::LEU2, SCP1/SCP1*	This study
AGY492*	*MATa/MATα, SAC6/SAC6, scp1Δ::HIS3/scp1Δ::HIS3*	This study
AGY493*	*MATa/MATα, sac6Δ::LEU2/sac6Δ::LEU2, scp1Δ::HIS3/scp1Δ::HIS3*	This study
BJ2168	*MATa, pep4-3, prb1-1122, prc1-407, trp1, ura3-52, leu2, gal2*	Jones, 2002
L4852	*MATa, ura3-1, leu2-3,112, his3-11,15, ade2-1, trp1-1, can1-100, GAL+*	Fink lab

* strains have the same genotype as AGY189, except at *SAC6* and *SCP1* loci.

Table 2.4 Plasmids generated in chapter 2.

Name	Insert	Vector
pAG3	*scp1(BglII)*	pRS316
pAG9	*GFP-SCP1*	pRS316
pAG16	*scp1S185A*	pRS316
pAG17	*scp1S185D*	pRS316
PAG18	*scp1 P156A, P159A*	PRS316
pAG20	*SCP1*	pRS316
pAG21	*SCP1*	pRS314
pAG22	*SCP1*	pTrcHisA (Invitrogen)
pAG37	*scp1S185D*	pTrcHisA (Invitrogen)
pAG50	*scp1S185A*	pTrcHisA (Invitrogen)
pAG179	*SCP1*	pRS426Gal1
pAG200	*scp1Δ12*	pRS314
pAG201	*scp1Δ28*	pRS314
pAG202	*scp1N(1-136)*	pProEX™HTa(GibcoBRL)
pAG203	*scp1C(136-200)*	pBAT4

Table 2.5 Oligonucleotides used for site-directed mutagenesis of Scp1 in Chapters 2 and 3

Name	Sequence (5'→3')	Resulting mutation
CPO5	CGGTTATATGAAAGGTGCA**G**CTCAG GCTACTGAAGGAGTGG	S185A
CPO6	CCACTCCTTCAGTAGCCTGAG**C**TG CACCTTTCATATAACCG	S185A
CPO7	CGGTTATATGAAAGGTGCA**GA**TCA GGCTACTGAAGGAGTGG	S185D
CPO8	CCACTCCTTCAGTAGCCTGA**TC**TG CACCTTTCATATAACCG	S185D
CPO9	GGACC ACAAC TGTCA ACAAA GAAG**G** CAAGA CCC**GC** TGTTA AGTCT AAACC AAAAC ATCTAC	P156A,P159A
CPO10	GTAGAT GTTTT GGTTT AGACT TAACA GCGGG TCTTG CCTTC TTTGT TGACA GTTGT GGTCC	P156A,P159A
CPO19	GGTGCATCTCAGGCTACTTAAGGA GTGGTGTTAGGACAACGG	Δ12
CPO20	CCGTTGTCCTAACACCACTCCTTA AGTAGCCTGAGATGCACC	Δ12
CPO21	CTACAAGATGGTACTGGATGAAGC ACTTTTGAATACGG	Δ28
CPO22	CCGTATTCAAAAGTGCTTCATCCA GTACCATCTTGTAG	Δ28
CAL3	G CGTAC AGCTAA AGTTT ACCGA GATCT TACGA TAAGA AGGC	*BglII* site at start
CAL4	GCCT TCTTA TCGTA AGATC TCGGT AAACT TTAGCT GTACG C	*BglII* site at start

amino-terminus and carboxyl-terminus of Scp1 were amplified by PCR and cloned into *NcoI* and *HindIII* sites of pProET™HTa (GibcoBRL) and pBAT4 (Peranen et al., 1996). All mutant *scp1* constructs were sequenced to verify that no additional mutations had been introduced.

Protein purification

Yeast actin was purified as previously described (Goode et al., 1999). His6-tagged Scp1 proteins were expressed in BL21/DE3 *E.coli* and purified on nickel resin as per manufacturer's instructions (Qiagen; Valencia, CA). Peak fractions eluted from the nickel column were pooled and fractionated on a monoQ (5/5) column using an AKTA FPLC (Amersham; Piscataway, NJ). Peak fractions were pooled, concentrated in a centricon 10 device (Millipore; Bedford, MA), and exchanged into $HEKG_5$ buffer [20 mM Hepes (pH 7.5), 1 mM EDTA, 50 mM KCl, 5% glycerol]. The proteins were aliquoted, frozen in liquid nitrogen, and stored at –80°C. Untagged carboxy-terminal fragment of Scp1 was expressed in *E. coli*. Cells were lysed in HEKG5 buffer using french press, and the lysate was clarified by centrifugation at 313,000x*g* at 4°C for 30 min (high-speed supernatant; HSS). HSS was fractionated on a 1ml HiTrap SP column (Amersham). Peak fractions were pooled, diluted in low salt buffer, and fractionated on a Mono S column. Peak fractions were concentrated and fractionated on a Superdex 75 (5/30) column (Amersham) equilibrated in $HEKG_5$ buffer. Peak fractions were pooled concentrated, aliquoted, frozen in liquid nitrogen, and stored at –80°C. Untagged full-length Scp1 was purified from yeast overexpressing *SCP1* (BJ2168 carrying pAG179). One liter of cells was grown to mid-log phase in SC-His medium with 2% raffinose. Then, 2% galactose was added to the medium, and cells were grown for an additional 12 hours and harvested by centrifugation. The cell pellet was resuspended in 0.3 volume of water and frozen in droplets in liquid nitrogen. Next, the frozen yeast cells were lysed in a coffee grinder using liquid nitrogen, described under 'lab protocols' on the Goode lab web page: www.bio.brandeis.edu/goodelab. A HSS was generated in $HEKG_5$ buffer supplemented with 0.5mM DTT and protease inhibitors as previously described

(Goode et al., 1999). The HSS was fractionated on a 1 ml HiTrap SP column (Amersham) and proteins were eluted with a linear salt gradient (50-500 mM KCl). Scp1 eluted at approximately 200mM KCl. Peak fractions were pooled and concentrated to 3 ml in a centricon 10 device, then fractionated on a Superdex 75 (26/60) column (Amersham) equilibrated in $HEKG_5$ buffer. Peak fractions were pooled, concentrated as above, aliquoted, frozen in liquid nitrogen, and stored at –80°C. Sac6 was purified as above for untagged Scp1 with the following exceptions: AAY1918 strain was used for galactose induction; after the HSS was fractionated on a HiTrap Q column, the Sac6-containing fractions were pooled, desalted, and fractionated on a monoQ (5/5) column. Peak fractions were pooled, concentrated in centricon 10 devices, and fractionated on a Superose12 (5/30) gel filtration column (Amersham) equilibrated in $HEKG_5$ buffer. Sac6 peak fractions were pooled, concentrated, aliquoted, frozen in liquid nitrogen, and stored at –80°C. Tpm1 was purified as previously described (Liu and Bretscher, 1989).

Actin filament binding and cross-linking assays

Yeast actin was assembled as follows. Actin (50 µM) in G-buffer [10 mM Tris (pH 7.5), 0.2 mM CaCl2, 0.2 mM DTT, 0.2 mM ATP] was thawed on ice, pre-cleared by centrifugation, and 20X initiation mix [10 mM ATP, 40 mM MgCl2, and 1 M KCl] was added to induce polymerization. Reactions were incubated for 1 hour at 25°C. Then, actin filaments were diluted in F-buffer [10 mM Tris (pH 7.5), 0.2 mM CaCl2, 0.2 mM DTT, 0.7 mM ATP, 2 mM MgCl2, and 50 mM KCl], purified proteins (Scp1, Sac6, Tpm1) and/or HEKG5 buffer were added, and the reactions were incubated at room temperature for 1 hour. For low speed pelleting assays, reactions were centrifuged for 10 minutes at 10,000 x *g*, 4°C. For high speed pelleting assays, actin filaments were pelleted by centrifugation for 30 minutes at 313,000x*g* in a TLA100 rotor (Beckman; Palo Alto, CA). In both assays, supernatants and pellets were fractionated on SDS-PAGE gels, stained with Coomassie, and bands were quantified by densitometry using NIH-Image (version 1.61, available at sippy.nimh.nih.gov). The binding constant (Kd) of Scp1

for actin filaments was defined as the concentration of Scp1 at which half maximal Scp1 binding occurred. For light scattering assays, yeast actin was thawed on ice, diluted in G-buffer, and mixed with 20X initiation mix plus Scp1 and/or HEKG5 buffer. Light scattering was monitored over time at 360 nm in a fluorescence spectrophotometer (Photon Technology International; Lawrenceville, NJ) held at a constant temperature of 25°C. Apparent viscometry of actin solutions was measured by the falling ball assay, performed as previously described (Pollard and Cooper, 1982). Rabbit muscle G-actin (Cytoskeleton) was clarified by centrifugation for 30 minutes at 313,000x*g* 4°C in a TLA100 rotor. Capillary tubes were loaded with actin (4.2μM), initiation salts, and varying concentrations of His6-Scp1 (0, 0.23, 0.46, 0.93, 1.4, 1.8, 3.7 μM) and incubated at room temperature for one hour before falling ball measurements. For electron microscopy, 2μl of reactions were spotted onto freshly ionized carbon-coated grids, stained with 1% uranyl acetate, and visualized using a Phillips EM410 transmission electron microscope.

Actin filament disassembly kinetics

Yeast actin (with 1% pyrene labeled rabbit skeletal muscle actin; Cytoskeleton Inc.; Denver, CO) was assembled at 35 μM as above. 7 μl of actin filaments in F-buffer was mixed with 52.5 μl F-buffer and 7 μl of Scp1 and/or HEKG5 buffer and incubated for 10 minutes at room temperature. Actin filaments were agitated by vortexing for 10 seconds, then mixed with 3.5 μl of latrunculin A (400 μM) in a cuvette. The final reactions contained 3.5 μM actin, 20 μM latrunculin A, and variable concentrations of Scp1 (0-2 μM). The depolymerization kinetics of pyrene-labeled actin filaments was monitored by excitation at 365 nm and emission at 407 nm in a fluorescence spectrophotometer held at a constant temperature of 25°C.

Fluorescence Light Microscopy

Images of cells were acquired using a Nikon TE300 inverted fluorescence microscope equipped with a Hamamatsu Orca CCD camera controlled by

Openlab software (Improvision, Inc.). The localization pattern of GFP-Scp1 fusion protein was examined in live yeast cells grown to log phase. To disrupt the actin cytoskeleton, cultures were treated with 200 μM latrunculin A for 5 minutes prior to imaging (Figure 2.2A). Co-localization of GFP-Scp1 and actin (Figure 2.2B) was performed essentially as described (Warren et al., 2002). Briefly, 1 ml of exponentially growing cells was fixed with 70% ethanol on ice for 10 minutes, and cells were pelleted by centrifugation at 3,000 x *g* and resuspended in 100 μl PBS buffer plus 1mg/ml BSA and 10 μL rhodamine-phalloidin (Molecular Probes, 300U in 1.5 ml methanol). After incubation on ice for 5 minutes, cells were washed three times in PBS buffer and mounted on a slide for imaging.

Antibody preparation and immunoblotting

Antibodies were generated by immunizing rabbits with either amino-terminal (DKKADVTSLDEDLRQGCL) or carboxy-terminal (GASQATEGVVLG QRRDIVGCL) synthetic peptides of Scp1 to generate anti-Scp1N and anti-Scp1C antibodies respectively (Covance Inc., Denver, CO). Antibodies were affinity purified with His6-Scp1 using QuickPure system (Sterogene). Both crude sera and affinity purified anti-Scp1N antibody recognized 25kDa band which was absent in protein extracts from *scp1*Δ cells and an additional 30kDa band which was present in *scp1*Δ cells and, therefore, was non-specific. AntiScp1C antibody also recognized non-specific epitopes in yeast cell extracts in addition to a weak 25kDa band. For immunoblotting, anti-Scp1N (0.3mg/ml) and anti-Scp1C (0.1mg/ml) antibodies were used at 1:1000 dilution. Anti-actin and anti-sac6 antibodies were a gift from B. Goode and were used at 1:1000 dilution.

For western blotting, 10 OD units of freshly grown yeast cells were harvested and lysed at 4°C using glass beads and buffer A (50mM HEPES pH7.5, 150mM KCl, 1mM EGTA, 1mM EDTA, 10% glycerol, 0.03% octyl-β-glucoside) supplemented with 1mM PMSF and 1X protease inhibitor coctail (Calibrochem). Protein extracts were clarified by centrifugation (20 min, 12,000x*g*), and protein concentration was determined using BCA assay (Pierce).

Protein extracts, along with purified protein standards, were separated by SDS-PAGE and analyzed by immunoblotting; the signal was detected using enhanced chemiluminescence (ECL; Amersham).

Two-dimensional gel electrophoresis

Protein extracts were prepared as described for immunoblotting and sent to Kendrick Laboratories for electrophoresis.

CHAPTER 3. SCP1 FUNCTIONS IN COMPLEX WITH ABP1.

BACKGROUND

In the previous chapter, I characterized biochemical and genetic functions of Scp1 and established that mutation of a single serine (S185) in the carboxyl terminus disrupts both actin binding and in vivo function of Scp1. I also determined that actin filaments were required for in vivo patch localization of Scp1. If Scp1 acts on actin alone, then the mutant Scp1 defective for actin binding (S185A) would be predicted to mis-localize in vivo. Surprisingly, the S185A Scp1 mutant localizes to cortical actin patches similar to the wild type Scp1 (see below). This observation prompted me to consider whether Scp1 might be anchored to the cortical actin patches by another protein. Several Scp1-interacting proteins were identified in a yeast two-hybrid screen (Brian Cali, unpublished observations; see Appendix 2). Among these proteins was the actin binding protein Abp1.

Abp1, a protein of 592 amino acids, contains multiple domains involved in interactions with actin filaments and actin-associated proteins (Figure 3.1A). Abp1 binds to actin filaments via its amino terminal ADF/cofilin homology domain (amino acid residues 1-144; Goode et al., 2001). At positions 200 and 435, Abp1 contains two acidic sequences involved in binding to the Arp2/3 complex (Goode et al., 2001). The proline-rich region of Abp1 (residues 448-533) interacts with the SH3 domain of another actin-binding protein Rvs167/amphyphysin, which functions in endocytosis (Lila and Drubin, 1997; Munn et al., 1995). Finally, the SH3 domain of Abp1 (amino acid residues 533-592) interacts with multiple binding partners, including several proteins that function in endocytosis and/or actin cytoskeleton organization (Srv2, Myo5, Ark1, Prk1, and Inp52; Cope et al., 1999; Fazi et al., 2002; Lila and Drubin, 1997).

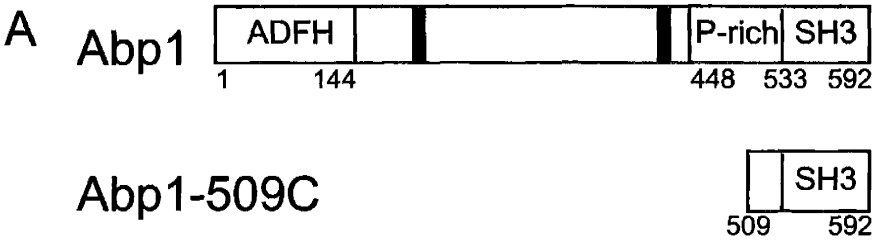
Abp1 was the first actin-associated protein identified in yeast (Drubin et al., 1988), but until recently, its in vivo functions were unknown. Abp1 localizes to cortical actin patches (Drubin et al., 1988). Strains carrying deletion of this gene on its own exhibit no obvious phenotype; however, *abp1*Δ synthetic lethal in

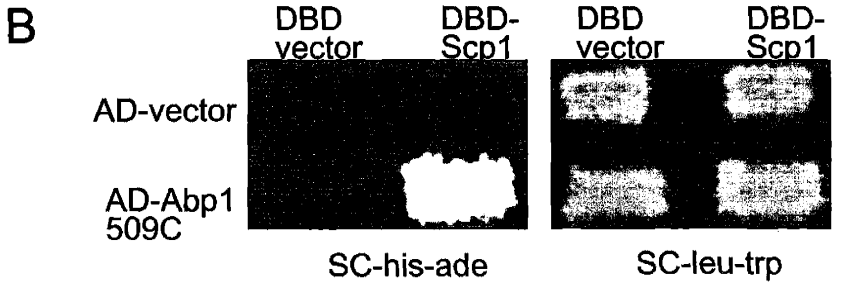
combination with deletions of other genes with actin-related functions (*SAC6*, *SLA1*, *SLA2*, and *PRK1*; Cope et al., 1999; Holtzman et al., 1993). Based on biochemical and genetic interactions, two functions have been proposed for Abp1. It functions in endocytosis and activates the Arp2/3-mediated actin nucleation (Goode et al., 2001; Lila and Drubin, 1997). Since the Arp2/3 complex is also involved in endocytosis (via a mechanism that remains unclear), the two functions of Abp1 may be related to each other. To understand how the functions of Scp1 may be linked with those of Abp1, I investigated the potential Scp1-Abp1 interaction suggested by the yeast two-hybrid screen.

RESULTS

Scp1 interacts with Abp1 by the yeast two-hybrid assay.

The carboxyl terminus of Abp1 (amino acids 509-592) was identified in a yeast two-hybrid screen with Scp1 (Brian Cali, unpublished observations). This fragment (Abp1-509C) contains a small portion of the proline-rich region and an entire SH3 domain of Abp1 (Figure 3.1A). Abp1-509C interacted specifically with Scp1 and not with the vector control strain (Figure 3.1B). It seemed likely that the SH3 domain of Abp1 was involved in the interaction with Scp1. Examination of the Scp1 sequence revealed a consensus SH3-binding sequence PXXP. I mutated the critical prolines of the consensus sequence to alanines (P156A, P159A) and tested the ability of this mutant, along with several other mutant Scp1 constructs, to interact with Abp1-509C in the yeast two-hybrid assay (Figure 3.1C). Scp1 carrying a point mutation in serine-185 was indistinguishable from the wild type Scp1. Mutation of the putative SH3-binding site (P156A, P159A) abolished the yeast two-hybrid interaction between Scp1 and Abp1. Deletion of the carboxy-terminal half of Scp1 (N118), containing the putative SH3-binding site, also abolished the interaction. On the other hand, nearly complete deletion of the amino terminal CH domain of Scp1 (121C) greatly enhanced the yeast two hybrid interaction with Abp1-509C. Therefore,





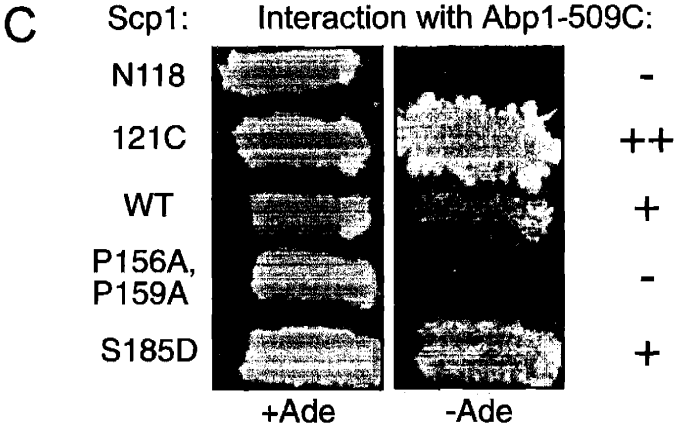


Figure 3.1 Scp1p interacts with the carboxyl terminus of Abp1p by the yeast two hybrid assay. A. Schematic representation of domain organization of Abp1p and truncated construct Abp1-509C identified in a yeast two hybrid screen with Scp1 as a bait. B. Yeast two hybrid interaction of Scp1 with Abp1(509C). Strains were grown in synthetic complete (SC) media or in SC media lacking histidine and adenine. C. Yeast two hybrid interaction of Scp1 mutants with Abp1(509C). Strains were grown in SC media or in SC media lacking adenine.

Scp1 interacts specifically with the carboxyl terminus of Abp1 and mutations in Scp1 alter the strength of this interaction.

Scp1 co-immunoprecipitates with Abp1 from the cell extracts.

To test whether the interaction between Scp1 and Abp1 can be observed in cell extracts, I overexpressed HA tagged Scp1 and V5-tagged Abp1 in the W303 yeast strain background and prepared cell extracts from these cells. Immunoprecipitation of Abp1 with anti-V5 antibody resulted in co-precipitation of Scp1; however, in the converse experiment, V5-Abp1 did not co-immunoprecipitate with anti-HA antibody and HA-Scp1 (Figure 3.2A). I tested the ability of the Scp1 carrying mutation in the putative SH3 binding site (P157A, P159A) to co-precipitate with Abp1. Although this mutation abolished the yeast two-hybrid interaction between Scp1 and Abp1, it had only a modest effect in the co-immunoprecipitation experiment (Figure 3.2B). Therefore, mutation of Pro-156 and Pro-159 is not sufficient to disrupt physical interaction between the full length Abp1 and Scp1 in yeast extracts.

Localization of Scp1 requires either its interaction with actin filaments or with the SH3 domain of Abp1.

To test whether the localization of Scp1 to cortical actin patches required Abp1, I expressed GFP fusions of Scp1 from a low copy plasmid (pAG9) in *ABP1* and *abp1*Δ mutant cells. To enhance the GFP signal, endogenous *SCP1* was deleted in these stains. In both cases, GFP-Scp1 localized to cortical actin patches (Figure 3.3A). Next, I tested whether mutations in *SCP1* affected patch localization of the GFP fusion. Mutation of the putative SH3 binding site (P157A, P159A) or serine-185 to aspartic acid (S185D) had no effect on localization of GFP-Scp1 (not shown). These mutations also exhibited wild type phenotypes in suppression assays (see chapter 2). However, mutation of serine-185 to alanine localized to patches in the wild type cells (Figure 3.3A), but not in the *abp1*Δ cells. This mutation disrupted in vitro actin filament binding and in vivo function

of Scp1 (see chapter 2). These results suggested that localization of Scp1 requires interactions of Scp1 either with actin filaments or with Abp1.

To test whether the SH3 domain of Abp1 was required for localization of the mutant Scp1, I examined GFP-Scp1 localization in the yeast cells carrying either a deletion of the SH3 domain (Abp1ΔSH3) or a point mutation W569A in the SH3 domain (Abp1 SH3*). This point mutation was previously shown to disrupt interactions of the Abp1 SH3 domain with its ligands (Fazi et al., 2002). As expected, wild type GFP-Scp1 localized to cortical patches in all strains tested, whereas the GFP-Scp1 S185A mutant failed to localize to actin patches when the SH3 domain of Abp1 was either missing or mutated (Figure 3.3B). Therefore, the SH3 domain of Abp1 is required for localization of the mutant Scp1 that is compromised for actin filament binding.

Scp1 inhibits Abp1-dependent activation of the Arp2/3 complex.

Abp1 activates nucleation activity of the Arp2/3 complex, thereby modulating the kinetics of actin filament assembly (Goode et al., 2001) . I tested whether Scp1 can alter this activity of Abp1 in a pyrene-actin assembly assay described in chapter 1 (Figure 1.3). As expected, in the absence of the Arp2/3 complex, actin polymerization was preceded by a long lag phase, corresponding to slow spontaneous actin nucleation (Figure 3.4, black curve). In the presence of the Arp2/3 complex, the lag phase was reduced, reflecting a basal nucleation activity of the Arp2/3 complex (Figure 3.4, green curve). Addition of Abp1 to actin polymerizing in the presence of the Arp2/3 complex further reduced the lag phase, reflecting activation of the Arp2/3 complex by Abp1 (Figure 3.4A, blue curve). This lag was increased when Scp1 was added the reaction containing actin polymerizing in the presence of Abp1 and Arp2/3 complex (Figure 3.4A, red curve). This result is consistent with the role of Scp1 in inhibiting Abp1-dependent activation of the Arp2/3 complex. To test whether this activity of Scp1 was specific for Abp1, we tested another activator of the Arp2/3 complex, Las17/WASP. The WA fragment of Las17 (amino acids 528-633) and Abp1

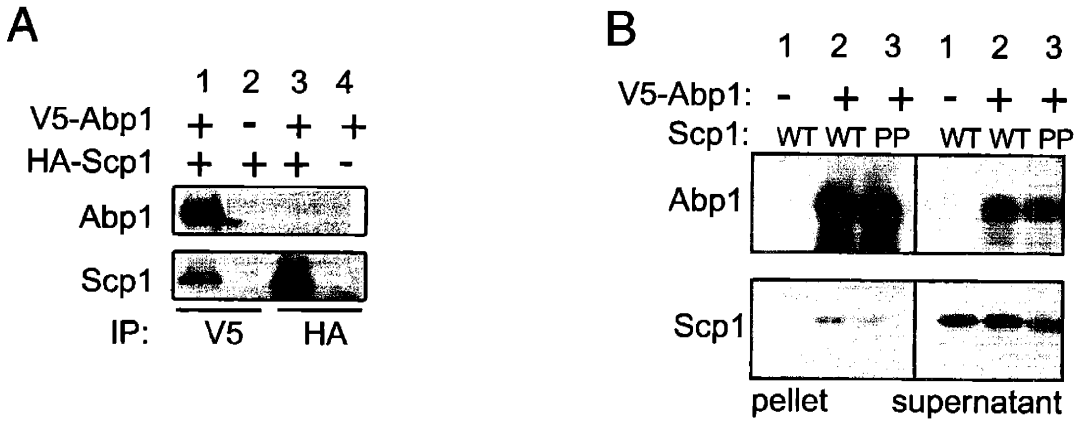


Figure 3.2 Co-immunoprecipitation of Scp1p with Abp1p. A. Western blot of proteins pelleted by immunoprecipitation. Protein extracts from the strains expressing either HA-Scp1 (lane 2) or V5-Abp1 (lane 4) or both (lanes 1 and 3) were used for immunoprecipitation with anti-V5 (lanes 1 and 2) or anti-HA (lanes 3 and 4) antibodies. Proteins in the pellet were detected by immunoblotting with anti-V5 (top) or anti-HA antibodies. B. Protein extracts from AGY322 (1), AGY425 (2) and AGY427 (3) were used for immunoprecipitation with anti-V5 antibody. Proteins in the pellet and supernatant were detected by western blotting with anti-V5 (top) or anti-Scp1N (bottom) antibodies.

stimulate Arp2/3 nucleation of the actin assembly to a similar extent (blue curves in Figure 3.4A and B). Addition of Scp1 to actin polymerizing in the presence of Arp2/3 and the WA fragment of Las17 had no effect on kinetics of actin polymerization (Figure 3.5B, red curve). Therefore, Scp1 specifically modulates the activation of the Arp2/3 complex by Abp1.

Figure 3.3 Localization of Scp1 mutant depends on Abp1. A. Localization of Scp1 in the presence and absence of Abp1. Yeast strains AGY197 and AGY390 expressing either GFP-Scp1 (pAG71) or GFP-Scp1S185A (pAG72) were grown to mid-log phase, observed and photographed. B. The SH3 domain of Abp1 is required for localization of Scp1 mutant compromised in actin binding. Yeast strains BGY628, BGY630 and BGY631 expressing either GFP-Scp1 (pAG71) or GFP-Scp1S185A (pAG72) were grown to mid-log phase, observed and photographed. Bar, 5μm.

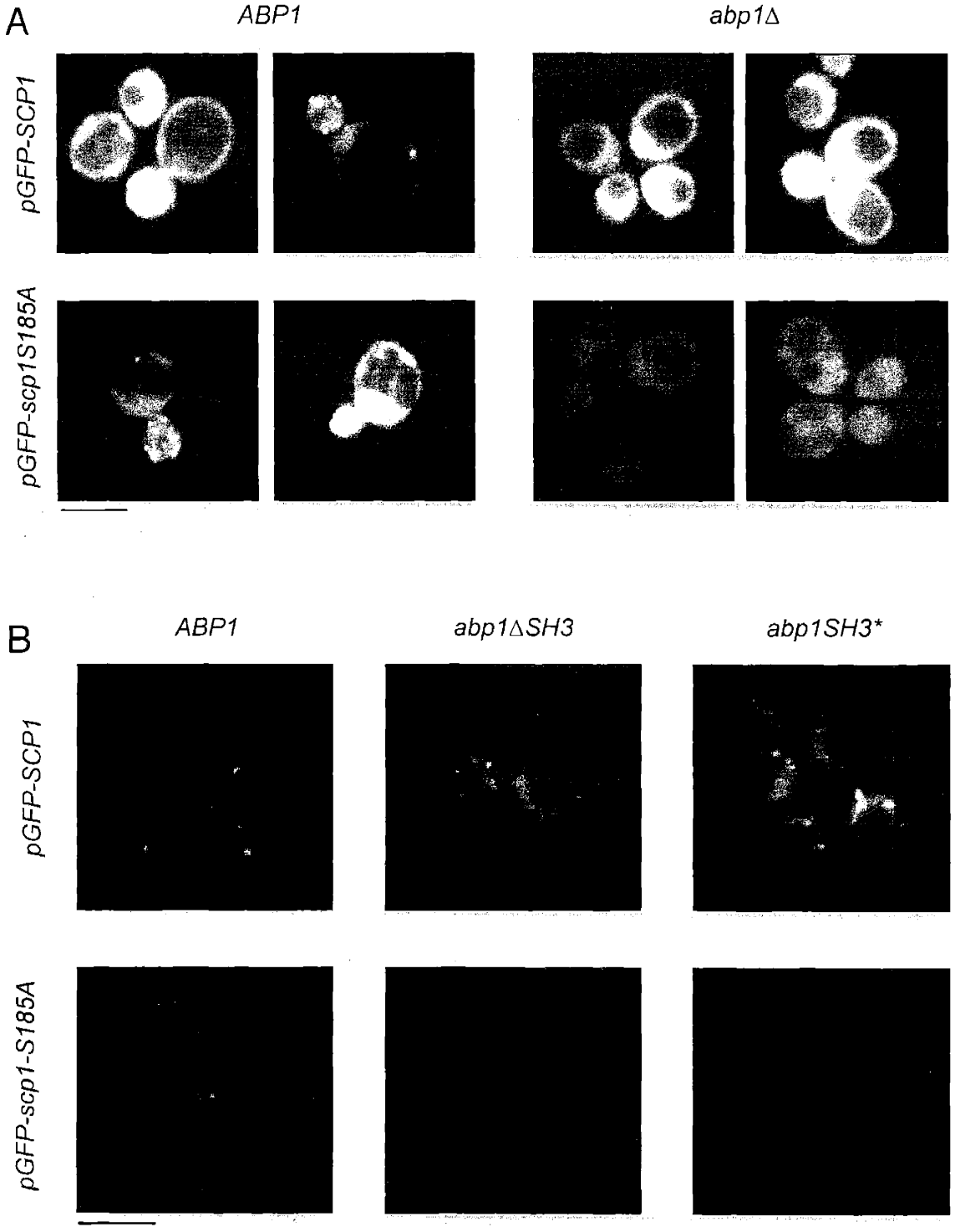

A
ABP1
abp1Δ
pGFP-SCP1
pGFP-scp1S185A
B
ABP1
abp1ΔSH3
abp1SH3*
pGFP-SCP1
pGFP-scp1-S185A

Figure 3.3

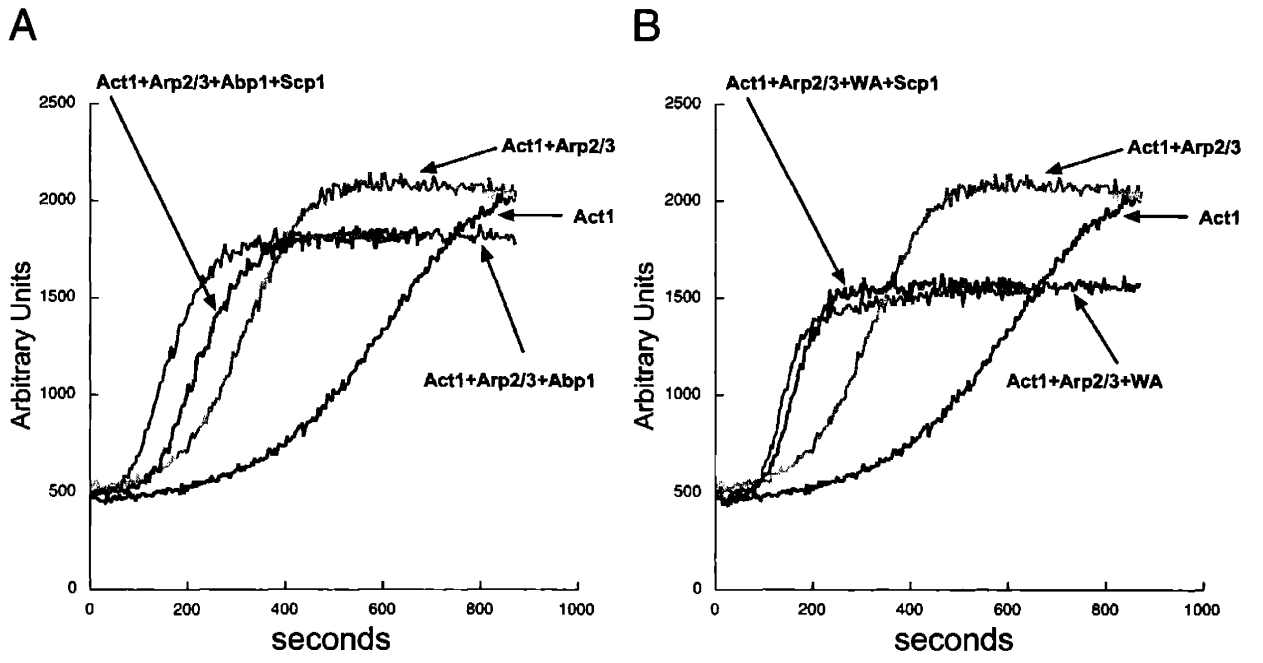


Figure 3.4 Scp1 modulates Abp1-dependent activation of the Arp2/3 complex. A. The effect of Scp1 on Abp1-dependent activation of the Arp2/3 complex in the actin filament assembly assay. The change in pyrene-actin fluorescence was monitored over time for actin alone (black), actin in the presence of Arp2/3 complex (green), in the presence of Arp2/3 and Abp1 (blue) and in the presence of Arp2/3, Abp1, and Scp1 (red). B. The effect of Scp1 on activation of the Arp2/3 complex by the carboxyl terminal fragment (WA) of Las17. The change in pyrene-actin fluorescence was monitored over time for actin alone (black), actin in the presence of Arp2/3 complex (green), in the presence of Arp2/3 and WA (blue) and in the presence of Arp2/3, WA, and Scp1 (red).

DISCUSSION

Regulation of the actin cytoskeleton in vivo involves intricate and dynamic network of protein-protein interactions (reviewed in Goode and Rodal, 2001). To understand the function of Scp1 in the context of other actin-associated proteins, we looked for Scp1-binding partners. One such partner, Abp1, was identified in the yeast two-hybrid screen for Scp1-intracting proteins. Biochemical evidence supports the interaction between Scp1 and Abp1. First, we demonstrated co-immunoprecipitation of Scp1 with Abp1 from cell lysates. A weakness of this experiment resides in the use of yeast strains overexpressing tagged Scp1 and Abp1. An independent method for demonstrating the existence of Abp1-Scp1 complex in vivo will be required (e.g. co-migration of native proteins in sucrose density gradients). Second, a recent high-throughput study of yeast protein complexes reported identification of Scp1 in a complex of seven proteins including Abp1 and Arp3 (Ho et al., 2002). Third, when actin-associated proteins from the yeast cell lysates are fractionated by gel filtration, Scp1 co-migrates with Abp1 and the Arp2/3 complex in the absence of actin filaments (Goode Lab, unpublished observation). Finally, actin assembly kinetics assays suggest that Scp1 inhibits activation of the Arp2/3 complex by Abp1.

In addition to biochemical data, in vivo localization data also support the existence of Abp1-Scp1 protein complex. Both Abp1 (Drubin et al., 1988) and Scp1 (chapter 2) localize to cortical actin patches and the SH3 domain of Abp1 is required for localization of the Scp1 mutant that has reduced affinity for actin filaments. This latter result suggests that actin filament binding of Scp1 is not required for its interaction with Abp1. However, I cannot rule out the possibility that another protein is required for the interaction between Scp1 and Abp1. Binding experiments with purified proteins will be required to demonstrate that the Scp1-Abp1 interaction is direct. In addition, the carboxy-terminal half of Scp1 should be tested in a biochemical assay for the ability to interact with Abp1. Deletion of the CH domain greatly enhanced the yeast two-hybrid interaction

between Scp1 and Abp1; therefore, the CH domain may conformationally mask the Abp1-binding site of Scp1.

Scp1 contains a consensus SH3-binding sequence PXXP near its carboxyl terminus and, therefore, may bind directly to the SH3 domain of Abp1. Recent characterization of the Abp1 SH3 domain specificity identified two types of ligands (Fazi et al., 2002). Ligands with a typical PXXP sequence conform to an extended consensus sequence +XXXPXXPXKPXXL. The atypical ligands of Abp1 SH3 domain contain long stretches of polyproline followed by a Lys-Pro dipeptide. While Scp1 contains a PXXP sequence, as well as two Lys-Pro dipeptides, it does not match either the typical or atypical ligand description. Nevertheless, the prolines in the predicted SH3-binding site are important for the Scp1-Abp1 interaction, since mutation of these amino-acid residues abolished the yeast two-hybrid interaction between Scp1 and Abp1. In contrast to the yeast two-hybrid assay, mutation of the prolines 156 and 159 had only modest effect on the interaction between Abp1 and Scp1 in the co-immunoprecipitation experiment. The differences in the protein concentrations in the two assays may account for the different results. In addition, full length Abp1 was used in the immunoprecipitation assay instead of the carboxy-terminal SH3 domain used in the yeast two-hybrid assay. It is possible that the full-length protein provides additional binding sites for Scp1 in addition to the SH3 domain; therefore, both full length Abp1 and the SH3 domain should be compared in the same assays in the future.

The interaction of Scp1 with Abp1 may be unique to yeast. When Scp1 is aligned with other members of the calponin family, the consensus SH3-binding site is found only in Scp1 and its orthologs from closely related species *S. castelli* and *S. kluyveri* (Figure 1.8 and data not shown). Sequences from other organisms, including *Neurospora crassa*, do not show conservation of the SH3-binding site. It remains to be tested whether the interaction between Scp1 and Abp1 is conserved in other organisms and whether other calponin family

members can interact with Abp1. It is possible that the Scp1-Abp1 interaction is indirect in other species.

What in vivo role might Scp1 play in a complex with Abp1? One proposed role of Abp1 is to serve as a scaffold for multiple proteins involved in actin organization and endocytosis (Lila and Drubin, 1997). It seems unlikely that Scp1 regulates interactions of Abp1 with its multiple ligands by direct competition, given the low expression levels of Scp1 (Chapter 2). An additional function of Abp1 in regulating actin nucleation by the Arp2/3 complex has been recently discovered (Goode et a., 2001). I obtained preliminary evidence supporting the role of Scp1 in modulating the activation of the Arp2/3 nucleating activity specifically by Abp1, but not by another activator (WA fragment of WASp/Las17). The yeast two-hybrid and GFP-Scp1 localization data support the importance of the SH3 domain of Abp1 in Scp1-Abp1 interactions. Whether the SH3 domain of Abp1 is required for the Scp1-dependent inhibition of the Abp1 activity in pyrene actin assembly assay remains to be tested.

Based on the data in figure 3.4, I propose that Scp1 regulates Abp1 by inhibiting its activation of the Arp2/3 complex. Since Scp1 and Abp1 physically interact via the SH3 domain of Abp1, the inhibition may also occur via the SH3 domain (Figure 3.5A). The regulation of the Arp2/3 complex via an SH3 protein domain is not unique to the Abp1/Scp1 pair, but has also been proposed for the WASP/SCAR protein family members (Higgs and Pollard, 2001; Rodal et al., 2003; Figure 3.5 B and C). The yeast member of this protein family, Las17, interacts with multiple SH3 domain-containing proteins, including Myo3, Myo5, Bbc1, Sla1 and others (Evangelista et al., 2000; Lechler et al., 2000; Li, 1997; Tong et al., 2002). Las17-mediated activation of the Arp2/3 complex is inhibited by the SH3 domains of Sla1 and by the SH3 domain-containing protein Bbc1 (Rodal et al., 2003; Figure 3.5B). In contrast to Las17, mammalian N-WASP is auto-inhibited in the absence of regulatory molecules, such as Cdc42, PIP_2, SH3 domain-containing proteins Grb2 and syndapin I (reviewed in Higgs and Pollard, 2001). One model for activation of N-WASP via the SH3 domains of Grb2 is

summarized in Figure 3.5C (Carlier et al., 2000). Whether other activators of the Arp2/3 complex are also regulated via SH3 domains is unknown, although some of them contain SH3 domains (yeast type I myosins Myo3 and Myo5) or interact with the SH3 domain containing proteins (Pan1/Eps15). SH3 domains are emerging as important regulatory domains for activation of the Arp2/3 complex, and the potential regulatory role of the Abp1 SH3 domain interaction with Scp1 will need to be examined more carefully.

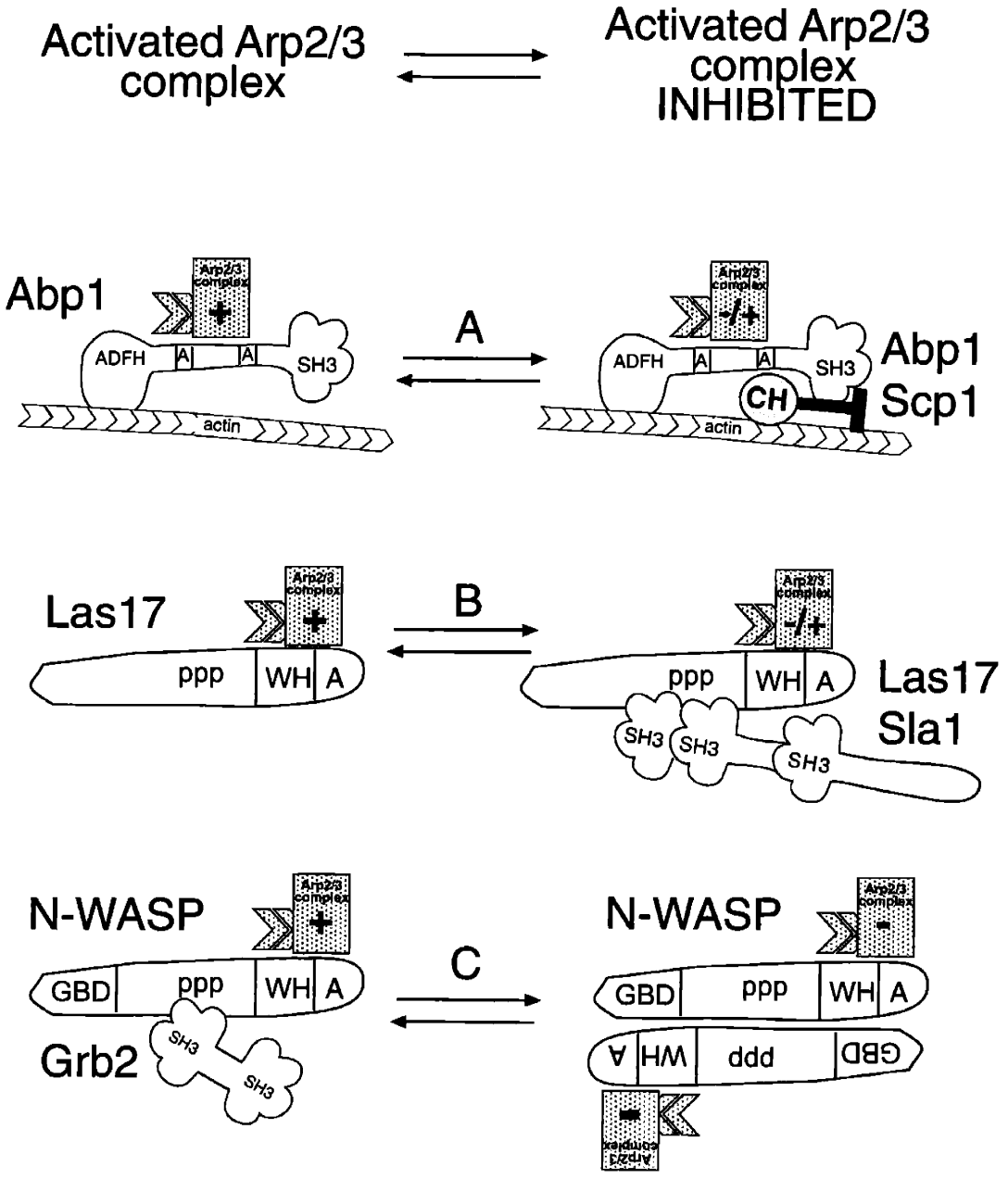


Figure 3.5 Models for the SH3 -dependent regulation of the activators of the Arp2/3 complex. A. Abp1-dependent activation of the Arp2/3 complex is inhibited by binding of Scp1 to the SH3 domain of Abp1 (this work). B Las17-dependent activation of the Arp2/3 complex is inhibited by binding of the SH3 domains of Sla1 to Las17 (Rodal et al., 2003). C. Binding of the SH3 domain of Grb2 to mammalian N-WASP keeps N-Wasp in monomeric active state, while dissociation of Grb2, leads to increased formation of N-Wasp dimers, that remain bound to Arp2/3 complex, but cannot activate it (Carlier et al., 2000). The Arp2/3 complex is shown in red, and its relative activation level is indicated with plus and/or minus signs.

MATERIALS AND METHODS

Yeast strains and growth conditions.

The yeast strains used in this study are listed in table 3.1. Standard methods were used for growing and manipulating yeast (Guthrie and Fink, 1991). Strains used in the immunoprecipitation experiments were generated by transforming wild type W303 strain with plasmids carrying either Scp1 or Abp1 under control of galactose-inducible promoter. For figure 3.2A, L4852 was transformed with pAG42 (Abp1p tagged with the V5 epitope) and pAG64 (Scp1 tagged with HA). Control strains carried either pAG42 or pAG64 in addition to an appropriate empty vector. Origin of the strains used in figure 3.2B is described in table 3.1. Yeast cultures were grown overnight in synthetic complete media lacking uracil and either histidine or tryptophan in the presence of 2% glucose; cells were washed in water; resuspended in media with 2% galactose and allowed to grow for additional 8-10 hours.

Plasmid construction

Plasmids used in these experiments are described in table 3.2. For the yeast two-hybrid assay pAG120 was generated using the Univector Plasmid Fusion System (UPS; Liu et al., 1998) described in detail in Appendix 3. pAG32 and pAG33 were generated by site directed mutagenesis (Quick Change™ Kit; Stratagene Inc,) using pAG47 as a template and the following pairs of oligonucleotides respectively: CPO7 and CPO8, CPO9 and CPO10 (Table 2.5). For immunoprecipitation experiments pAG38 was described in chapter 2, pAG40 was generated by site-directed mutagenesis using pAG38 template and CPO9 and CPO10 oligonucleotides. pAG64 was generated using UPS as described in more detail in Appendix 3. To generate *GFP-SCP1* fusion on a plasmid marked with *TRP1* (pAG71), I switched the marker of pAG9 (Chapter 2) by gap repair. pAG72 was generated by site-directed mutagenesis using pAG71 as a template and CPO5 and CPO6 oligonucleotides.

Table 3.1 Strains used in Chapter 3.

Name	Genotype	Source
L6456	*MATa, ura3-52, leu2-3,112, his3Δ200, trp1-901, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*	Fink Lab (Jones et al., 1996)
L6457	*MATα, ura3-52, leu2-3,112, his3Δ200, trp1-901, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*	Fink Lab
AGY401	L6456 transformed with pDBD-SCP1/URA/2μ	B. Cali
AGY403	L6457 transformed with pAD-ABP1-509C/LEU2/2μ	B. Cali
AGY405	L6456 transformed with pDBD-scp1-N118/URA/2μ	B. Cali
AGY551	L6456 transformed with pDBD-scp1-121C/URA/2μ	B. Cali
L4852	*MATa, ura3-52, leu2-3,112, his3-11,15, ade2-1, trp1-1, can1-100, GAL+*	Fink Lab
AGY322	L4852 transformed with pRS426Gal1 and pAG38	This study
AGY425	L4852 transformed with pAG42 and pAG38	This study
AGY427	L4852 transformed with pAG42 and pAG40	This study
AGY543	L4852 transformed with pRS414Gal1	This study
AGY544	L4852 transformed with pAG64 *(Gal1-HA3-SCP1/TRP1/CEN)*	This study
AGY391	*MATα, his3Δ200, leu2-3,112, ura3-52, trp1, scp1Δ::LEU2, abp1Δ::URA3*	This study
AGY334	*MATα, his3Δ200, leu2-3,112, ura3-52, trp1, scp1Δ::LEU2*	This study
BGY628	*MATα, his3Δ200, leu2-3,112, ura3-52, trp1-1, ade2-1, ssd-, GAL+ABP1::HIS3*	Goode lab
BGY630	*MATα, his3Δ200, leu2-3,112, ura3-52, trp1-1, ade2-1, ssd-, GAL+abp1ΔSH3::HIS3*	Goode lab
BGY631	*MATα, his3Δ200, leu2-3,112, ura3-52, trp1-1, ade2-1, ssd-, GAL+abp1SH3*::HIS3*	Goode lab

Table 3.2 Plasmids used in Chapter 3.

Name	Insert	Vector
pAG32	*DBD-scp1S185D*	pDBU-C
pAG33	*DBD-scp1P156A,P159A*	pDBU-C
pAG120	*DBD-SCP1*	pASlox
pAG38	*SCP1*	pRS423Gal1
pAG40	*scp1P156A,P159A*	pRS423Gal1
pAG42	*Gal1-His6-V5-ABP1*	Invitrogen
pAG47	DBD-SCP1	pDBU-C*
pAG64	*Gal1HA-SCP1*	pRS414Gal1
pAG71	GFP-SCP1	pRS314
pAG72	GFP-scp1S185A	pRS314

*James et al., 1996

Yeast two hybrid assay

The yeast two-hybrid assay was performed as described (James et al., 1996). In figure 3.1B, *MATα* L6457 strain was transformed either with empty vector (pGAD) or with pGAD-ABP1-509C (which was identified in the yeast two hybrid screen; Brian Cali, unpublished observations); *MATa* L6456 strain was transformed with empty vector (pAS2lox) or pAG120.; pair wise mating of these four strains resulted in diploids that were assayed for expression of *HIS3* and *ADE2* genes by comparing growth on synthetic complete (SC) media and on SC lacking histidine and adenine. Strains in figure 3.1C were generated and assayed similarly, with the following *MATa* strains used for mating: AGY401, AGY405, AGY455, and L6456 transformed with pDBU-C, pAG32 or pAG33 plasmids.

Co-immunoprecipitation assays

For immunoprecipitation, cell lysates were prepared by harvesting 10-20 OD units of freshly grown yeast cells and disrupting cells at 4°C using glass beads and buffer A (50mM HEPES pH7.5, 150mM KCl, 1mM EGTA, 1mM EDTA, 10% glycerol, 0.03% octyl-β-glucoside) supplemented with 1mM PMSF and 1X protease inhibitor cocktail (Calibrochem). Protein extracts were clarified by centrifugation (20 min, 12,000x*g*), and protein concentration was determined using BCA assay (Pierce). For each immunoprecipitation reaction 100µg total protein in 100µL of buffer A was mixed with 400µl IP buffer (50mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 0.25% gelatin, 1%NP40) supplemented with protease inhibitors (same as above). Non-specific proteins were pre-absorbed with Protein A/G Plus agarose beads (Santa Cruz Biotech) for 1 hour at 4°C, then, 10µg of anti-V5 (Invitrogen) or anti-HA (12CA5) antibody was added to each reaction. After one hour, Protein A/G Plus agarose beads (Santa Cruz Biotech) were added, and incubation was allowed to continue for an additional hour. Beads were pelleted and washed three times in IP buffer, while proteins in

the supernatant were precipitated with 10% TCA. Samples were then separated by SDS-PAGE and analyzed by immunoblotting.

Fluorescence Light Microscopy

Images in figure 3.3 were acquired as described in chapter 2.

Actin filament assembly kinetics

Purification of His6-Scp1 used in these assays was described in chapter 2. Abp1, WA fragment of Las17, and Arp2/3 complex were purified by Avital Rodal and acquired through generosity of Dr. Bruce Goode (Brandeis University). Actin assembly was monitored by the pyrene-actin fluorescence assay as described (Goode et al., 2001), using 2μM final yeast actin (with 1% pyrene labeled rabbit skeletal muscle actin; Cytoskeleton Inc.; Denver, CO) in 70μL reactions. Briefly, 56.5μL of pre-cleared actin in G-buffer was mixed with actin-binding proteins diluted in 10μL HEKG5 buffer (see chapter 2) or with buffer alone. Final concentrations of the actin-binding proteins were: 20nM Arp2/3 complex, 67nM His6-Scp1,70nM Abp1, 500nM WA. Reactions were then mixed with 3.5 μL of 20X initiation mix [1M KCl, 40mM $MgCl_2$ 10mM ATP] in a quartz fluorimetry cuvette (3mm light path; Hellma). Change in pyrene fluorescence was monitored at constant temperature (25°C) by excitation at 365nm and emission at 407nm in a fluorescence spectrophotometer (Photon Technology International).

CHAPTER 4. FUTURE DIRECTIONS

In this thesis, I have addressed several open questions about the calponin protein family. I have established that Scp1, the yeast transgelin-like member of the calponin family, binds and cross-links actin filaments. I have characterized the effects of Scp1 on actin dynamics in vitro. Moreover, I obtained preliminary evidence supporting a novel activity of a calponin family member in modulating actin nucleation by the Arp2/3 complex. Genetic analysis suggested that in vivo functions of Scp1 partially overlap with Sac6/fimrin functions. This genetic interaction is supported by two biochemical activities shared between Scp1 and Sac6, actin filament stabilization and cross-linking. In this chapter, I discuss several remaining questions regarding Scp1 and other proteins in the calponin family, as well as the new questions that emerged in the course of these studies.

Interaction of Scp1 with actin

Several questions regarding biochemical activities of Scp1 remain unanswered. First, although I demonstrated the ability of Scp1 to assemble actin filaments into higher order structures, I have not carefully characterized these structures. Using electron microscopy, I observed tangled networks of actin filaments in the presence of Scp1. Additional assays (i.e. falling ball viscometry) will be required to establish that the tangled actin filaments are, in fact, cross-linked actin filament gels. Moreover, electron microscopy assays should be repeated using different ratios of Scp1 to actin and different assay buffers, since some proteins bundle or gel actin filaments depending on reaction conditions

(Otto, 1994). In addition, actin filament organization should be observed in the presence of Scp1 and Sac6 simultaneously, as well as in the presence of Scp1 and Abp1, since these proteins co-exist and may function together in vivo.

The mechanism of actin filament cross-linking by Scp1 and other calponin family members remains unknown. Several steps can be taken to identify this mechanism. First, the controversy surrounding the possible role of the CH domain in actin filament binding needs to be resolved. Molecular models of the calponin-actin interaction (Hodgkinson et al., 1997; Bramham et al., 2002) can be tested using site directed or random mutagenesis. Identifying specific mutations in the CH domain of Scp1 that affect either actin filament binding or cross-linking by Scp1 would suggest the involvement of the CH domain in actin binding. Second, if the CH domain does not function in actin binding, then either the second actin binding site or the dimerization site must be identified. Narrowing down the region involved in actin cross-linking may be possible using truncated Scp1 constructs. In addition, identification of Scp1 mutations that interfere with actin filament cross-linking, but not actin binding will be informative in elucidating the mechanism of actin cross-linking. Either the random or site directed mutagenesis can be used to generate such mutations. Positively charged residues near the carboxy-terminal CLR provide a good target for site-directed mutagenesis, since the analogous charged region of mammalian calponin has been shown to bind actin filaments.

Another biochemical activity of Scp1 that requires further characterization is the effect of Scp1 alone on the rate of actin filament assembly. I observed a

decreased rate of assembly at low concentrations of Scp1 and loss of the lag phase of assembly at high (micromolar) concentrations of Scp1 (unpublished data). The loss of lag phase suggests an actin nucleation activity that has not been reported for any other member of the calponin family. Proteins known to nucleate actin filaments do so by binding actin monomers (Arp2/3 complex; Welch et al., 1997) and/or capping actin filament ends (capping protein, formins; Caldwell et al., 1989; Sagot et al., 2002). Could Scp1 have a weak actin monomer binding or actin filament capping activity? These activities await further testing. To separate the effects of Scp1 on actin filament nucleation and actin filament elongation, actin assembly should be monitored in the presence of actin filament seeds.

Finally, competition of Scp1 with Sac6 for binding to actin filaments suggested that the Scp1 and Sac6 have overlapping binding sites on actin. This hypothesis may be tested using biochemical and genetic tools. Many mutant actin alleles are available in yeast, and some of these are defective for binding to Sac6. Assays of Scp1 and Sac6 binding to mutant actin filaments may provide further evidence for similar binding sites on actin. Additional evidence may come from a genetic assay based on the overexpression phenotype of Scp1. This phenotype requires intact binding of Scp1 to actin (Chapter 2). It should be informative to compare the effects of Scp1 overexpression and Sac6 overexpression on viability of various actin alleles. Identification of actin alleles that are defective for binding to Scp1 and Sac6 and are resistant to

overexpression of Scp1 and Sac6 will support the model in which fimbrin and calponin bind to overlapping sites on actin.

Identification of in vivo relevant binding partners of calponin protein family.

In recent years, many in vitro ligands of calponin have been reported: caldesmon, calcineurin, caltropin, myosin, tubulin, desmin, ERK kinase, PKC, and phospholipids. Many interactions have not been tested rigorously and, more importantly, their function and in vivo relevance have not been demonstrated. Yeast and other genetically tractable organisms may provide in vivo evidence supporting the biochemical data. For example, during analysis of Scp1 function, we discovered that cells lacking Scp1 and fimbrin become sensitive to microtubule depolymerizing agents benomyl and nocodazole. This phenotype is not common among genes with actin cytoskeleton-related function. Moreover, since deletion of fimbrin alone confers barely detectable sensitivity to these agents, Scp1 function may be specifically related to microtuble cytoskeleton function and consistent with the physical interaction reported for mammalian calponin. I obtained preliminary data on binding of Scp1 to microtubules, although I have not tested for the specificity of this interaction. Further characterization of this interaction will be required to establish with confidence that Scp1 binds microtubules.

Another novel genetic interaction suggests that the physical interaction between calponin and phospholipids may be relevant in vivo. Deletion of inositol phosphotase Inp51 has been previously reported to suppress partially the

temperature sensitivity of *sac6*Δ yeast strains (Srinivasan et al., 1997). I found that the ability of *inp51*Δ to suppress *sac6*Δ defects is abolished in the absence of Scp1 (unpublished data). This result is consistent with the inhibitory function of *INP51* upstream of the *SCP1* in a pathway that is redundant (parallel) with the *SAC6* pathway. The next step is to test the ability of phospholipids to bind Scp1 and to regulate actin binding of Scp1 in vitro. Further genetic analysis may reveal a role for phospholipids in the regulation of Scp1 functions. One should test, for example, whether *SAC6* and *SCP1* exhibit genetic interactions with other genes in the *INP51* pathway and whether the partial suppression *sac6*Δ phenotypes by *inp51*Δ and by low level *SCP1* overexpression is additive.

The interaction between Scp1 and Abp1

Preliminary evidence points to a possible role of Scp1 in regulation of the Abp1-dependent activation of the Arp2/3 complex. Further characterization of this interaction will be necessary to establish if binding of Scp1 to actin, or to the SH3 domain of Abp1, or to both is required for the modulation of the Arp2/3 complex activity. In addition, providing evidence for the relevance of the Abp1-Scp1 interaction in vivo will be important. First, the interaction between Scp1 and the Abp1-Arp2/3 complex in vivo should be demonstrated. This can be achieved by fractionation of the cell lysates on sucrose gradients or by gel filtration and probing the fractions for co-migration of the interacting proteins. Second, it will be important to establish that the interaction in cell extracts occurs via the SH3 domain of Abp1, and not through binding of both Abp1 and Scp1 to actin

filaments. To demonstrate the specificity of the interaction, one can take advantage of the Scp1 mutations that impair actin binding, as well as of the Abp1 mutation in the SH3 domain. Finally, it will be important to obtain genetic evidence supporting the function of Scp1 in modulation of the Arp2/3 complex. This evidence may emerge from testing for genetic interactions between Scp1 and specific mutations in the subunits of the Arp2/3 complex and in the known regulators of the Arp2/3 complex.

The interaction between Scp1 and the SH3 domain of Abp1 is not likely to be conserved in other organisms, since the SH3-binding site is unique to Scp1 (Figure 1.2). However, the function of this interaction in modulating activity of the Arp2/3 complex may be conserved and executed via different binding partners. Good candidates for the alternative binding partners are found among the proteins from the epsin family. Interactions of Scp1 with the yeast epsins Ent1 and Ent2 were identified in the yeast two hybrid screen, but were not pursued further (Appendices 2 and 4). Epsins physically interact with Pan1, which like Abp1, activates the Arp2/3 complex. The initial steps in characterizing the Scp1-epsin interaction should include demonstration of direct binding between Scp1 and epsins, as well as testing for the regulatory effects of Scp1 and epsins in the kinetic actin assembly assays.

Summary: Molecular functions of Scp1

Synthesis of the data presented in chapters 2 and 3, as well as preliminary results mentioned in this chapter, lead me to propose a model for the molecular

functions of Scp1 (Figure 4.1). According to this model, Scp1 stabilizes and cross-links actin filaments at the cortical actin patches (Chapter 2). These two functions of Scp1 are shared with fimbrin/Sac6. In addition, Scp1 functions with Abp1 in modulation of the actin nucleation by the Arp2/3 complex (Chapter 3). Moreover, through interactions with Ent1 and Ent2, Scp1 may also regulate Pan1-dependent activation of the Arp2/3 complex (Appendices 2 and 4). The Arp2/3 complex is involved in multiple functions in the cell and exactly which functions are regulated by Scp1 is not clear.

How the functions of Scp1 are regulated also remains unknown. Potential roles of phosphorylation at the conserved serine (S185) and of phospholipids will need to be addressed. Finally, how the molecular functions of Scp1 affect biological processes in the cell is not clear. Deletion of *SCP1* results in no detectable phenotype, with the exception of a weak latrunculin sensitivity. Maybe the functions of Scp1 are so important that fail-safe mechanisms were built into the system. This view is supported by the synthetic interactions between *SCP1* and *SAC6*. It appears that, at least under artificial laboratory growth conditions, *SCP1* is not required for cell growth. However, conservation of Scp1 sequence in diverse organisms suggests that it does have an important function.

Conservation of Scp1 functions among other calponin family members

The functions of mammalian calponin family members outside the smooth muscle have not been defined. Based on synthetic interactions between *scp1Δ*

Figure 4.1 Model for partially overlapping functions of Scp1 and Sac6. Each circle encloses proposed actin-related functions of Scp1 and Sac6; intersection of the two circles indicates overlapping functions. Arrows indicate known physical interactions of Scp1 and Sac6 probably involved in the actin-related functions. Scp1 exhibits its effects on actin either directly or via its interactions Abp1 and Ent1. (1) Drubin et al., 1988; Adams et al., 1991; (2) actin binding and cross-linking assays in chapter 2; (3) Drubin et al., 1988; (4) Welch et al., 1997; (5) yeast two-hybrid assays, co-immunoprecipitation, and localization data in chapter 3; (6) Goode at al., 2001; (7) yeast two hybrid data in Appendices 2 and 4; (8) Wendland et al., 1999; (9) Duncan et al, 2001.

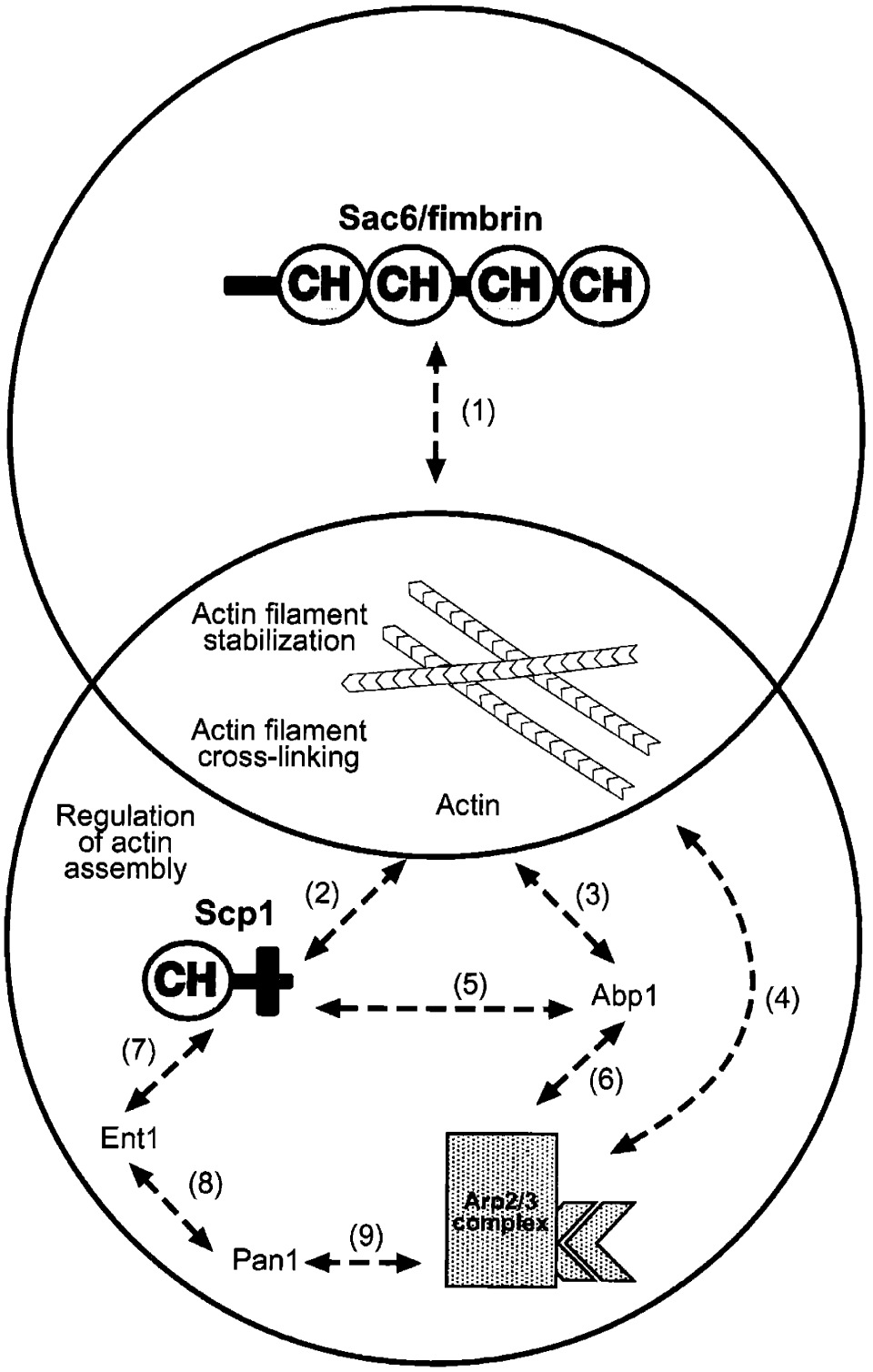

Sac6/fimbrin
CH
CH
CH
CH
(1)
Actin filament stabilization
Actin filament cross-linking
Actin
Regulation of actin assembly
Scp1
CH
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
Abp1
Ent1
Pan1
Arp2/3 complex

Figure 4.1

and *sac6Δ*, we developed the first in vivo assay for the function of transgelin-like proteins in yeast. The next step will be to test whether the function of Scp1 that is shared with Sac6/fimbrin has been conserved in transgelins and calponins in other organisms. This test can be achieved by comparing growth phenotypes of the sac6Δscp1Δ strains expressing wild type Scp1 and other calponin family members under control of Scp1 promoter from a low copy plasmid. Lack of suppression will not be very informative, as there are many reasons why this assay may not work with proteins from other organisms (i.e. other calponin family members may be unstable in yeast, may fail to bind yeast actin, may not undergo proper post-translational modifications). However, if suppression is detected, it will suggest conservation of functions shared with fimbrin.

The model for molecular functions of Scp1 (Figure 4.1) should also be tested with respect to other calponin family members. For example, calponin family members should be tested for the ability to bind to Abp1 and/or epsins, as well as for regulation of the Arp2/3 complex in the presence of known mammalian Arp2/3 regulators. It will be important to test not only mammalian, but also invertebrate calponin family members (i.e. MP-20) in the in vivo and in vitro assays, since phylogenetic analysis suggests that Scp1 and other invertebrate transgelin-like proteins form a subfamily distinct from vertebrate transgelins and calponins.

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APPENDICES

APPENDIX 1. Summary of phenotypes of the *sac6Δ* mutant cells

	Phenotype enhanced in the *sac6Δ scp1Δ* double mutant?
sac6Δ mutants are sensitive to:	
High temperature (34, 36, 37°C)/YPD	+
Caffeine (5mM YPD)	+
Zymolyase	+
Salt stress (NaCl, KCl)	+
Nocodazole (4µg/mL)	+
Benomyl (10µg/mL)	+
Latrunculin A	+
NaF (20mM)	+
G418 (0.05µg/mL)	-
Hygromycin (50µg/mL)	-
Stationary phase (slow recovery)	+
Morphology/polarity phenotypes of *sac6Δ*:	
random budding in diploids	
large, round cells	
depolarized actin cytoskeleton	
increased % of multinucleated cells at 37°C	
Endocytosis defect of sac6Δ:	
Lucifer yellow internalization	
Myc-ste6p internalization	
Other phenotypes of *sac6Δ*:	
Invasion	
Pseudohyphal growth	
Mating efficiency	
***sac6Δ* mutants have wild type phenotype on:**	
cold (12oC, 16oC)/YPD	
osmotic stress: 1.5M sorbitol	
Low pH (pH=3)	
YPGlycerol	
formamide (3%)	
Low phosphate/YPD	
staurosporine (0.1µg/mL)	
EGTA (10mM)	
CaCl2 (100mM)	
Rapamycin (0.1µg/mL)	
Paromomycin (0.2 and 1µg/mL)	
axial budding pattern in haploids	
alpha factor secretion	
growth on sucrose	

APPENDIX 2. Genes identified in a yeast two-hybrid screen with Scp1

Identified gene	Description
*SAC6**	Actin bundling protein, contains 4 CH domains
*ABP1***	Actin binding protein, cryptic function in endocytosis
PHO5	Secreted acid phosphatase
APE3	Vacuolar enteropeptidase
*ENT1****	Related to clathrin-binding protein AP180
ENT2	Related to clathrin-binding protein AP180
YMR244w	Related to *SUN4* gene family involved in aging

Yeast two hybrid screen was carried out by Brian Cali as described in Jones et al., 1996. * Multiple SAC6 fusions were identified, all of which contained truncations of the frist CH domain. The full length Scp1 did not interact with Scp1 by the yeast two hybrid assay. Therefore, interaction of truncated Sac6 may be an artifact resulting from the natural affinity of the three CH-domains of fimbrin for the fourth CH domain. **Carboxy-terminal fragment of Abp1 was identified starting with amino acid 509. ***Ent1 identified in this screen was missing amino-terminal ENTH domain (162-C).

APPENDIX 3. SCP1 constructs generated using Univector Plamid Fusion System

To simplify transitions between different constructs, I took advantage of the Univector Plamid Fusion System (UPS; Liu et al., 1998). This system relies on CRE-LOX recombination to generate precise fusions between a Univector plasmid (pUNI), in to which a gene of interest is cloned by conventional method, and a pHOST plasmid, which carries regulatory information for the gene of interest, as well as any desired fusion tag. I used this system to generate a number of plasmids that may be usefull in future Scp1–related studies.

To clone Scp1 into the Univector (pUNI10), I first introduced an NdeI clonning site at the start codon of Scp1 (pAG59) using QuickChange PCR mutagenesis (Stratagene; pAG21 template and CPO17 (GCGTACAGCTAAAGT TTACATATGAGTTACGATAAGAAGGC) and CPO18 (GCCTTCTTATCGTAACTC ATATGTAAACTTTAGCTGTACGC) oligonucleotides. Scp1 from pAG59 was cloned into NdeI/HpaI sites of pUNI10. Resulting plasmid pAG138 was used for fusions with the host vectors listed in the table below.

I also generated a host vector carrying Scp1 promoter, followed by a 3HA tag (pAG107). To accomplish that, I excised Gal promoter of p1217 (Liu et al., 1998) with KpnI and EcoRI, and replaced it with a the SCP1 promoter amplified by PCR with CPO23 (GGAATTCCGGTAAACTTTAGCTGTACGCTTACAAAG) and CPO24 (CCTCACTAAAGGGAACAAAAGCTGGGTACCGGG) primers. pAG107 will be useful for testing whether other calponin family members complement *scp1*Δ growth phenotypes (in sac6Δ background). Once, other calponin member is cloned into pUNI10, it can be rapidly fused to this construct to express a tagged protein under control of Scp1 promoter in yeast. Additional benefit in cloning other calponin family members into pUNI is the ability to rapidly generate other fusions and test for conservation of Scp1 function in other assays (e.g. yeast two hybrid interactions, overexpression phenotype, localization) as well as for expression in bacteria or mammalian cells.

Univector plasmid fusions with *SCP1*

pHOST	Description (base vector)	*pUNI*		
		pAG138 *SCP1*	pAG116 *(scp1Δ12)*	pAG118 *(scp1Δ24)*
1206	*E. coli* expression of His6-tagged protein (pET15b(T7))			pAG165
1207	*E. coli* expression of FLAG-tagged protein (pET15b(T7))	pAG166		pAG164
1216	*CEN TRP1 GAL* expression of untagged (pRS414)	pAG169 pAG170		
1217	*CEN TRP1 GAL* expression of 3XHA tagged (pRS414)	pAG64		
1220	*CEN URA3 GAL* expression of untagged protein (pRS416)	pAG167 pAG168		
1221	*2μ TRP GAL* expression of untagged (pRS424)	pAG135	pAG136	pAG137
1231	*2μ LEU2 ADH-GAL4 AD* fusion vector (2 hybrid system)	pAG156 pAG157	pAG158 pAG159	
1232	*2μ TRP1 ADH-GAL4 DBD* fusion vector (2 hybrid system)	pAG1210	pAG121	pAG122
pAG107	*CEN TRP SCP1Prom* for expression of 3XHA tagged proteins under *SCP1* promoter	pAG103*, pAG104* pAG132	pAG133	pAG134

pHOST column lists ID numbers of plasmids received from S. Ellege. Plasmids resulting from Cre-catalysed fusion reaction are listed at the intersection of each univector column and pHOST row. For some constructs, multiple independent fusions are listed. Star indicates plasmids that suppress *scp1Δ* temperature sensitive and caffeine sensitive growth phenotypes in *sac6Δ* background.

APPENDIX 4. Putative Scp1-binding protein Ent1

Carboxyl termini of Ent1 and Ent2 (missing the amino-terminal ENTH domain) were identified in the yeast two hybrid screen (Appendix 2). These proteins function in endocytosis and physically interact with Pan1, a protein involved in endocytosis and regulation of the Arp2/3 complex. I cloned *ENT1* into pUNI (see appendix 5 for more details on pUNI), intending to pursue the connection between Scp1 and Ent1 further.

I amiplified the coding region of *ENT1* using YDL1 (GCAGAACAACCATG GCGAAACAATTTGTTAGATCTGC and YDL2 (GCGGATCCTCATAAATCAATTA GAGTATATCCCCG) primers and it cloned into NcoI/BamHI of pUNI10 resulting in pAG82 an pAG84. Clonning of *ENT1* introduced a mutation (*NdeI* site) that resulted in change of ser-2 to alanine. Sequencing revealed that pAG82 carried an additional mutation that changed methionine-297 to theonine. However, both pAG82 and pAG84 plasmids suppressed lethality of *ent1*Δ *ent2*Δ in a plasmid shuffle assay. For this assay, I transformed AGY555 (a.k.a. BWY502 ent1Δent2Δ pENT1/URA; from Beverly Wendland) with pAG141 and pAG144 (see below); these strains were grown on SC-Trp+2% Galactose and on 5FOA+2%Galactose. Both plasmids were able to suppress lethality of AGY555 on 5FOA and, therefore, were functional. My efforts to overexpress Ent1 in yeast using plasmids pAG141, 142, 144, and 145 were successful, as verified by immunoblotting with anti-Myc or anti-HA antibodies. However, low solubility of Ent1 prevented me from pursuing the physical interaction between Scp1 and Ent1 by conventional biochemical methods.

When tested in the yeast two-hybrid assay, both pAG123 and pAG125 activated gene expression in the presence of empty vector. This result agreed with B. Wendland's finding (personal communication). However, the construct identified in the yeast two hybrid screen, missing the first 161 amino acids, interacted specifically with Scp1 and did not self-activate. Moreover, this interaction was disrupted by specific mutations of Scp1. S185D or 121C constructs did not interact with Ent1-162C, while P156A/P159A mutant ineraction were indistinguishable from wild type. Note that interactions with Abp1 were reversed, with PP mutant being defective, while the other two mutants maintaining interactions. This leads me to believe that the interaction between Scp1 and Ent1 is real and will be worth pursuing (also see chapter 4 for discussion of the potential role of Ent1 in regulating the functions of the Arp2/3 complex). The plasmids listed in the table below may be useful in this pursuit.

Univector plasmid fusions with *ENT1*

pHOST ID#	Description (base vector)	pAG82 pUNI/ENT1	pAG84 pUNI/ENT1
1216	*CEN TRP1 GAL* expression of untagged (pRS414)	pAG140	pAG143
1217	*CEN TRP1 GAL* expression of 3XHA tagged (pRS414)	pAG141	pAG144
1218	CEN TRP GAL expression of 3XMYC-tagged protein (pRS414)	pAG142	pAG145
1224	2μ URA GAL expression of untagged protein (pRS426)	pAG91 pAG92	
1231	*2μ LEU2 ADH-GAL4 AD* fusion vector (2 hybrid system)	pAG90 pAG123	pAG125
1232	*2μ TRP1 ADH-GAL4 DBD* fusion vector (2 hybrid system)	pAG154 pAG155	

APPENDIX 5. Screen for multicopy suppressors of *sac6*Δ mutant phenotypes

Background

Fimbrin is an actin binding and bundling protein conserved from yeast to humans. In yeast fimbrin is not essential for growth under optimal conditions; however fimbrin mutants fail to grow at elevated temperatures or in high salt, have depolarized actin cytoskeleton, and are blocked for endocytosis. I have found additional phenotypes of fimbrin mutants: sensitivity to caffeine and to microtubule depolymerizing drugs benomyl and nocodazole. In order to understand the functions of yeast fimbrin I initiated a screen for multicopy suppressors of fimbrin mutant phenotypes.

Methods

Diploid cells where used in the screen as they exhibit more severe phenotypes. AGY611 (*sac6*Δ/*sac6*Δ) was transformed with 2μ genomic library (B2339; pRS202 vector; Fink Lab collection) and plated on SC-URA plates. After 2-3 day incubation at 30oC, transformants were collected and frozen. These transformants were later screened for different phenotypes by plating under selective conditions (YPD/37°C, YPD + 5mM caffeine, YPD + 10μg/mL benomyl). Individual colonies were re-patched under selective conditions again. Patches were replica plated onto YPD and YPD+5FOA. Strains unable to grow on 5FOA were discarded since they were incapable of loosing URA plasmid. Patches grown on 5FOA were replica plated on YPD and selective media. Strains resistant to selective condition before 5FOA and sensitive after passage over 5FOA were considered candidate suppressors. Genomic DNA from these strains was used to transform *E.coli* and recovered plasmids were re-transformed into AGY611 for re-testing. Plasmids that suppressed sensitivity of *sac6*Δ/Δ were sequenced using T3 and T7 primers.

Results

In a pilot screen, I have isolated 10 different plasmids capable of suppressing at least one *sac6*Δ/Δ mutant phenotype (see table below). So far, the most interesting suppressor identified is sfm4. This plasmid encodes a kinase domain of uncharacterised kinase Akl1, as well as several other ORFs. I have deleted the other ORFs and found that sfm4Δ still suppressed sac6Δ phenotypes. In addition, I cloned full length ALK1 into a high copy plasmid and also found suppression of sac6Δ ts phenotype. Scp1 is not required for suppression, since sfm4 also suppresses ts phenotype of *sac6*Δ *scp1*Δ double mutant. Deletion of this gene exhibited no growth phenotype or synthetic interactions with *sac6*Δ and *scp1*Δ. The mechanism of suppression by *ALK1* is unclear, but it is interesting that the other two kinases similar to Alk1 (Ark1 and Prk1) are involved in the reglation of the actin cytiskeleton and endocytosis, localize to actin via Abp1, and phosphorylate Pan1. As discussed in chapters 3 and 4, both Abp1 and Pan1 may be functionally linked to Scp1.

plasmid	Genes	CHR#: start*	End*	1	2	3
sfm3	*YCR024c, PMP1, YCR025c, YCR026c*	3: 158776	166615	+	+	-
sfm4	*AKL1*	2: 358565	365189	+	+	-
sfm9	*HSF1*	7: 363459	370116	+	+	+
sfm6	*HSF1*	7: 366254	373702	+	+	+
sfm9-22	*HSF1*	7: 362960	369976	-	+	+
sfm8-2	*YAP1, GIS4, tRNA*	13: 252268	259709	-	++	+++
sfm9-18	*NMT1, PWP1, SIK1*	12: 541615	547849	-	+	+
sfm8-7	*HIR1, YBL009w, YBL010c*	2:205219 (T3)	212082(T7)	-	-	+
sfm9-6	*ALG9, ADE12, YNL217w, 218w*	14: 234886 (T7)	241887(T3)	-	-	+
sfm9-44	*LOS1, CBT1, YKL207w, 206c*	11:46761 (T7)	53628(T3)	-	-	+

Plasmids identified in a dosage suppressor screen for suppression of *sac6Δ* phenotypes: temperature sensitivity (1), caffeine sensitivity (2), benomyl sensitivity (3). *columns indicate chromosome number and coordinates of the start and end of the insert.