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Multiple domains of human CLASP contribute to microtubule dynamics and organization in vitro and in *Xenopus* egg extracts

by

Kieren Jay Patel

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

in

Molecular and Cell Biology

in the

Graduate Division

of the

University of California, Berkeley

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Professor Rebecca Heald, co-Chair

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Fall 2011

ABSTRACT

Multiple domains of human CLASP contribute to microtubule dynamics and organization in vitro and in *Xenopus* egg extracts

by

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Doctor of Philosophy in Molecular and Cell Biology

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Professor Rebecca Heald, co-Chair

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Cytoplasmic Linker Associated Proteins (CLASPs) comprise a class of microtubule (MT) plus end-binding proteins (+Tips) that contribute to the dynamics and organization of MTs during many cellular processes, including migration and division. Human CLASP contains multiple MT-binding domains, including Tumor Overexpressed Gene (TOG), as well as Ser-x-Ile-Pro (SxIP) motifs known to target some +Tips though interaction with End-Binding Protein 1 (EB1), but how individual domains contribute to CLASP function is poorly understood. We generated full-length recombinant human CLASP and a series of truncation mutants and found that the two N-terminal TOG domains make the strongest contribution to MT polymerization and bundling and mediate binding to the MT lattice, while plus end tracking requires interaction with EB1. The C-terminal coiled-coil mediates dimerization and association with many other factors, including kinetochore motor CENP-E, while other regions near the S/R region interact with the spindle mid-zone protein PRC1 and chromokinesin Xkid. Importantly, only the full-length protein was able to rescue spindle assembly in *Xenopus* egg extracts depleted of endogenous CLASP. Even minor amounts of CLASP that was missing its C-terminal domain, led to dramatic spindle phenotypes, indicating that proper localization of CLASP is essential for its activity during mitosis.

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ABBREVIATIONS

APC	Adenomatous Polyposis Coli
CHD	calponin homology domain
CLASP	Cytoplasmic Linker Associated Protein
Clip-170	Cytoplasmic linker protein
crTOG	cryptic TOG
c-terminus	carboxy terminus
EB1	Ending Binding Protein 1
EBH	EB homology domain
GSK3b	Glycogen synthase kinase beta
GST	Glutathione-S-transferase
HEAT	huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor
HEK293T	Human embryonic kidney cells subclone 293T
MACF	MT-actin cross linking Factor
MAP	Microtubule Associated Protein
MT	Microtubule
MTOC	Microtubule Organizing Center
PEI	polyethyleneimine
SxIP	Ser-x-Ile-Pro
TOG	tumor over-expressed gene

ACKNOWLEDGEMENTS

This body of work marks an important place in an intellectual journey that I have been on for much of my life. It would not be possible without the help and support of numerous people that I have had the privilege to work with along the way. First, I would like to thank my graduate advisors Rebecca and Eva. Their patience and generosity to allow me to work on such interesting questions for past six years has left an invaluable mark on my professional development. Without their help and support, this would not be possible. I'd also like to thank my committee members, David and Dan for suggestions and guidance in helping me complete this work.

Additionally, a great many thanks must go out to my fellow lab members, staff members and students, past and present, for their help, advice and effort in supporting my project and helping me conduct my experiments. .

Lastly, I'd like to thank my family and friends for their unwavering support. I'd like to especially thank my father for teaching me to always be curious.

Chapter 1

Introduction

In the first edition of the Molecular Biology of the Gene in 1965, J.D. Watson wrote "The regular lining up of chromosomes during the metaphase stage is accompanied by the appearance of the spindle …. Much of the spindle region is filled with long, thin protein molecules, which some people think are similar to the contractile proteins of muscle" (Watson et al., 1965). 50 years later, we now know that these thin protein molecules are microtubules (MTs), structures that are indispensable to many cellular functions, including cell division.

During mitosis, faithful segregation of chromosomes to daughter cells requires the assembly of the spindle, a complex macromolecular machine assembled from MTs and MT associated proteins (MAPs). This complex machine employs a dynamic set of proteins that are capable of generating forces, probing the cellular space and responding to cues from the cell(Amos, 2005; Saleem, 2010). Proteins regulating these dynamic processes are critically important, including the subject of this work, CLASP1. CLASP (Cytoplasmic Linker Associated Protein), represents a class of key MT regulatory proteins with a variety of functions in the assembly and maintenance of the spindle and its ability to segregate chromosomes.

Microtubule Structure and Function

MTs are composed of α tubulin and β tubulin dimers that assemble in a head-to-tail fashion. These protein dimers form linear chains called protofilaments that further assemble, via lateral contacts, into sheets and then into hollow tubes about 25 nm in diameter. MTs observed *in vivo* appear to contain 13 protofilaments and adopt a B lattice type, a configuration of the MT where adjacent protofilaments associate in a staggered pattern with two α tubulins of one protofilament interacting with two *b* tubulins of another protofilament (Nogales, 1999).

Due to the polar nature and head-to-tail assembly of α and β tubulin dimers, the MT itself is polar. One end is classified as the minus end, which is defined as the end with a comparatively slower rate of polymerization and de-polymerization. The minus ends of MTs are often found near the nucleus in the cell, closely associated with centrosomes or MT organizing centers (MTOC). MT plus ends, however, emanate into the cell and are characterized by rapid transitions between assembly and disassembly. The conversion of the MT from a growing state to a shrinking state is termed a catastrophe, while the opposite switch from shrinking to growing is termed a rescue. Additionally, the MT can experience a third state called a pause, in which the MT neither grows nor shrinks. The rapid switching of MTs from one state to another is called dynamic instability, a process that is critical to the function of MTs in the cell. The dynamic properties of MTs allow them to probe various parts of the cell, growing and shrinking to respond to different stimuli and changing conditions (Heald, 2002; Howard, 2009). Polymerization and de-polymerization can also generate force, used to push and position organelles and smaller cellular vesicles or to pull chromosomes during mitosis (Inoue, 1997).

Role of +TIPs in regulating MT behavior

The +TIP family of proteins was first characterized by observation of "comet like" tracking behavior on the plus ends of MTs. Time lapse video microscopy of the +TIP EB1 (End Binding Protein 1), covalently labeled with Alexa-488 dye, shows an example of this unique tracking movement, as the protein travels from areas near the MTOC out toward the periphery, similar to exploding fireworks (Morrison, 2002; Tirnauer, 2002). Fixed and live imaging of cells both *in*

vitro and *in vivo* have revealed many more +TIPs that share this unique and interesting behavior, including the CLASP family of proteins (Maiato, 2003).

While the +TIP family is highly diverse, there are several similar functional characteristics of these proteins. Their affinity for the MT plus-end makes them inherent regulators of dynamic instability. It has been proposed that +TIPs can associate with specific features of the MT, including the curved sheet of the plus end as well as the MT seam. An ability to selectively recognize these features allows them to accumulate selectively at the plus end (Nogales, 2006). Via these specific interactions, many +TIPs such as EB1 and Cytoplasmic linker protein-170 (Clip-170) can promote MT rescues and decrease catastrophe frequency (Bieling, 2008; Katsuki, 2009). Furthermore, another +TIP, XMAP215, can act as a 'MT polymerase,' greatly increasing the rate of MT assembly while others, such as CLASP and the Adenomatous polyposis coli protein (APC) appear to selectively stabilize certain MTs in the cell (Brouhard, 2008; Kirik, 2007; Louie, 2004). Interestingly, it has been observed that for many +TIP proteins, the 'comet' formed by +TIP binding is much longer than 1 µm, the typical length of curved tubulin sheets found at a then tip of growing microtubule. This has been observed for nearly all members of the +TIP family, including CLASP proteins (Galjart, 2010; Jiang, 2011; Mathe, 2003; Pereira, 2006) .Thus, while +TIPs appear to be enriched at the plus ends of microtubules, they are not found there exclusively and are capable of binding elsewhere on the MT lattice.

+TIP regulation of tubulin assembly occurs in many different functional contexts. A common theme is that +TIPs act as linking factors between MTs and various intracellular structures. Regulated processes include cell motility and lead edge dynamics, organelle and membrane vesicle positioning and transport, and mitotic spindle function, among others (Ambrose, 2007; Kirik, 2007; Kumar, 2005) . +TIPs can act to physically link different cellular components, for example MACF links actin to MTs, or they can act as force couplers, as in the case of Clip-170 which helps to tether membrane vesicles to the growing ends of MTs during vesicular transport (Bernier, 2000; Lomakin, 2009; Pierre, 1992).

CLASP and the +TIP family

Although CLASPs and other members of the +TIP family have the ability to accumulate at the growing plus ends of MTs, there is no common structural motif responsible for this behavior and consequently there seem to be multiple molecular mechanisms of +TIP-MT interaction. Crystal structures, bioinformatics and biochemical analyses from numerous studies have identified a few key domains conserved among some family members (Plevin, 2008; Slep, 2005; Slep, 2007). In addition to having one or more of these domains, almost all +TIPs interact with other members of the family, forming multi-subunit protein complexes containing multiple MT binding domains that must somehow be coordinated for specific functions (Niethammer, 2007). The CLASP family of proteins represents a class of large multi-functional proteins that contains multiple MT binding domains as well as binding sites for other +TIP proteins. While in some instances they appear to share some functional similarities or overlap with proteins containing similar domains such XMAP215, CLASP appears to coordinate a unique combination of MT binding domains to perform its various functions (Galjart, 2005).

EB1 and the +TIP family

One the most extensively characterized members of the +TIP family is the EB1-like protein family. These relatively small proteins are found across multiple organisms and are able to bind to a host of other +TIPs, including CLASP (Tirnauer, 2002). Association occurs through a conserved motif in its carboxy terminus (C-terminus) called the EB homology domain (EBH). This domain is capable of recognizing various domains of other +TIPs including the SxIP motif (discussed in the next section) and Cap-Gly domains, which are found in the Clip family. At the amino terminus, EB1 proteins contain a highly conserved calponin homology domain (CHD) that is responsible for recognizing the end of growing MTs by interacting with tubulin through electrostatic and hydrophobic forces (Honnappa, 2009; Plevin, 2008; Vaughan, 2005).

From a functional standpoint, EB1 proteins play a critical role in +TIP tracking behavior. In vitro studies have shown that these proteins can act as "master trackers" capable of selectively binding the plus-ends of MTs in reconstituted systems. EB1 is therefore thought to be a 'core' +TIP especially when complexed with any number of +TIPs including CLASP. It is thought that EB1 proteins recognize distinct structural elements of the MT lattice, including the seam and portions of the open sheet formed at the end. EB1s appear to promote growth of MTs, perhaps by stabilizing the growing ends. In the context of proteins that can associate with EB1, it is thought that these larger +TIPs 'hitchhike' along with EB1, which may increase affinity or specificity of associated +TIPs for certain sites on the MT lattice (Vaughan, 2005; Vitre, 2008).

CLASP contains a SxIP motif

As it became evident that EB1 is often a critical component of +TIP complex formation and function, biochemical and crystallization studies have revealed a small conserved Ser-x-Ile-Pro (SxIP) polypeptide motif responsible for interaction with the EBH domain. This motif is found in many large multi-domain proteins such as CLASPs, adenomatous polyposis coli (APC) and the MT-actin cross linking Factor (MACF). This small motif, generally embedded within a stretch of basic residues such the Ser/Arg region in CLASP, is thus proposed to act as a "localization signal" for these +TIP proteins. It is uncertain however, how this signal is coordinated with other MT binding domains either in a +TIP such as CLASP or from other +TIPs in a particular complex (Honnappa, 2009). In the CLASP family, CLASP1 appears to have one SxIP motif while CLASP2 has acquired two, thus enabling it to bind to two EBH domains and inherently increase its affinity for EB1 . Interestingly, it has been found that SxIP interaction with EB1 is negatively regulated by phosphorylation of the region by a protein kinase, Glycogen synthase kinase beta (GSK3b), providing a potential mechanism or switch for controlling the CLASP-EB1 interaction and possibly CLASP TIP tracking behavior (Galjart, 2005; Watanabe, 2009).

CLASP may contain TOG domains

In addition to the presence of the SxIP domain, the CLASP family also contains multiple putative TOG (tumor over-expressed gene) domains which are found in a closely related +TIP ch-TOG (*Xenopus* homolog XMAP215) (Widlund, 2011). On a cellular level XMAP215 and CLASP appear to share some functional similarity, localizing to MT plus ends in interphase while promoting MT growth (Gergely, 2003; Kronja, 2009; Mimori-Kiyosue, 2005). Knock

downs of *Drosophila* homologs of these proteins produce similar mitotic defects, including smaller spindles with dramatically reduced MT content (Brittle, 2005 ; Maiato, 2005). Structurally, it is thought that this activity is due to the function of multiple arrayed TOG domains, which have been studied in detail for XMAP215 and other homologs of this family of +TIPs. In XMAP215, there are 5 TOG domains arrayed in a single monomer, alternating between net positive and net negative domains (Slep, 2009). In the yeast S. cerevisae, the homolog of XMAP215, STU2 , behaves as a dimer, with 2 TOG domains on each monomer coordinated to produce a functional complex for 4 TOG domains (Al-Bassam, 2006). Despite this divergence in sequence, it is clear that arrays of multiple of TOGs, found in either a *cis* or *trans* arrangement is critical to function. Crystal structures of some of the individual domains show them to be large paddle like structures with defined 'faces' that may be important for tubulin interactions (Al-Bassam, 2007). TOG domains are characterized by repetition of six HEAT (huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor). From crystal structures, each canonical HEAT repeat consists of two anti-parallel *α* helices of roughly the same chain length and the net linear arrangement of these repeats produces a TOG domain of dimensions of roughly 20 x 30 x 60 Å (Slep, 2007).

Studies investigating the nature of interaction between TOG domains and tubulin have shown that at least two TOG domains are required for tubulin binding. Additionally, mutations within the conserved loops that separate the domains can drastically decrease affinity for the MT. From the relative size of the domain and tubulin subunit (~40 Å), it is proposed that when at least two TOG domains are arrayed in tandem, one is specific for *α* tubulin and one for *b* tubulin. This might help to partially explain the pattern observed in XMAP215 of alternating net charges of the 5 TOG domains (Slep, 2009; Slep, 2010; Slep, 2007).

Single molecule studies have revealed interesting functional aspects of XMAP215. In simple reconstituted systems, XMAP215 acts as a "MT polymerase," capable of increasing the rate of growth of the MT plus ends by facilitating the processive addition of tubulin dimers. The mechanism by which this occurs is complex; the arrayed TOG domains appear to bind a single tubulin dimer, which is ferried to the MT plus end via the C-terminus by diffusion along the MT lattice where the free tubulin incorporated. XMAP215 concentrates at both the plus and minus ends of the MT, but this selectivity appears to be EB1 independent. It is proposed that the molecule is able to bind a single tubulin dimer while surfing on the MT lattice. This model suggests that parallel TOG domains coordinate binding similar to the function of formins in actin polymerization. One set of domains binds the polymer, while the other binds free tubulin and incorporates it into the lattice (Brouhard, 2008).

In the context of CLASP, it is unclear how many TOG domains these proteins contain and if they share a similar function with XMAP215. Interestingly, CLASP at first appears to only have one canonical TOG domain in the amino-terminus region, suggesting a different purpose for the TOG than the arrayed configuration of XMAP215. However, with closer inspection, it is thought that CLASP contains two other cryptic TOG (crTOG) domains elsewhere in its sequence. While the sequence of these domains is somewhat diverged from XMAP215 TOG domains, there are homologous sequence elements similar to canonical TOG domains that contain predicted sets of twelve *α* helices and conserved intra HEAT residues in adjacent loops. These initial predictions make it likely that while XMAP215 and CLASP both contain TOG domains there remains fundamental differences in how these molecules regulate MT behavior (Al-Bassam, 2010; Galjart, 2005).

Mechanisms of CLASP-MT regulation

Recent evidence using single molecule techniques has shed some light on possible mechanisms of action of the S. pombe CLASP homolog in MT regulation. Experimental evidence reveals that CLASP is able to bind free tubulin. CLASP wraps around tubulin dimers, producing rounded complexes, reminiscent of similar complexes formed between XMAP215 monomers and free tubulin (Al-Bassam, 2010). In reconstituted systems however, there are key differences in regulation of MT dynamics. Whereas XMAP215 increases the rate of polymerization of MT ends, CLASP promotes rescue events. Furthermore, there are fundamental differences in the activity of the C-termini of either protein. This domain in XMAP215 allows the protein to diffuse along the lattice and eventually accumulate at the plus end of the MT, while CLASP is observed to bind stably along the lattice (Brouhard, 2008). From other studies, it has been shown that the C-terminus of CLASP is important in the recruitment of other factors, including the +TIP Clip-170 and other MT binding proteins (discussed in more detail in next section) (Grallert, 2006).

It is proposed that when MTs undergo catastrophe and protofilaments peel back from the lattice, bound CLASP molecules can act to stop de-polymerization. Studies of MTs both in vitro and in vivo suggest that CLASP is able to promote the pause state after a catastrophe. Through an unknown mechanism, CLASP is thought to release free tubulin during the pause state to facilitate growth of the plus end, acting to increase the local concentration of tubulin in the space surrounding the MT end. Despite this early model of how CLASP might work, there are still significant questions surrounding the mechanism, especially with regard to domain architecture and how this relates to activity as well as possible mechanism for CLASP plus end tracking (Akhmanova, 2001; Al-Bassam, 2010; Ambrose, 2008).

CLASP is a Multifunctional +TIP

CLASPs were originally identified using yeast two-hybrid assays with the CLIP family of +TIPs, indicating direct binding. There are two major isoforms expressed in humans, with CLASP1 ubiquitously expressed and CLASP2 enriched in the brain. While there are other minor variants of the two proteins, both CLASP1 and CLASP2 appear to be functionally similar (Galjart, 2005; Goodson, 2006). Previous studies have shown CLASP to be located throughout the cell, participating in wide variety of cellular activities. In HeLa cells, it has been found to co-localized with membranes, including the vesicles of the Golgi apparatus, mitochondria as well as the cellular cortex, where it is thought to be involved in organelle movement and positioning. It has also been found at the lead edge of migrating fibroblasts, acting to bundle and stabilize MTs as well as linking MTs to actin filaments (Chiron, 2008; Lansbergen, 2006; Mimori-Kiyosue, 2005; Wittmann, 2005). Perhaps CLASP's most studied functional aspect is its role in various phases of mitosis. In multiple systems, CLASP has been found to be involved throughout mitosis, contributing to chromosome congression, spindle bipolarity, MT attachment to kinetochores and central spindle function in late anaphase/cytokinesis (Hannak, 2006).

Roles of CLASP in mitosis

Although CLASP appears to play numerous roles in the cell, the majority of studies to date have focused on various aspects of its function in cell division. CLASP has been observed to

have a direct regulatory role on MT dynamics, acting as a rescue factor. While the mechanism is unknown, it is possible that CLASP can aid in tubulin addition at the MT plus-end. It is also found at the kinetochores, large protein complexes that facilitate attachment of MTs to chromosomes. It is here that CLASP is thought to assist in attachment of MTs to other kinetochore proteins, while promoting MT growth. CLASP is thought to stabilize a specific set of MTs that interact with the kinetochore (k-fibers) and help promote attachment in spindle assembly checkpoint pathway (Mimori-Kiyosue, 2006; Pereira, 2006; Sousa, 2007). Depletion of CLASP homologs from *Xenopus* eggs produces dramatic chromosome congression defects, whereby chromosomes are mis-aligned during metaphase. This is also seen in mammalian cells where CLASPs are found to be critical in the prevention of aneuploidy in knock-out animals. It is unclear mechanistically however, how this protein is functioning during chromosome congression (Hannak, 2006).

Interestingly, CLASP is also found both at the spindle poles with MT minus ends, where it is thought to stabilize short MTs before they are lost from the MTOC. It appears that CLASP MT stabilizing activity can be differentially localized to act on different subsets of MTs in the spindle (Lansbergen, 2006; Stramer, 2010).

Additionally, CLASP has also been observed to be associated with specific highly stable subsets of MTs found in overlapping arrays, especially at the spindle poles and in the central spindle during anaphase. CLASP is thought to be important in regulating not only dynamic MTs, but also highly stabilized MT bundles that are important for spindle maintenance and integrity. What determines CLASP activity and how it is differentially localized throughout the spindle remain important questions (Drabek, 2006; Laycock, 2006; Maiato, 2005; Maiato, 2003).

***Xenopus* egg extract as model system**

In order to address questions regarding CLASP's role in mitosis, I used the *Xenopus* egg extract system as an experimental tool. This system provides a powerful biochemical model in which spindle assembly can be reconstituted and manipulated *in vitro*. A reconstituted system has many advantages, including the ability to control progression through mitosis and arrest at metaphase, and the ease with which conserved factors can be depleted from or added to the extract.

In brief, extract is prepared through the centrifugation of unfertilized *Xenopus* eggs and isolation of the crude cytoplasmic fraction. Prior to fertilization, the *Xenopus* egg is arrested in metaphase of the second meiotic division. The metaphase-arrested state of the cytosol is preserved and the system can be driven into interphase by calcium, which promotes the replication of added sperm chromosomes. Return to metaphase can be induced and mitotic spindles assemble from endogenous proteins stockpiled in the egg. The system is fundamentally based on biochemistry and is not accessible to genetic manipulation as are most other systems. To remove gene products from the system, immune-depletion via protein specific antibodies is used. To add gene products, they must be made exogenously, such as through expression of recombinant proteins (Hannak, 2006).

To address questions regarding the activities of CLASP domains and the ability of CLASP to function in spindle assembly I had to first successfully produce recombinant versions of CLASP protein and various CLASP domains (Chapter 2). After overcoming various technical hurdles, I was able to assay various activities in *Xenopus* egg extract with these reagents, observing their behavior in depletion/rescue experiments to assay function as well as general

roles in regulating MT dynamics on assembled spindles. Additionally, *Xenopus* egg extract was also used as a medium to generate dynamic MTs with human centrosomes to assay tracking on the plus ends of MTs (Chapter 3).

Electron microscopy as a tool

In addition to functional experiments in *Xenopus* egg extract, I also attempted to use electron microscopy (EM) to gain structural information about CLASP and its interaction with MTs. EM is a tool with a specific advantage in providing structural data about high molecular weight complexes, such as MTs or MAPs bound to MTs (Nogales, 2010). While X-ray crystallography has produced interesting structures of TOG domains, it is unknown how these domains interact with tubulin and MTs and how this relates to their function. High resolution cryo-electron microscopy could provide a detailed picture of how CLASP interacts with tubulin lattice on the molecular level, yielding direct clues to its mechanism of action. Unfortunately, cryo-electron microscopy requires samples with crystalline like properties, or regular repeating units that can be averaged (Downing, 2010). Despite generating the reagents to do this, CLASP samples never behaved in a way suitable for this method. While this was a setback and perhaps represents a goal for the future, I used negative EM and other biochemical based assays, specific for MTs and MAP regulators, to generate some other clues about molecular interactions between CLASP and MTs, complementing our functional studies in *Xenopus* egg extract (Chapter 4).

Chapter 2

Biochemical Purification of CLASP and CLASP Domain Fragments

Introduction

To date, the majority of studies of CLASP have been performed using a combination of genetic and *in vivo* approaches, providing evidence of function in the context of many other factors in the cell. In order to study CLASP structure and function outside the cell, for experiments *in vitro* and the *Xenopus* egg extract system, full length CLASP protein had to be produced through recombinant expression and biochemical purification. At the time, CLASP protein had never been successfully purified for these types of analyses. Furthermore, little was known about the domain architecture of CLASP and how different domains contribute to the various functions of the protein. To address this experimental goal, domain mapping was performed through expression and purification of numerous fragments of CLASP. Each fragment had to be designed based on hypothetical placement of various domains using different informatics tools. Successful generation of these reagents would be essential for almost all the experiments performed.

Generation of these reagents proved not to be trivial, given both the biochemical nature of CLASP and that no known prior purification scheme existed. As a large, basic protein, and as later learned, one highly susceptible to proteolytic degradation, CLASP proved to be extraordinarily difficult to purify in an active form and without noticeable degradation products. Effort was directed at optimizing both expression and purification conditions, ranging from testing different expression systems, affinity tags and polylinkers to a variety of purification schemes to help preserve function of the protein. Ultimately, CLASP and a small, but diverse library of CLASP fragments were successfully produced for downstream analysis.

Results

Purification of Recombinant CLASP from HEK293T Cells

Previous efforts attempted to isolate recombinant Xorbit protein using baculovirus/insect cell systems. Xorbit was the logical choice, as the C-terminus had already been characterized and the protein would be used for experiments in *Xenopus* egg extract. While expression of the C-terminal domain of Xorbit had proved successful in E. coli, the full length protein was found not to express in bacteria, due either to the size of the theoretical protein (~180 kd) or requirement of various chaperones or accessory proteins not found in prokaryotic cells for proper expression or folding. Surprisingly, attempts in insect cells also proved unsuccessful, for unknown reasons. The protein was successfully expressed as an N-terminal fusion with a Glutathione-S-transferase (GST) affinity tag, however subsequent purification yielded only small quantities of protein, with little or no activity. The protein appeared to be highly degraded. Possible factors included: instability of the Xorbit protein itself, as has been observed for various *Xenopus* proteins in comparison to other homologs; unusually high proteolysis in insect cells; or instability of the protein as a result of the GST n-terminal fusion.

To address these potential issues a new strategy was adopted that included focus on the human homolog CLASP1, and recombinant transient expression with smaller affinity tags in HEK293T cells. To address the degradation, a vector containing two affinity tags, one at the N-terminus and one at the C-terminus were chosen. The bio-ease tag and polyhistidine tags were chosen for their small size and specificity, potentially preventing interference with activity of

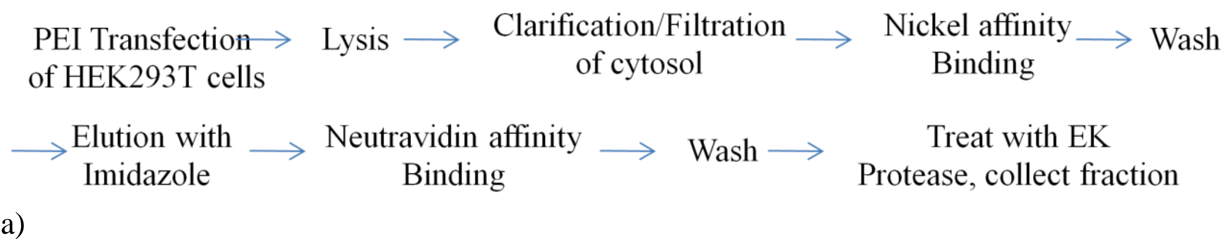
domains located at either terminus. HEK293T cells were chosen as an expression system due to several potential advantages over insect cells. There was the possibility of improved stability of the protein using a human cell line. Additionally, transient transfection afforded greater speed to express and purify constructs and evaluate stability and solubility of different constructs. Lipid mediated transient transfection allowed for rapid screening of different affinity tag fusions, various length polylinkers, and variable length CLASP fragments, as compared to the time needed to generate a large number of baculo-viruses for insect cell expression (Raymond, 2011).

Based on alignment of amino acid sequences using informatics tool PRALINE (http://www.ibi.vu.nl/programs/pralinewww/), the CLASP family shares a high degree of homology across multiple species, ranging from yeast to human. Specifically, Xorbit and CLASP1 share 85% homology, providing basis for the decision to use CLASP for biochemical characterization as well applications with *Xenopus* egg extract. It was later demonstrated that CLASP is able to rescue spindle formation defects in *Xenopus* egg extracts depleted of Xorbit validating this decision (See Chapter 3).

The gene for full length CLASP (AAI32724.1) was obtained from an I.M.A.G.E clone library and subsequently cloned into pMT/BioEase-DEST vector (Invitrogen), generating a fusion construct with N-terminal bio-ease tag and C-terminal six poly-histidine tag. The bio-ease tag is a 72-amino acid sequence from *K. pneumoniae* that directs *in vivo* biotinylation of a specific lysine residue. DNA of this fusion construct was transfected into HEK293T (Human embryonic kidney cells) (ATCC CRL 1573, sub-clone 293 (EBNA) (Inivtrogen)) cultured in 10 cm plates to a density of 6-10 x $10e^5$ cells/ml in RPMI 1640 medium supplemented with 5% fetal bovine serum (Life technologies). DNA was prepared for transfection by mixture in 10:1 wt/wt ratio with 60 kDa polyethyleneimine (PEI) 50% wt/vol solution (Sigma-Aldrich) in unsupplemented media. DNA-PEI complexes were allowed for form for 30 min, added drop-wise, and allowed to mix with cells for 24-48 hours. Cells were harvested from plates by gentle pipetting, washed once in cold phosphate buffered saline (PBS buffer), pelleted (1,000 RPM, JS-4.0 rotor; Beckman) and flash-frozen in liquid nitrogen.

To purify CLASP protein, a double affinity purification was used based on the bio-ease tag and polyhistidine tags. The HEK293T cell pellet was lysed using a glass homogenizer and lysis buffer (100 mM Tris, pH8.0, 500 mM NaCl, 5% glycerol, 1 mM DTT, EDTA protease inhibitor cocktail, 1 tablet per 50 ml volume of lysate, Roche). Lysate was further clarified by centrifugation to remove nuclei, organelles and membranes by centrifugation (10,403 RCF avg) for 20 min at 4 C. The supernatant was filtered through 0.4 µM PES membrane filter (Nalgene) and applied to HIS-Select Nickel Affinity Gel (Sigma), pre-equilibrated with lysis buffer. The clarified cell lysate was incubated for 3 hours, sedimented (5000 RPM, SX4250; Beckman-Coulter) and washed three times with wash buffer (PBS buffer, 1 mM DTT, EDTA free protease inhibitor cocktail). Protein was eluted from the affinity gel in elution buffer (50 mM Tris pH 8.0, 500 mM NaCl, 1 mM DTT, 5% glycerol, 200 mM Imidazole) (Fig. 2.1a).

In the subsequent steps, CLASP protein was further isolated via affinity purification with Neutravidin Agarose Resin (Pierce). Fractions from the Nickel column containing protein were pooled and batch bound to NeutrAvidin resin for 1 hour. The resin was sedimented (500 RPM, SX4250; Beckman-Coulter) and washed 3 times with wash buffer). Protein was eluted in batch by incubating with elution buffer supplemented with enterokinase (EK) protease (Fig. 2.1b).



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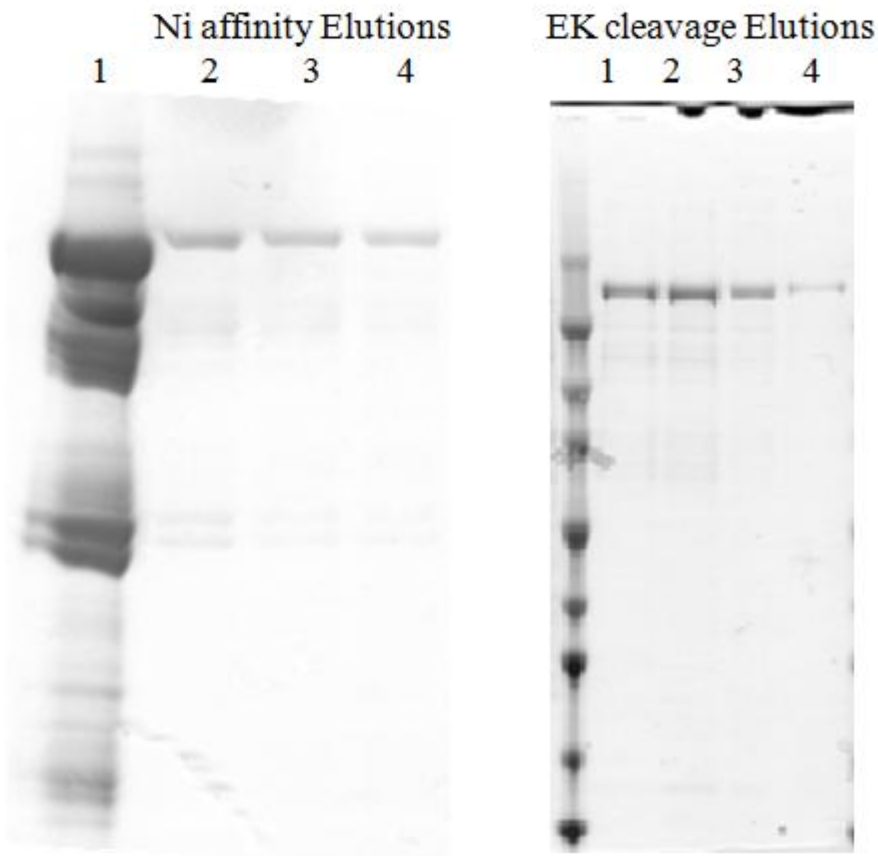


b)

Figure 2: a) Purification scheme b) gel products after Nickel (Ni) purification and subsequent purification via enterkinase protease (EK) cleavage off of Neutravidin affinity beads separated by SDS PAGE 10% Bis-Tris.

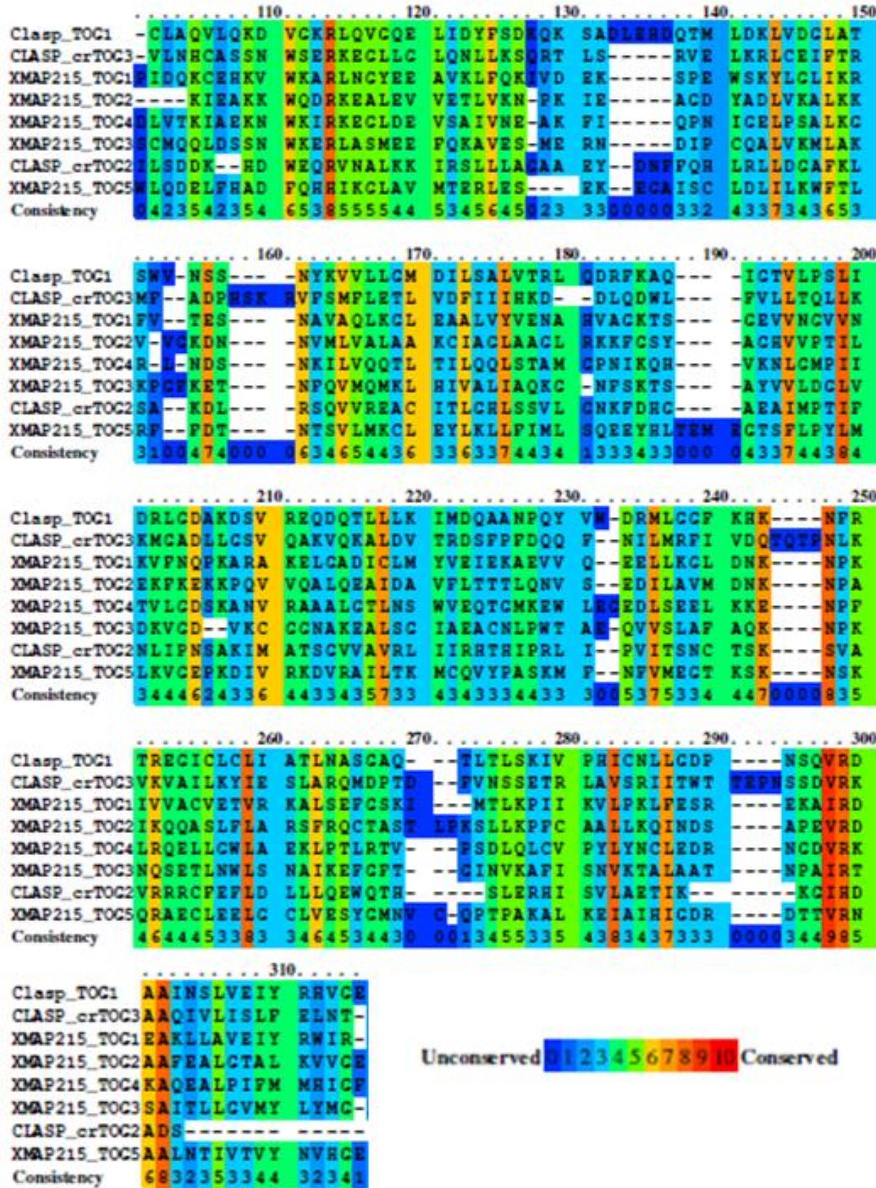
Design of CLASP fragments

Successful purification of active CLASP allowed the possibility of in-depth domain mapping of the full length protein. Given the lack of information about the structure of CLASP and where putative domains might exist, informatics tools were used to provide a general framework for a domain-mapping strategy.

As with most +TIPs, there was high likelihood that CLASP contained various coiled-coiled elements in its secondary structure. The program COILS (http://www.ch.embnet.org/software/COILS_form.html) was used as a tool to analyze different ranges of sequence (14, 21, 28 residues) and compares them to a database of known parallel two-stranded coils. A probability score was then generated based on score differences between globular and coiled-coil proteins. While this information did not reveal specific identification of domains in CLASP, it did provide clues to certain boundary areas. From the plot (Fig 2.3a) there is a high probability of coiled-coil region in the C-terminus of the protein. This area corresponds to a region in Xorbit previously characterized and shown to have functional activity, binding a number of other factors including the +TIP Clip-170. Furthermore, there appear to be short sequences with high probability throughout the protein that may correspond to other domain boundaries, especially in the N-terminal region of the protein.

Further informatics analysis of sequence alignment of CLASP and other another known TOG domain containing protein XMAP215 appear to indicate the presence of multiple TOG domains throughout CLASP. The first TOG domain (TOG1) appears to share the greatest homology with sequences from XMAP215 family. However, two other TOG like domains, or cryptic TOGs (crTOG) also seem to be present in CLASP1. While the residues of the α helices of the HEAT repeats share little conservation, there is some conservation of residues in putative loop regions that are spaced similarly as canonical TOG domains. Only domain mapping and functional testing of these regions would indicate whether these are actual TOG domains (Fig 2.2a).

Another multiple sequence alignment between proteins thought to contain the EB1 binding motif SxIP also indicates the present of 1 or more of these sequence elements in CLASP1 (Fig 2.2b)



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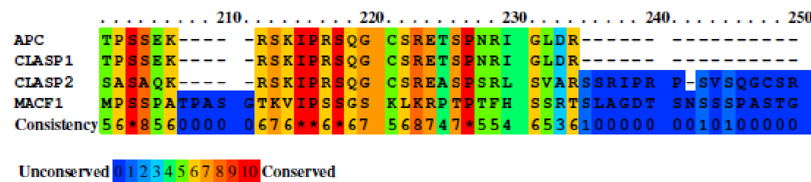
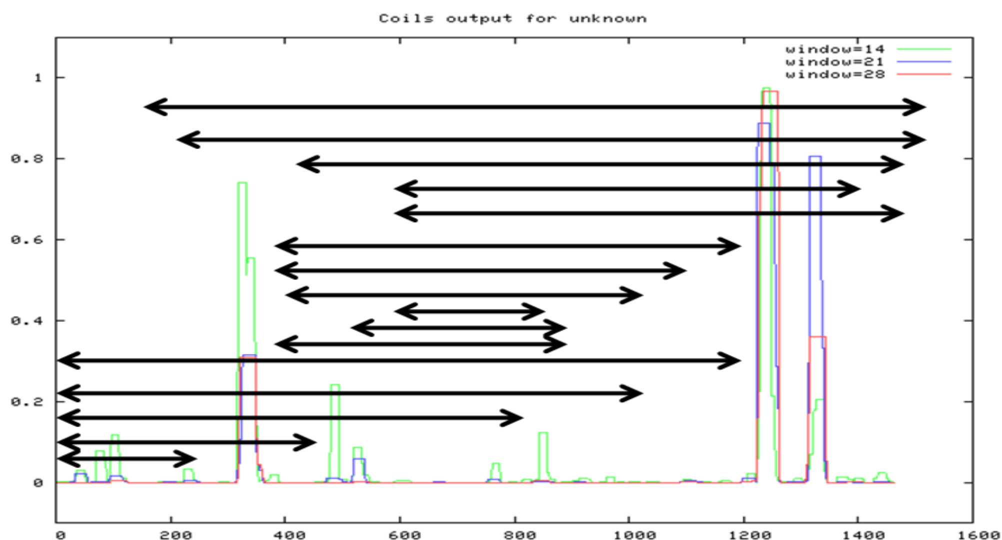


b)

Figure 2.2: Multiple Sequence Alignments of a) TOG and b) SxIP domains (PRALINE). Degree of sequence homology is indicated by color (blues – unconserved; green/yellow moderately conserved; reds – conserved).

This basic informatics analysis provided a general framework for the design of multiple CLASP constructs. Systematically, different fragments of CLASP were cloned based on rough predicted boundary regions (Fig 2.3b). Multiple fragments were cloned into identical pMT/BioEase-DEST vectors, and expressed and purified in a similar fashion to the full length protein (described above). The full length protein was either analyzed by gel electrophoresis or by western blot to determine solubility, purity and relative stability. Constructs with the highest expression and apparent stability were chosen for downstream analysis.



a)

Construct	AA length	Expected Protein size	Predicted PI
CLASP662-850	188	19.26	10.86
CLASP510-804	294	30.88	10.6
CLASP450-871	421	45.40	10.04
CLASP450-1051	601	66.00	9.8
CLASP391-1171	780	85.03	9.59
CLASP125-1171	1046	114.44	9.42
CLASP662-1171	509	55.70	9.15
CLASP125-998	873	114.54	9.42
CLASP1-1051	1051	115.75	9.42
CLASP1-1171	1171	128.44	9.42
CLASP1-871	871	95.26	9.48
CLASP1-804	804	87.60	9.49
CLASP1-662	662	72.77	9.22
CLASP1-998	998	109.94	9.41
CLASP391-1463	1072	117.82	9.15
CLASP450-1463	1013	111.49	9.03
CLASP510-1463	953	104.52	9.08
CLASP662-1463	801	88.50	8.36

b)

Figure 2.3: Design and expression testing of various CLASP fragments. a) Prediction of Coiled-Coil regions of CLASP1 using COILS. Arrows indicate fragment in relationship to predicted Coiled-Coil regions b) Table of protein fragments generated and tested for expression/purification. Fragments (highlighted in red) were chosen for further characterization based on sequence coverage and stability/expression levels.

Purification of CLASP from Insect Cells

Despite the fact that HEK293T expression appeared to produce active protein, yields of almost all constructs were very low. Producing protein in enough quantities for biochemical and *Xenopus* egg extract experiments would have been both cumbersome and costly. Typical yields from 20 large dishes of cells produce only 200 – 250 µg of protein for the most stable constructs. Due to this limitation in generating enough material for downstream experiments, it was important to test purification of CLASP and other protein fragments using baculovirus/insect cell expression where yields several orders of magnitude greater could be possible.

Full length CLASP and fragments, selected based on data from HEK293T expression tests, were re-cloned into insect cell expression vectors. These constructs were produced using customized pFAST-BAC vectors (Invitrogen), in which a small 10 amino acid StrepII tag and polylinker had been inserted for N-terminal fusion, while a 10 polyhistidine tag and polylinker were inserted for C-terminal fusion. The StrepII tag was chosen for several reasons. While the bioease tag had worked well for expression in the HEK293T cells, no existing tag has been used previously in insect cell expression. It is not certain if insect cells have the same type of machinery for *in vivo* biotinylation. The StrepII tag is also a relatively small tag, which would avoid interference with domain function at the N-terminus. It is also an affinity tag with high specificity for binding ligand during purification thus capable of yielding highly pure protein (Mueller, 2003). In addition to changing the N-terminal tag, different buffer components were also tested. Downstream applications, especially experiments involving microtubules or *Xenopus* egg extract are somewhat sensitive to addition of Tris buffer. Purification of full length CLASP was purified in various sample buffers to determine conditions for optimum protein stability, activity and compatibility. Additionally, CLASP proteins were cloned with similar StrepII tag and 10X polyhistidine tags along with a C-terminal eGFP gene, creating fusion products: StrepII – CLASP-eGFP-10XHIS.

CLASP proteins affinity purified were using both StrepII and polyhistidine tags. Baculovirus for each construct was generated using published methods. For expression SF9 insect cells (Expression Systems) were infected with virus and left to incubate for 48 – 56 hours. Insect cells were harvested, washed once in cold PBS buffer, pelleted (1,000 RPM, JS-4.0 rotor; Beckman) and flash-frozen in liquid nitrogen.

The cell pellet was lysed using a glass homogenizer and lysis buffer (100 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.2% Tween-20, 1 mM DTT, EDTA protease inhibitor cocktail, 1 tablet per 50 ml volume of lysate, (Roche)). Nickel affinity purification was performed as described above. StrepII affinity purification was performed by applying Streptactin affinity gel (Qiagen) to pooled fractions of eluted protein from the nickel gel elutions. After 1 hour incubation at 4 C, the gel was washed with cold 1X PBS and eluted with elution buffer (100 mM HEPES, pH 7.5, 150 NaCl, 0.2% Tween-20, 1 mM DTT, EDTA, 10 mM d-biotin (Sigma). Fractions were analyzed by SDS-PAGE, stained with Sypro-Ruby and imaged using an *A*-Imager (Bio-rad) (Fig. 2.4a,c).

Discussion

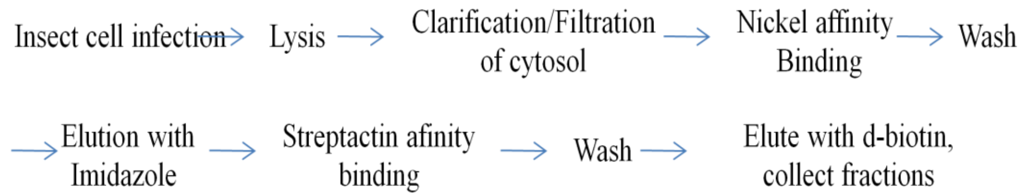
Successful generation of full length CLASP protein was a critical step in the development of this project. After numerous strategies and attempts at purification, I was able to isolate active

full length protein, in addition to various protein domains. CLASP proved to be a difficult protein to purify, not only due to its size but also its tendency to aggregate or degrade. At nearly all points during the purification process, various parameters had to be optimized to circumvent these technical obstacles.

Early in the process, degradation appeared to be significant problem and more traditional methods to remove contaminating degradation products such as gel filtration proved to be difficult with this protein. Using a double tag affinity purification system, especially with the small but specific polyhistidine and StrepII tags was a critical decision in producing full length protein. Double affinity purification allowed for more rapid isolation of highly pure protein that could be concentrated during the purification process (Fig. 2.4c).

Another critical aspect of the purification process was the use of a mammalian expression system with HEK293T cells to test the stability of different constructs. This method ultimately produced high quality protein without the need for generation of numerous baculo-viruses for insect cell based expression. While it ultimately proved to be insufficient for producing protein in quantities needed for downstream assays, it did provide a necessary starting point and complement system for successful purification of CLASP protein.

Successful generation of CLASP protein should provide opportunities to study aspects of the protein not previously pursued, as nearly all previous studies have been based in a cellular context. The reagents I produced could be instrumental to future studies, even outside the scope of this project, providing the tools necessary for in-depth structural, single molecule and biophysical experiments needed to help probe the function of CLASP.



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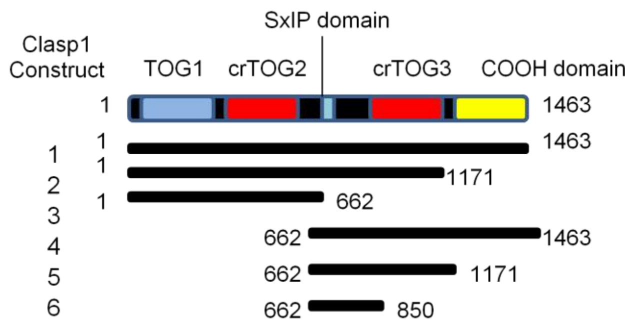

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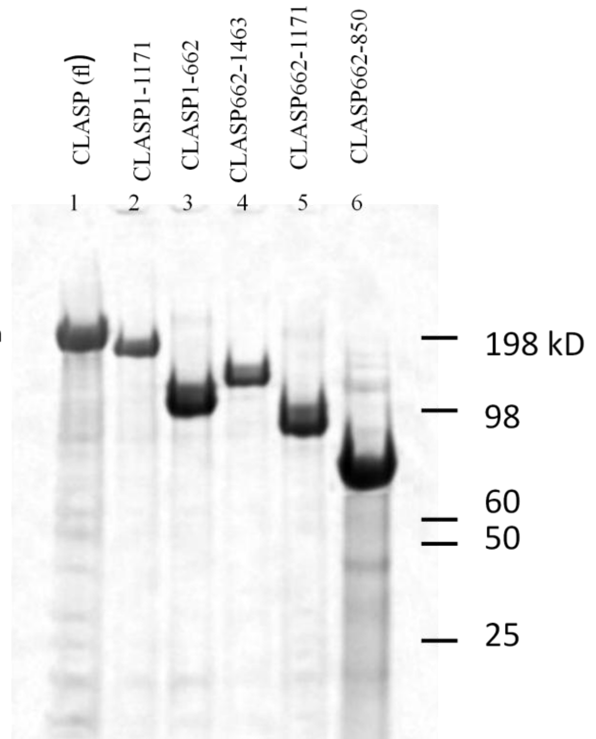


c)

Figure 2.4: Purification of CLASP domains from Insect Cells. a) Purification scheme using double affinity tagging with StrepII and 10X Histidine tags. b) depiction of CLASP fragments and domains selected for downstream characterization. c) SDS-PAGE of purified proteins (10% Bis-Tris).

Chapter 3

Functional Analysis of CLASP Domains in *Xenopus* Egg Extract

Introduction

After successful purification of full length CLASP and various domains, a major goal was to test for activity of the proteins. While the samples appeared to be pure (>95%) and in sufficient quantities, it was important to further characterize both the nature of their activities as well as the relative levels. To address these questions, *Xenopus* egg extract was used to investigate CLASP's role in spindle assembly as well its MT plus end tracking ability.

Some of the first functional experiments to be done were to test whether CLASP or its domains could rescue spindle assembly in reactions depleted of the endogenous Xorbit protein. These tests were critical to determine if CLASP is a functional homolog of Xorbit and thus relevant to this type of analysis. In addition to depletion/rescue experiments, CLASP proteins were also assayed at varying concentrations in non-depleted extracts to screen for potential dominant- negative effects on spindle assembly and morphology.

To dissect the TIP tracking functionality of CLASP and its domains, a time-lapse fluorescence microscopy assay was used in extract supplemented purified centrosomes to serve as MT nucleation sites together with GFP fusions of various CLASP proteins. This assay was designed to distinguish which domains are important for plus-end tracking behavior as well as the dependence on EB1 to localize CLASP to plus ends.

Results

Determination of Endogenous Levels of Xorbit

Before assaying various CLASP domains in the context of spindle assembly, I determined the endogenous concentration of Xorbit in extract to establish how much CLASP protein to add back to Xorbit-depleted egg extracts. Fortunately, an antibody that recognizes the Xorbit C-terminus, previously described, was found to also recognize the C-terminus of CLASP (Hannak, 2006). A comparison by Western blot of recombinant C-terminal domains of Xorbit and CLASP demonstrated a similar affinity of the α-Xorbit antibody for both proteins (Fig 3.1a). Increasing known amounts of full length CLASP were fractionated by SDS PAGE and compared to 5 μl of CSF egg extract by Western blot using the α-Xorbit antibody (1:1000 dilution), revealing the physiological concentration of Xorbit to be approximately 1.5 μM (Fig 3.1b).

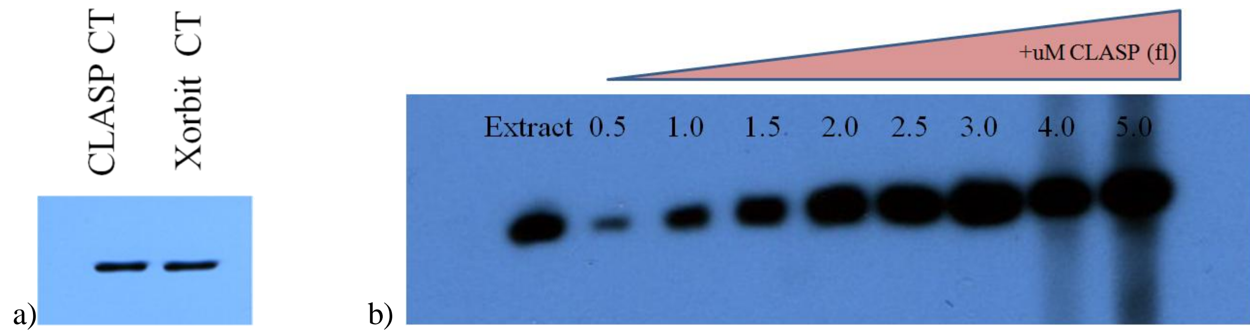


Figure 3.1: Estimation of the endogenous level of Xorbit by Western blot. a) comparison of α-Xorbit antibody recognition of recombinant purified CLASP and Xorbit C-terminal domains. b) Titration of Xorbit in extract. α- Xorbit antibody was used to compare the amount of protein in 5 μl of egg extract to known amounts purified, full length CLASP protein.

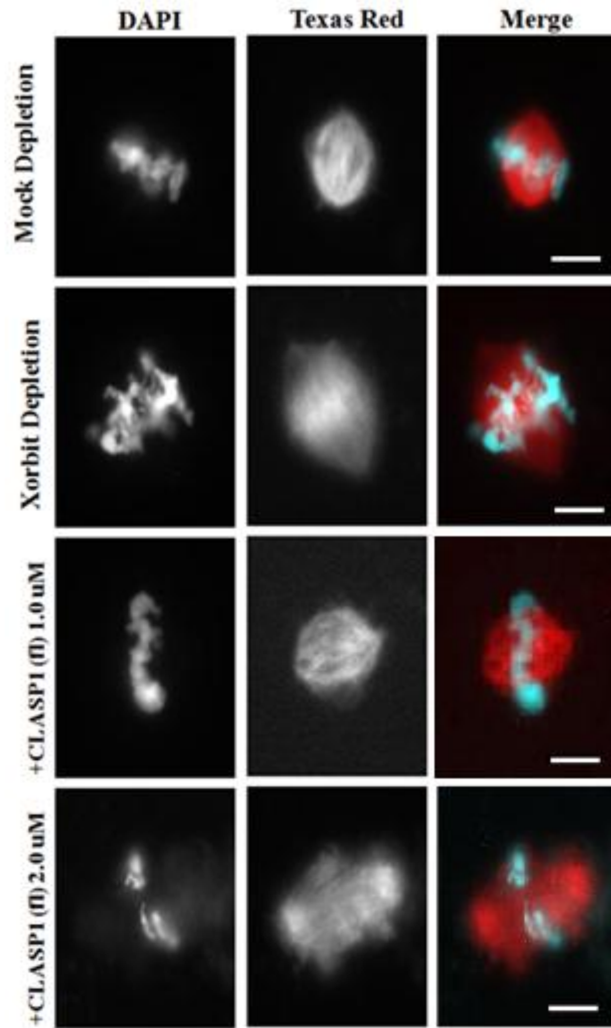
Full Length CLASP is Required for Proper Spindle Assembly in *Xenopus*

Given the nature of the *Xenopus* system, in which genetic manipulation is extraordinarily difficult, a biochemical method was employed in which antibodies coupled to beads are used to immuno-deplete specific proteins from the extract, while preserving activity for assays such as spindle assembly reactions.

Xenopus egg extract reactions were prepared using established protocols for immuno-depletion and spindle visualization. Endogenous Xorbit was first removed from extract using specific α-Xorbit antibody cross-linked to magnetic beads. Successive rounds of immune-depletion, incubation with beads and subsequent recovery of bound beads via magnets, produced egg extract with little to no Xorbit protein. Reactions were then supplemented with the bovine tubulin, chemically labeled with the fluorophore rhodamine. Addition of sperm and calcium were used to drive the reaction into mitosis to observe spindle formation and function. I observed spindles generated in the presence or absence of recombinant CLASP. Incorporation of labeled tubulin with endogenous tubulin during microtubule polymerization and DNA specific dye (DAPI) allowed for visualization of spindle morphology via standard fluorescence microscopy techniques.

As previously described, depletion of Xorbit produces dramatic phenotypes with small, distorted spindles containing mis-aligned chromosomes (Hannak 2006; Fig 3.2). Full length CLASP protein added at the endogenous concentration of ~1.5 µM rescued spindle and chromosome congression defects as did 1.0 µM (data not shown) suggesting that the recombinant full length CLASP protein is active. Added at levels higher than 1.5 µM, full length CLASP caused MT bundling and spindle fusion. 2.0 µM CLASP increased MT density as measured by fluorescence intensity of polymerized fluorescent tubulin, especially at the poles (Fig 3.2a), indicating dominant effects of CLASP on spindle morphology.

Unlike with full-length protein, the addition of different CLASP domains yielded no clear rescue of the ΔXorbit phenotype. For some constructs such as CLASP1-662 and CLASP1-1171, addition of endogenous levels of protein produced dramatic dominant negative effects that made it difficult to analyze chromosome alignment defects. These effects are described in more detail in Section 3.3. To look specifically at rescue of chromosome congression defects, the amount of protein was reduced to ~1/3 (0.5 µM) to prevent these dominant-negative effects from obscuring observation. At lower concentrations the percentage of spindles affected by dominant negative effects was markedly lower, providing clearer images to assess rescue. Upon addition of either construct, similar Xorbit depletion phenotypes were observed. In order to rule out that failure to rescue was due to the decreased amount of protein, the effects of full length CLASP at similar concentrations (0.5 µM) were also observed for comparison. At lower concentrations, more than 50% of spindles had been rescued with respect to chromosome congression, indicating that protein at lower concentrations can still produce rescue effects. With CLASP1-662 and CLASP1-1171, a high percentage of spindles with chromosome congression defects were still observed, comparable with Xorbit depletion alone (Fig 3.2). In experiments with CLASP662-1463 and CLASP662-1171 that produced less dramatic dominant negative phenotypes, 1.5 µM protein also failed to rescue chromosome congression defects, and spindles displayed the Xorbit depletion phenotype.



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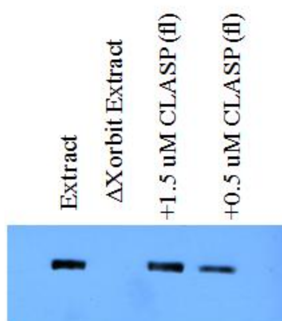

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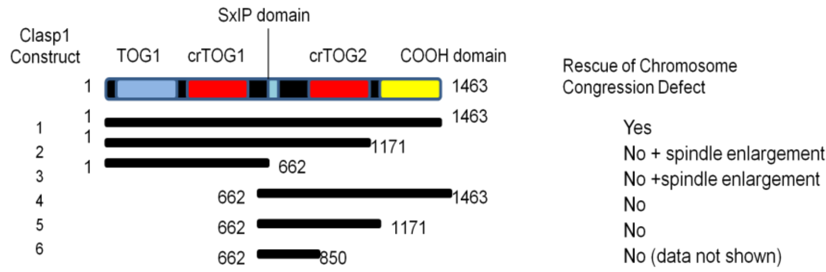

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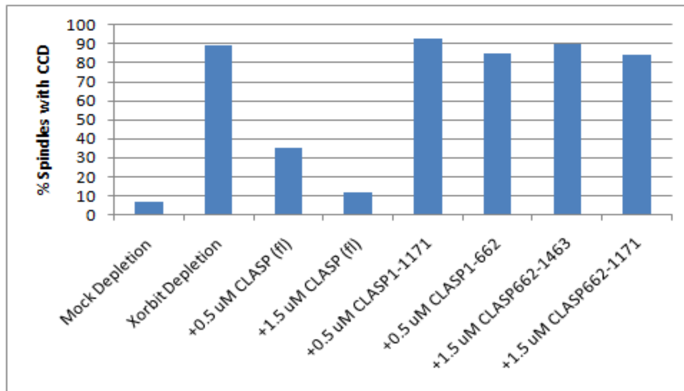

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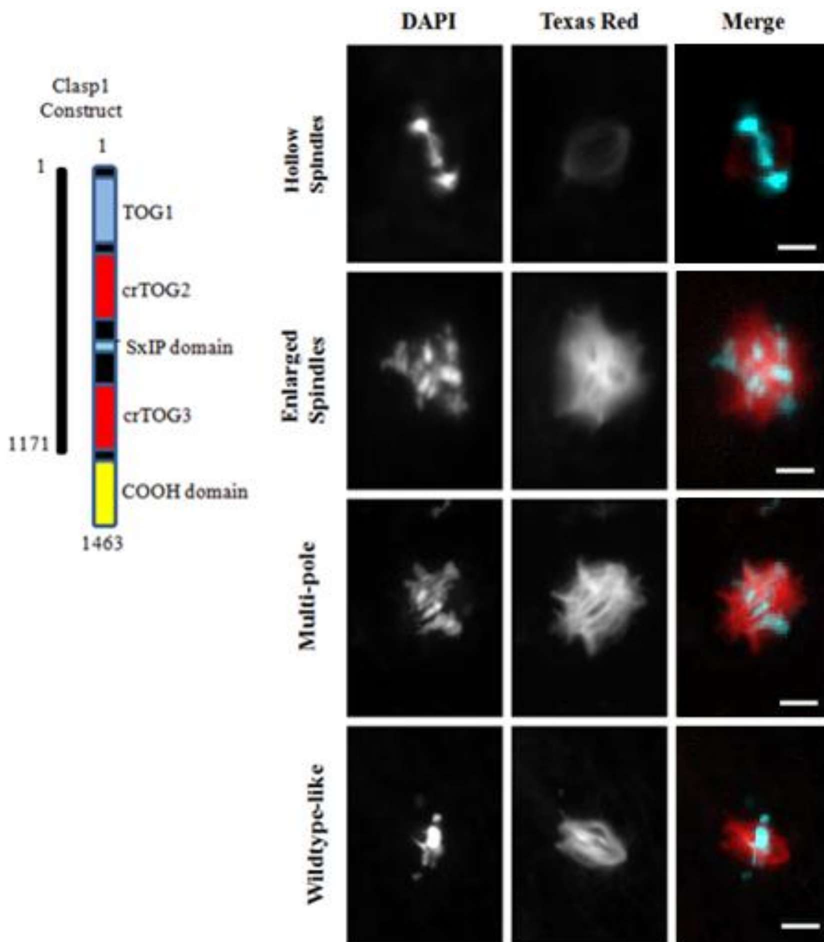
Figure 3.2: Rescue effects of CLASP proteins. a) fluorescence images of spindles in egg extracts that were depleted with control antibodies (Mock depletion) or depleted with Xorbit antibodies followed by addition of buffer control (Xorbit depletion) or full length CLASP protein, either 1.5 or 2.0 µM. b) Western blot analysis of depletion and add-back reactions. c) diagram of CLASP protein domains and rescue effects in Xorbit depleted extracts. d) Counts of spindles with chromosome congression defects with addition of various CLASP domains.

CLASP domains differentially affect spindle assembly and morphology in *Xenopus* egg extracts

Based on preliminary observations that higher amounts of full length CLASP affected spindle morphology, differing amounts of exogenous protein were added to egg extract during spindle assembly reactions. CLASP-GFP proteins were added at 0.2 µM, 0.5 µM and1.5 µM concentrations, based upon activity levels initially observed during rescue experiments. Protein was added either upon entry in mitosis or at metaphase with very similar results (metaphase data not shown). As a control, equivalent volumes of CLASP elution buffer were added and spindle reactions were setup and visualized in a similar fashion as previously described. In quantifying different spindle phenotypes, ~115 spindles were counted in 2 different egg extracts.

Exogenous addition of full length CLASP protein produced a mixed distribution of various spindle phenotypes. The most common phenotypes at this concentration were similar to wild type spindles in size, while some possessed rounded poles with increased MT density (not shown). Three other spindle phenotypes appeared, including multi-polar spindles with 3 or more poles centered around the metaphase plate, and enlarged spindles with a longer average spindle length and greater tubulin polymerization. Even upon addition of endogenous levels (1.5 µM), although more prevalent at higher concentrations of full-length protein, a more homogenous and dramatic phenotype was observed in which the MT density increased dramatically throughout the spindle, from the pole to the metaphase plate. At 2.0 µM and even higher additions (not shown), MTs enveloped and obscured the chromosomes. Additionally, many of these spindles bundled together, making it difficult to distinguish one spindle from another. Differential effects of several different exogenous proteins disappeared at higher concentrations when the MT over-polymerization and bundling phenotype predominated.



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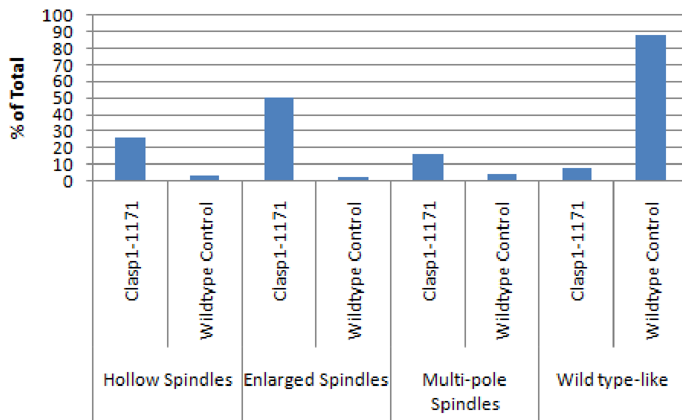

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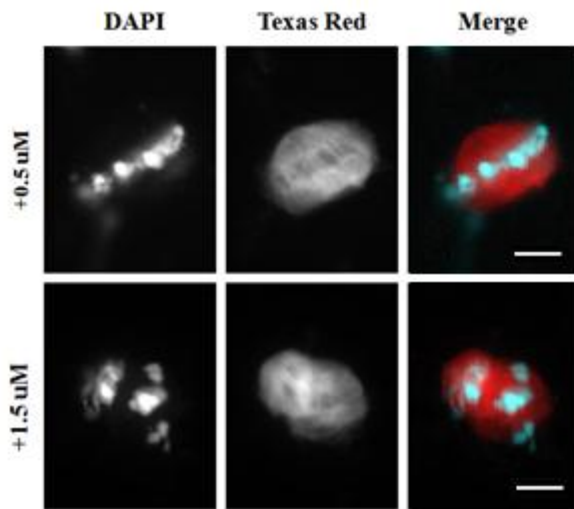
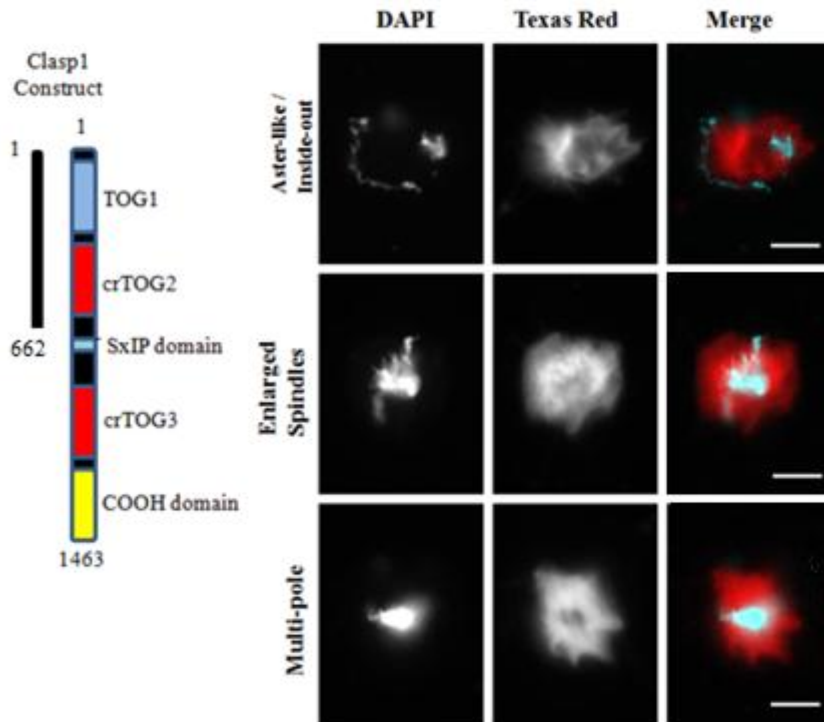


c)

Figure 3.3: Major spindle phenotypes after exogenous addition of higher concentrations of CLASP1-1171 at entry into mitosis. a) representative images of spindle phenotypes with 0.2 µM of protein added. b) distribution of phenotypes after exogenous addition of 0.2 µM CLASP1-1171 at entry of mitosis. Numbers of phenotypes are compared to wild type and shown as % of total spindles counted (~115 spindles each). Scale bar = 10 µM c) representative images of spindle phenotypes with 0.5 and 1.0 µM of protein added. Scale bar = 20 µM

The spindle phenotypes observed with CLASP1-1171 appear to be morphologically similar to exogenous addition of full length with subtle differences (Fig. 3.3a) CLASP1-1171 domain lacks the C-terminal domain, but has the full complement of putative MT binding domains. The C-terminus is thought to localize CLASP to the metaphase plate. One interesting phenotype class observed were ‘hollow spindles.’ The morphology of these spindles suggestd that exogenous protein is mis-localized, perhaps with more CLASP accumulating near the pole. This caused a more dramatic effect of shifting tubulin distribution away from the metaphase plate (Fig 3.3a). The number of hollow spindles is also in greater abundance with addition of this CLASP fragment. Other phenotypes, such as enlarged spindles and multi-pole spindles are also found in large quantities. While there were some wild type spindles, the dominant negative effect of CLASP1-1171 appears to be much stronger than full length CLASP. The majority of spindles were characterized as non-wild type in comparison to a buffer only control (Fig. 3.3b)

As with full length CLASP, addition of higher concentrations also produced larger spindles with increase MT density from pole to metaphase plate (Fig 3.3c). For this construct, lower concentrations (0.5 µM and 1.5 µM) produced a homogenous distribution of these spindle types similar to full length CLASP at higher concentrations (1.5 µM and 2.0 µM).



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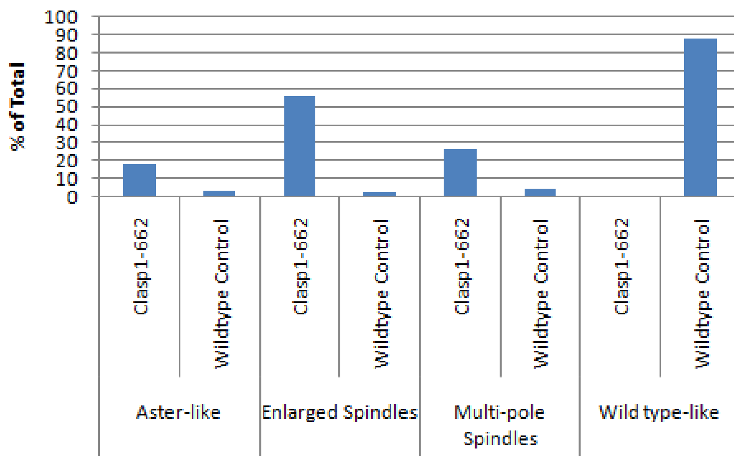

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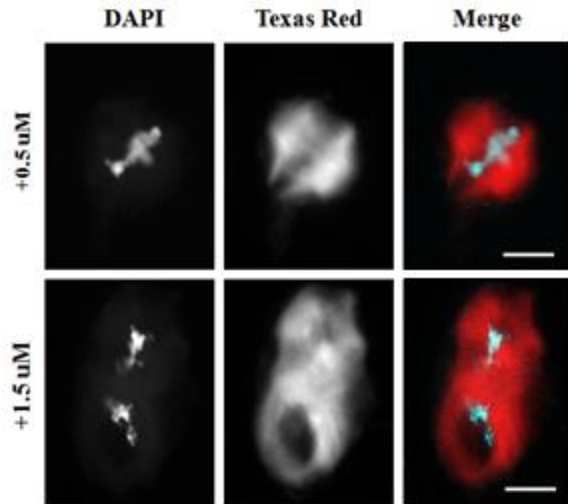
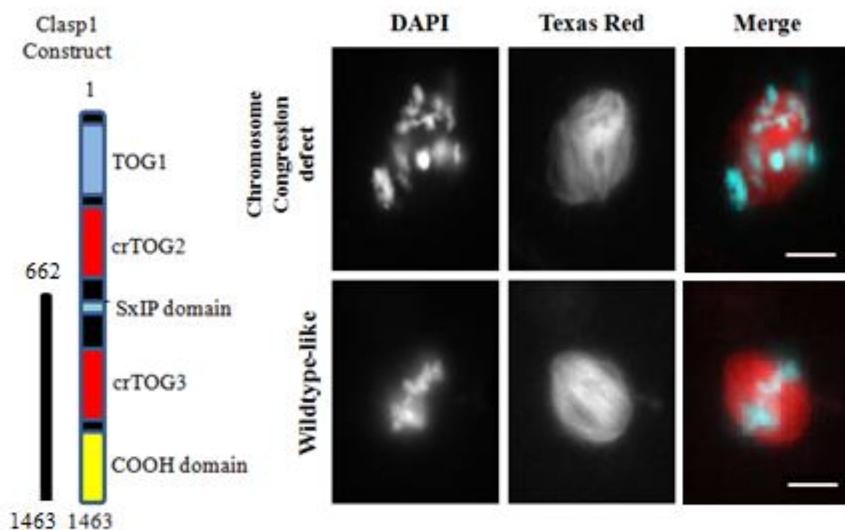


c)

Figure 3.4: Major spindle phenotypes after exogenous addition of higher concentrations of CLASP1-662 at entry of mitosis. a) representative images of spindle phenotypes with 0.2 µM of protein added. Scale bar = 10 µM. b) distribution of phenotypes after exogenous addition of 0.2 µM CLASP1-662 at entry of mitosis. Numbers of phenotypes are compared to wild type and shown as % of total spindles counted (~115 spindles each). c) representative images of spindle phenotypes with 0.5 and 1.0 µM of protein added. Scale bar = 20 µM.

CLASP1-662 produced perhaps the most dramatic dominant negative effects when added to spindle assembly reactions. This smaller CLASP fragment contains TOG1, crTOG2 and a portion of the S/R rich region. Addition of this protein produced massive MT bundling and polymerization throughout the spindle. Fewer categories of spindle phenotypes were seen, possibly masked by accumulation of MTs in all regions of the spindle (Fig 3.4a). Surprisingly, almost no wild type spindles were observed, with distribution across multi-polar spindles, enlarged spindles, and a few rare spindles that resembled asters (Fig 3.4b). These spindles looked as if they were deconstructed, or flipped inside out, with chromosomes being ejected from a large density of MTs. At higher concentrations of this protein, MTs further accumulated in the spindle with massive bundling obscuring basic spindle shape (Fig. 3.4c).

CLASP662-1463 contains several putative MT binding domains such as portions of the S/R domain, a SxIP domain and a crTOG3 domain as well as the critical C-terminal domain region. This protein had considerably less activity with respect to domains containing TOG1 and crTOG2. Interestingly, spindle phenotypes were only observed at higher concentrations (1.5 μM). Few effects were seen at lower concentrations. While some spindles were somewhat larger, with an increase in MT density at the metaphase plate, a majority appeared to have chromosome congression defects (CCDs) (Fig. 3.5b). Nearly 60% of spindles were characterized with CCDs and defects in the spindle itself (SD) (Fig 3.5b). Others contained only CCDs. This CCD phenotype is similar to a dominant negative effect seen with addition of the C-terminal domain, as previously characterized. While the effects on MTs is not as dramatic as those seen with domains containing TOG1 and crTOG2, there does seem to be some effect on MTs and spindle morphology.



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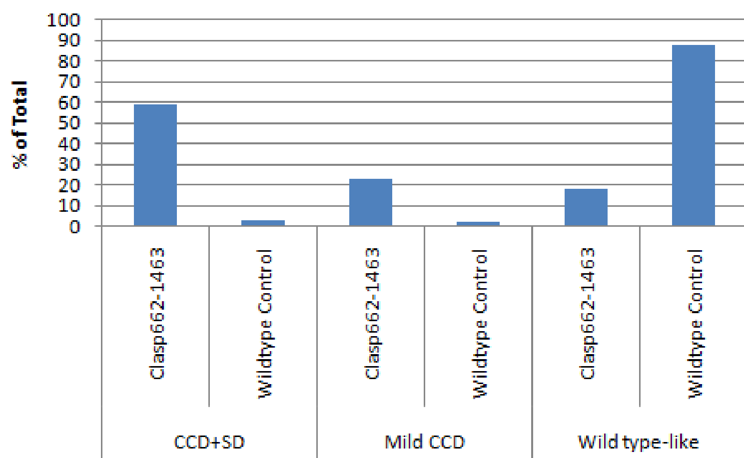

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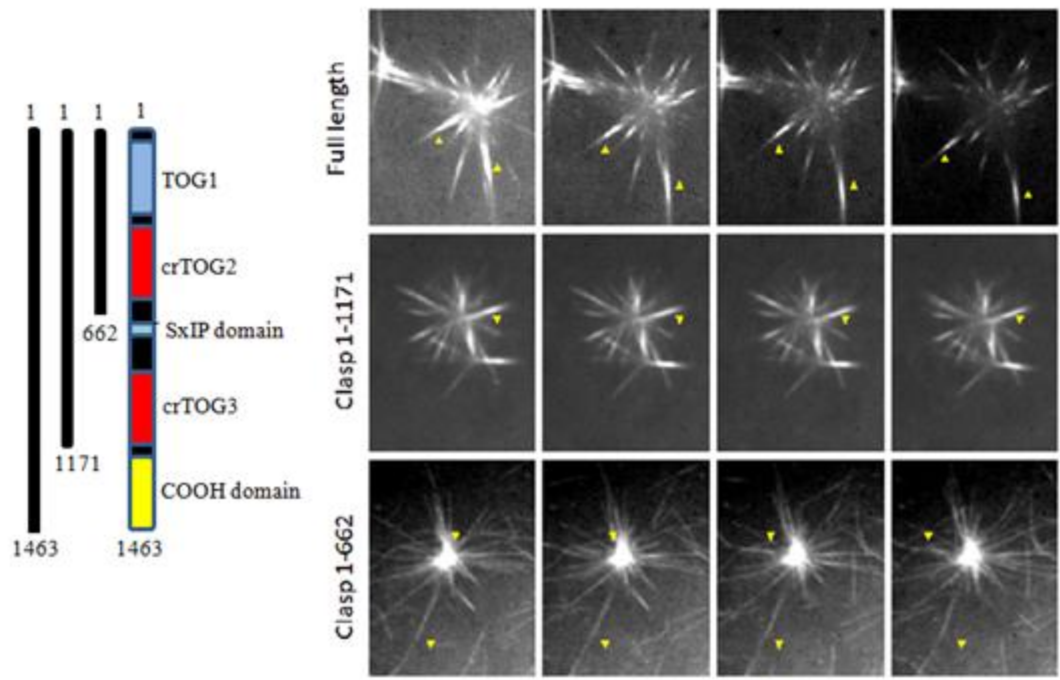
Figure 3.5: Major spindle phenotypes after exogenous addition of higher concentrations of CLASP662-1463 at entry of mitosis. CCD (Chromosome congression defect), SD (Spindle defect). a) representative images of spindle phenotypes with 0.2 μM of protein added. Scale bar = 20 μM. b) distribution of phenotypes after exogenous addition of 0.2 μM CLASP662-1463 at entry of mitosis. Numbers of phenotypes are compared to wild type and shown as % of total spindles counted (~115 spindles each).

CLASP SxIP Motifs Mediate MT Plus Tip Tracking

Given that CLASP shares features (TOG domains) with the XMAP215 family that tracks MT plus ends autonomously, and also contains an SxIP motifs that mediate EB1-dependent tip tracking of many other +TIPs, CLASP proteins were tested for the ability to track the plus ends of MTs. Furthermore, the requirement for EB1 was also tested. GFP-tagged CLASP fragments were added to metaphase-arrested egg extracts together with centrosomes that served as MT nucleation sites, and MT growth was imaged by time-lapse fluorescence microscopy (Figure 3.6). Recombinant EB1 was purified, as previously described, and added back at levels equal to or in excess of its physiological concentration (1.5 μM).

Upon addition of CLASP proteins containing TOG1 and crTOG2 domains, tracking was difficult to observe. These proteins bundle MTs at even moderate concentrations, obscuring MT ends and stabilizing MTs in the extract. For larger constructs, such as CLASP1-1171 and full length CLASP, while these proteins do appear to be enriched toward the tips of MTs, a concentration could not be found at which plus-end tracking was not confounded by MT bundling and stabilization. Despite limitations for the larger constructs, CLASP1-662 was not observed to track, even at lower concentrations. Individual MTs were seen to grow and shrink indicating that a low enough concentration of protein was used so as to prevent MT-over-polymerization and bundling. Even in the presence of dynamic MTs this construct was not seen to track exclusively to the plus-ends of the MT, but rather bind along the lattice.



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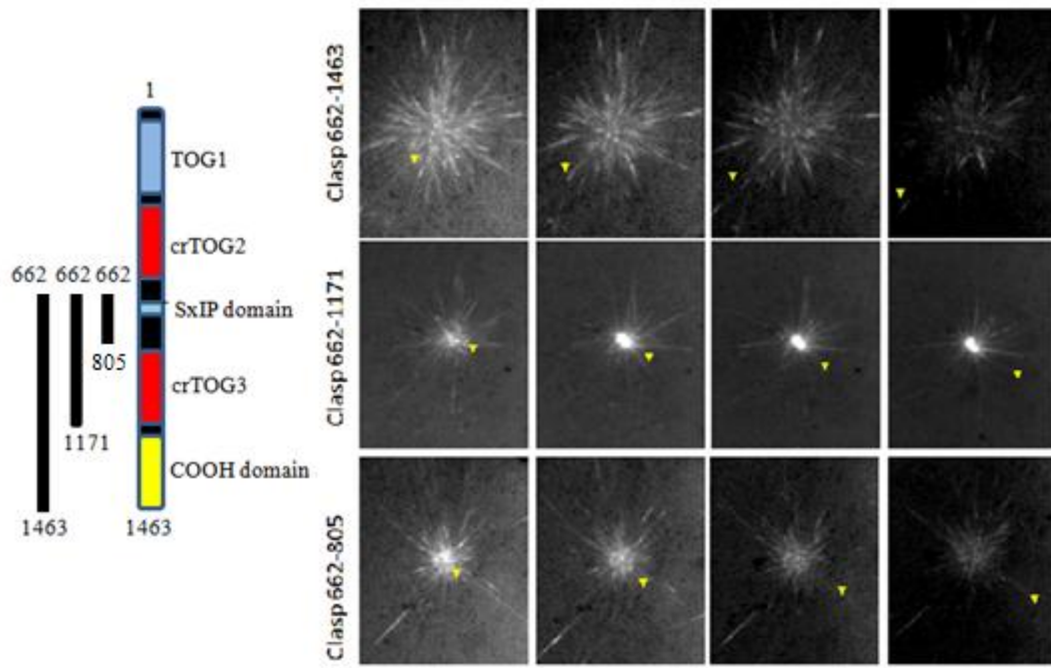

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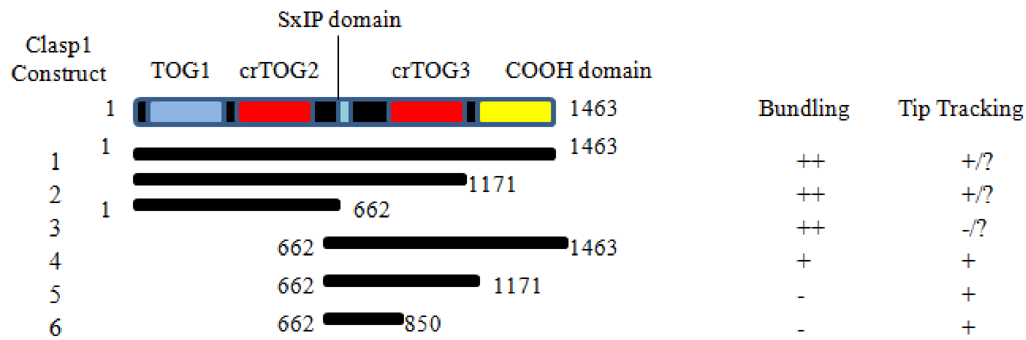


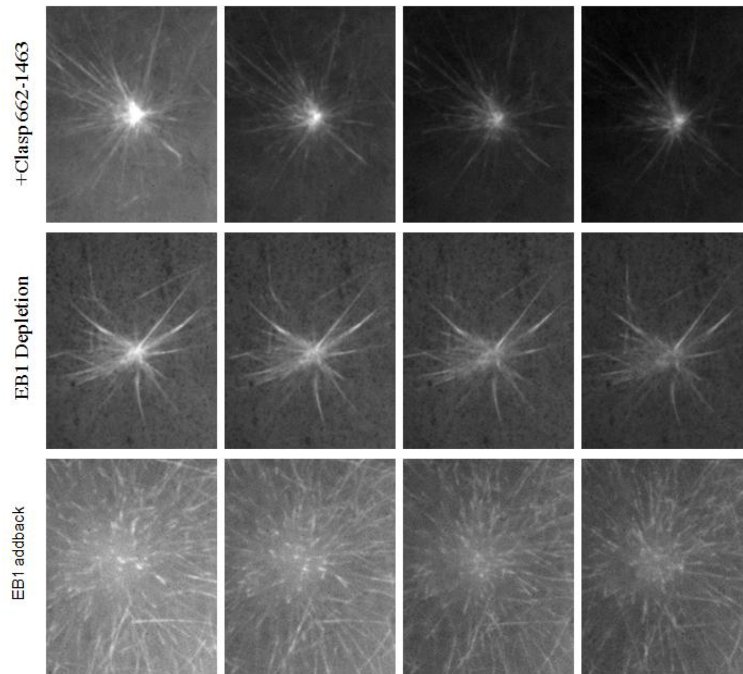
c)

Figure 3.6 CLASP-GFP fusion proteins added to human centrosomes in metaphase-arrested *Xenopus* egg extract. a) Representative time-lapse images of protein constructs containing TOG1 and crTOG2 domains. b) representative time-lapse images of constructs lacking TOG1 and crTOG2 domains. c) summary of tracking experiments. Arrows reflect individual MTs or bundles. Images taken at 2.5 sec intervals.

Upon addition of CLASP proteins lacking TOG1 and crTOG2 domains, tracking was much easier to observe. All of these fragments contain the SxIP domain and appear to track the plus ends of MTs. CLASP662-1463 added at higher concentrations did produce some MT bundles, but with lower concentrations, tracking was observed. While it was difficult to observe tracking with the larger CLASP proteins, it appears that constructs with the EB1 interacting SxIP domain have the ability to track MT plus-ends. To test this further, tracking assays were performed in EB1 depleted extracts.

EB1 is required for CLASP MT plus tip tracking

Data are shown for CLASP662-1463, as it is the largest fragment that clearly tracks along plus-ends of MT and CLASP1-1171 or full length do not produce that activity under the conditions tested. When similar amounts of CLASP662-1463 are added to extract depleted of EB1, we observe the protein bound bind along the MT lattice but not specifically at the plus ends. When recombinant EB1 is added back to this depleted extract, MT plus-end tracking was observed, demonstrating CLASP's dependence on EB1 MT for tip tracking.



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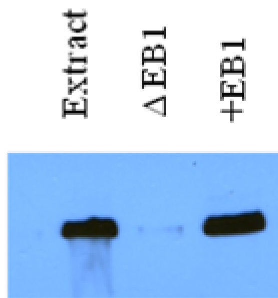


b)

Figure 3.7 Centrosome tracking assay of CLASP662-1463. Added CLASP662-1463 is observed to bind the lattice and also tip-track (top panel). Tip-tracking is abolished upon depletion of EB1 (middle panel). Tracking is restored with addition of recombinant EB1 (bottom panel). Images taken at 2.5 sec intervals. b) Western blot control of depletion and add back of EB1 protein.

Discussion

The observation that only full length human CLASP was capable of rescuing Xorbit-depleted egg extracts illustrates that the purified recombinant proteins are fully active. It also reflects the high degree of conservation between the frog and human proteins and the necessity for the full complement of CLASP domains for proper function in the spindle. At levels higher than 1.5 µM of the full length protein, additional phenotypes appeared, including MT bundling and spindle clustering. While it is possible that CLASP and Xorbit have different relative activities, CLASP rescue activity demonstrates overall functional similarity.

Interestingly, we observed morphological effects in spindle assembly reactions in the presence of added CLASP fragments at concentrations as low as 0.2 µM. Fragments containing residues 1-662 (TOG1 and crTOG2) caused massive MT bundling and polymerization, enlarging spindles that often became round and multi-polar. Thus, the strong MT polymerizing activity of this region must be localized correctly for proper spindle function. Similar but less dramatic effects were observed in larger constructs containing additional domains. CLASP1-1171 lacking only the C-terminal domain, showed somewhat higher MT polymerization and bundling activity compared to full length CLASP at the same concentration. This activity however produced a much less dramatic spindle phenotype than even CLASP1-662. This suggests that while CLASP1-1171 might have more MT binding domains than CLASP1-662, this larger construct might also contain other regulatory domains that promote more specific localization and thus a less dramatic phenotype. Addition of physiological concentrations did not cause an increase in overall density of MTs but rather a shift in tubulin density from the spindle midzone to the poles. This suggests another role of CLASP at spindle poles outside of MT polymerization at the kinetochores. The shift in tubulin density could be driven by bundling activity by the cryptic TOG3 domain or recruitment of other binding partners through another sub-domain flanking the S/R region.

CLASP fragments lacking TOG1 and TOG2, but containing the C-terminal domain, such as CLASP662-1463 produced dominant negative spindle phenotypes similar but less penetrant than those observed with the C-terminal domain alone, suggesting differential regulation of the fragment either through localization or by another domain in the fragment (Hannak, 2006). This protein, like the C-terminal domain alone, localized not only to kinetochores but also to the spindle poles. Interestingly there appeared a subtle difference in activity between CLASP662-1463 and CLASP662-1171. While the latter lacks the C-terminal domain, which does bind MTs directly, it also has a lower affinity for MTs and less of an effect on spindle MTs. This suggests that the C-terminal domain might affect the activity of the crTOG3 domain.

Addition of CLASP662-1171 produced the fewest effects on spindle morphology (data not shown). At 0.2 µM the majority of spindles were wild type- like. Some spindles appear to be enlarged, but were much smaller than enlarged spindles seen after addition of proteins with TOG1 and crTOG2 (Fig 3.5). Even at higher concentrations, the effects of this protein were limited, producing spindles very similar to wild type. CLASP662-1171 is thought to contain all the domains of CLASP662-1463 except for the C-terminal domain. While no chromosome congression defects were seen, spindle enlargement is also reduced suggesting that the C-terminus might affect MT polymerization or bundling activity, either by localization or through an intra-molecular interaction.

Only those fragments containing SxIP motifs tracked the growing ends of MTs, including a minimal domain containing the only this motif. Analysis was somewhat complicated by the fact that CLASP fragments containing TOG1 and crTOG2 domains bundled MTs to such a great extent that tracking was nearly impossible to observe. However, bundles did occur nearer to the ends of MTs rather than distributed throughout the centrosome. CLASP1-662 containing 2 arrayed TOG domains, bound to the MT lattice but failed to track plus ends, indicating that CLASP's MT +TIP tracking ability is mediated not by its series of TOG domains, but rather by the SxIP motif, presumably through binding of EB1. Under these conditions, fragments lacking the SxIP motif bound to the MT lattice but failed to tip track (Fig. 3.7). A similar bundling effect was seen with fragments containing SxIP and TOG1 and TOG2 domains making observations of tracking difficult with EB1 added back. CLASP fragments containing TOG domains could still bind to the MT lattice in the absence of EB1, suggesting that while the TOG domains do not confer affinity for the plus end, they play important roles in MT binding.

The results from experiments in *Xenopus* egg extracts demonstrates that CLASP is a potent MT polymerizing and bundling protein, the activity of which must be localized to specific regions for proper spindle formation and maintenance. The activity of CLASP appears to be affected by multiple regions in the molecule. While TOG1-crTOG2-S/R regions promote MT assembly and bundling, multiple regions in the rest of the molecule help regulate this activity.

It is unclear however, how modulation of activity of this protein changes function in the context of the spindle. From tracking assays, it is interesting that CLASP activity can change based on the presence of interacting partners. In the presence of EB1, CLASP is found to be enriched on the plus-ends of MTs, while without it, it is found bound across the lattice. In this one context, it appears that CLASP function can change due to specific interactions with binding partners. How this regulation takes place is not known. One model might suggest that CLASP activity is regulated by promoting or negating these interactions, perhaps through pos-translational modification, as has been previously studied in the context of filopodia formation (Ambrose, 2008; Wittmann, 2005). At one point in spindle assembly, CLASP might be enriched on dynamic MTs through this type of regulation, where +TIP binding is enhanced, while bundling is reduced through modifications of the TOG domains. This might occur during steps leading to kinetochore attachment to chromosomes. As the spindle progresses to anaphase, perhaps this regulation is changed, in which CLASP binding properties are altered to promote more bundling, as is seen in the central spindle (Hannak, 2006).

Another potential feature of this regulation is that differential localization of the protein also contributes to activity. In extract experiments, the C-terminus localizes CLASP to the kinetochores and spindle poles. At kinetochores, this localization is thought to be mediated by interaction with the +TIP Clip-170 and the kinetochore based motors XCENP-E and perhaps XKID (Cheeseman, 2005; Galjart, 2005; Tanenbaum, 2006). Additionally, a region flanking these TOG domains appears to bind PRC1, a known MT bundler and scaffold protein of the central spindle. CLASP can be viewed as bi-functional, both as a +TIP for dynamic MTs as well as a potent stabilizer helping to stabilize and induce MT bundles (Kapitein, 2008; Liu, 2009). Localization of the protein helps direct activity for specific functions. From experiments in extract for example, mis-localization of certain fragments produced dominant negative effects that promoted MT regulation in unbalanced manner, driving bundling and polymerization at the poles and altering tubulin distribution across the spindle. Perhaps there exists a balance of this

activity in the different regions of the spindle, mediated through localization with various partners. It will be interesting for future studies to investigate how these 2 functions of CLASP contribute to spindle assembly and moreover how these activities are modulated in different regions of the spindle.

Materials and Methods

Rescue/Depletion

Xenopus laevis egg extracts arrested in metaphase of meiosis II by cytostatic factor (CSF) were prepared as described (Hannak, 2006). Immuno-depletion of Xorbit was accomplished using 10 μg of α-Xorbit antibody coupled to 50 μl protein A-Dynabeads (Dynal) and incubated in 150 μl of CSF extract. Non-specific rabbit IgG antibody (Sigma-Aldrich) was used as a mock-depletion control (Fig 3.2a). 2 rounds of depletion were performed. The level of depletion was tested by western blot with 1 μg/ml of a-Xorbit antibody (Fig 3.2e). Full length CLASP proteins, and proteins CLASP662-1463 and CLASP662-1171 were then added at 1.5 μM or 2.0 μM concentrations to Xorbit depleted extracts, either upon entry in mitosis or at metaphase (metaphase data not shown). For CLASP proteins CLASP1-1171 and CLASP1-662, only 0.4 μM was added. As a negative control, an equivalent volume of CLASP elution buffer was added to Xorbit depleted extract reactions. For data shown, CLASP proteins used were all C-terminal GFP fusions.

Spindle Imaging

Spindle reactions were spun onto coverslips, fixed and mounted for imaging, as previously described (ref). Images were collected with a fluorescence microscope (model BX51; Olympus) with a dry 40 x NA (0.75) objective, a cooled CCD camera (model Orcall; Hamamatsu) and Metamorph software (Molecular Devices).

Centrosome Plus end Tracking Assays

2 μl of CSF extract was incubated with centrosomes purified from KE37 cells as previously described (ref), and incubated with various amounts of protein on coverslips. Exogenous purified EB1 was also added. Coverslips were imaged using a Spinning Disk Confocal (Zeiss). Similar experiments were also performed using metaphase-arrested egg extracts depleted of EB1 protein, using similar immune-depletion methods (using 25 μg of antibody) with Xorbit as described above.

Chapter 4

Biochemical Characterization of CLASP Domains

Introduction

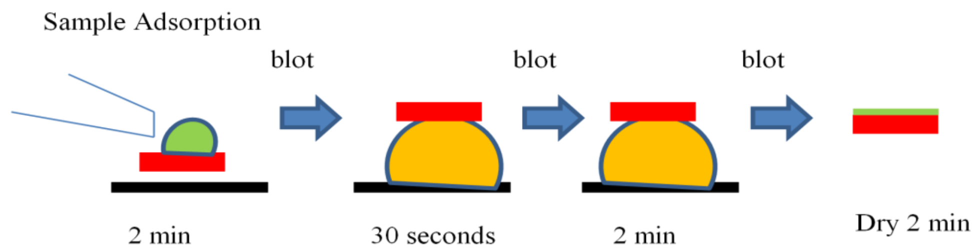
After examining various activities of CLASP in *Xenopus* egg extracts, especially in the context of spindle formation, the next step was to look more closely at biochemical and mechanistic aspects of the protein in a reconstituted in vitro system. Given the difficulty in producing purified CLASP, the majority of previous studies have focused on CLASP function *in vivo*. The availability of purified full-length recombinant CLASP and associated domain fragments allowed for a detailed biochemical characterization of this protein, especially concerning the roles of specific domains and their interaction with tubulin and other cellular factors. Using various assays common to the study of microtubule regulators, the activity of different CLASP constructs were determined to provide evidence for the existence of putative functional domain structure of the protein.

One of our original goals was to determine the structure of CLASP bound to microtubule by electron microscopy. While this ultimately proved to be challenging, electron microscopy (EM) should still be a valuable tool to characterize structure/function of CLASP in the future.

Results

CLASP has a thin, elongated structure

I examined full length CLASP protein by electron microscopy using negatively stained samples. Micrographs reveal CLASP as fairly mono-dispersed particles , thin and about ~10 nm in length (Fig. 4.1b). Thus, it is likely that the predicted functional domains are arrayed linearly in an extended conformation . In fact, in instances of optimal staining and imaging conditions, CLASP images suggests the appearance of sub-domains along the length of the molecule. However, the molecule is highly flexible, making image processing and the generation of class averages impossible. Despite this limitation, these first mages of CLASP do provide a general shape for the molecule. They also confirmed that purified CLASP in selected buffer conditions appears to be stable and not found in large aggregates. One important question yet answered by our EM studies, is whether CLASP exists as a dimer or monomer. Based on the apparent length of the CLASP particles, there seems to be a mixture of monomers and dimers, although it is difficult to distinguish between actual dimerization and particles that are close together. This led us to use of a biochemical method to address this aspect of CLASP structure.



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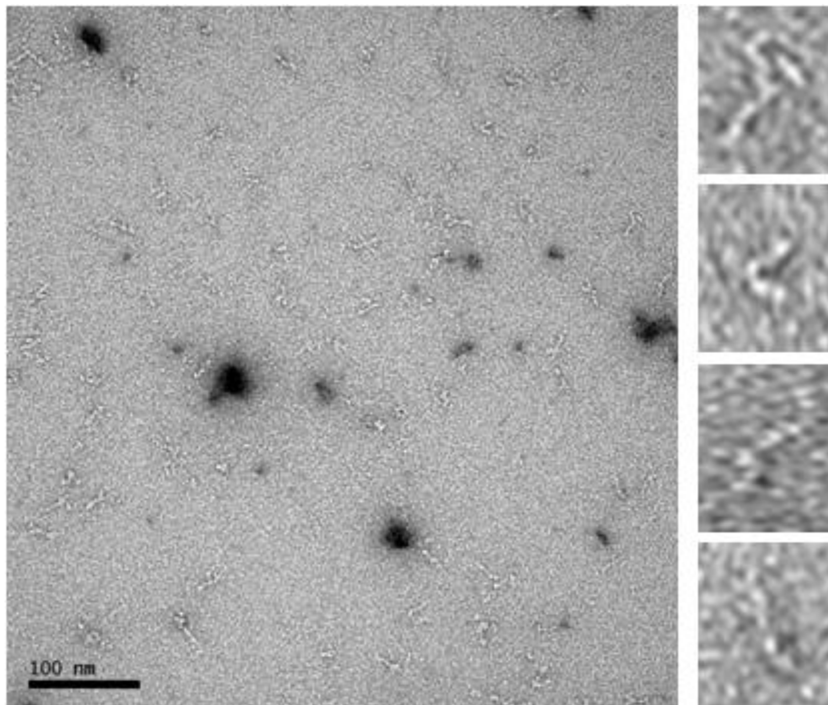


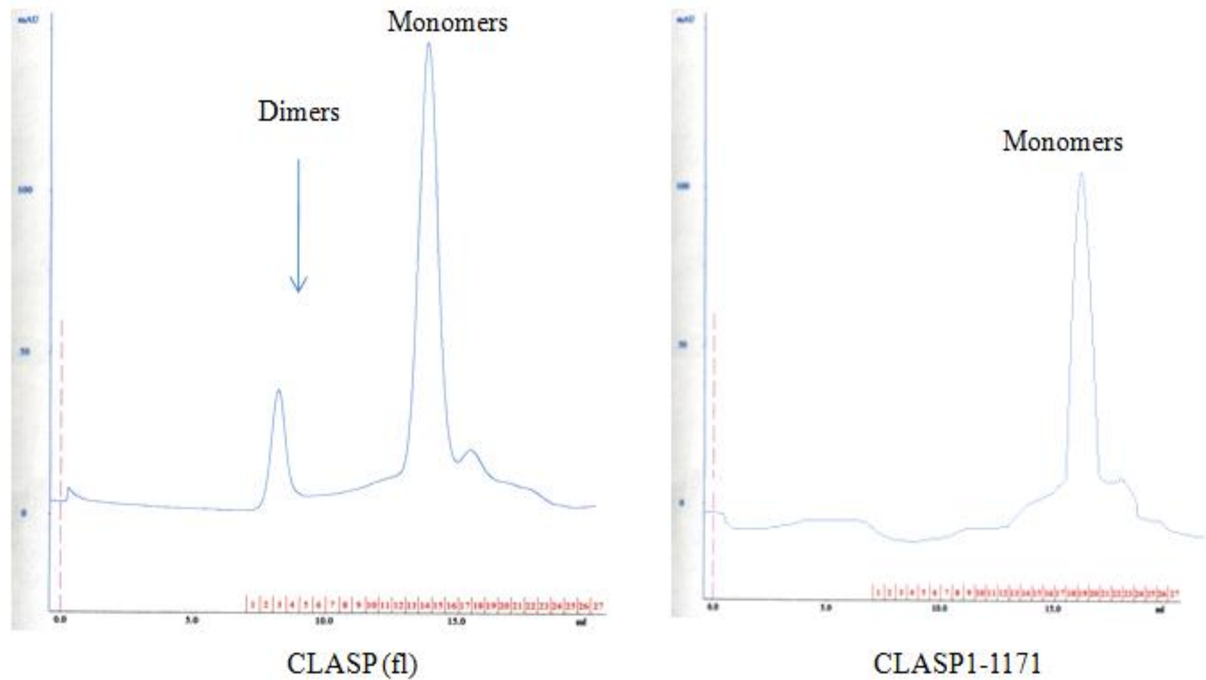
b)

Figure 4.1: Electron microscopy of negatively-stained CLASP. a) Diagram of sample preparation for EM studies. b) Typical micrograph of CLASP (left) and selected images of individual CLASP particles illustrating the extremely flexible character of this protein (right).

CLASP is able to homo-dimerize via the C-terminal domain

EM images of CLASP raised the possibility that the molecule could exist as a dimer. Given the presence of a predicted coiled-coil region at the C-terminus, it has been proposed that CLASP could dimerize through this domain, similarly to what has been shown for a CLASP homolog found in S. pombe. To determine if CLASP can associate as a dimer through the C-terminal domain, full length protein and CLASP1-1171 were run through gel filtration columns. CLASP1-1171 is identical to full length CLASP except that it lacks the last 292 C-terminal residues (Fig. 4.2).

The gel filtration elution profile of full length CLASP shows two major peaks, with the second peak not present in the elution profile for CLASP1-1171, around fractions 2-5. This region of the elution profile corresponds to a molecular weight of roughly ~350 kD, suggesting the presence of an equilibrium between monomers and dimers for the full length CLASP. In contrast, CLASP1-1171 elutes in a single peak, a few fractions later than the monomeric full length, protein reflecting a slightly smaller molecular weight. The fact that dimers form with full length CLASP and not CLASP1-1171 indicates that the C-terminus is responsible for dimerization.



a) b)

Figure 4.2: Elution profiles of CLASP proteins after gel filtration. A) CLASP full length protein Arrow indicates a peak at fractions 2-5 which corresponds to a protein MW of ~350 kD and presumably dimers of CLASP. Elution peak at fractions 12-18 represent protein monomers with a MW of 180 kD. b) CLASP1-1171 fragment. There is an absence of a second peak at fractions 2-5.

Different CLASP domains promote an increase in rate and net tubulin polymerization

Based on our experiments in extract, CLASP appears to be a potent regulator of microtubule assembly, having dramatic polymerization and bundling activities. To test these activities *in vitro*, a 90˚ light scattering assay was used to measure bulk polymerization of tubulin in the presence of different CLASP domains. Light scattering showed interesting differences between the various CLASP constructs. Proteins containing TOG1 and crTOG2 (CLASP (fl), CLASP1-1171, CLASP1-662) appear to induce the same steep initial rate of tubulin polymerization, much faster than the rate of polymerization observed in the presence of other constructs or for tubulin alone. However, full length protein appears to generate greater bulk polymerization at steady state (plateau) than CLASP1-1171 or CLASP1-662. It is interesting that CLASP1-1171 behaves differently from wild type, as the C-terminal domain (CTD) lacking in these constructs has little if any activity on MT polymerization (Fig. 4.3). These results suggests that the C-terminal domain is indirectly affecting microtubule polymerization, most likely through the dimerization of CLASP via its C-terminal coiled-coiled domain. On the other hand, the higher steady state polymerization in the presence of CLASP1-1171 with respect to CLASP1-662 suggests increased binding activity of the former and/or stabilization of the MT lattice, which can be explained by the presence of an additional microtubule binding domain. Such extra microtubule-binding region could correspond to an extra segment of the S/R region, or the putative crTOG3 domain, which are present only in the larger of the 2 constructs.

A surprising result is that even CLASP662-1463 appears to have some positive effect on tubulin polymerization. While the effect is not as dramatic as for CLASP constructs containing the TOG1 and crTOG2 domains, the initial rate of tubulin polymerization and overall bulk polymerization are greater than that observed in the presence of CLASP662-1171 or the CTD alone. Again, this difference in activity appears to be due to the presence or lack of the C-terminus, which appears to affect a putative MT binding region in CLASP662-1463.

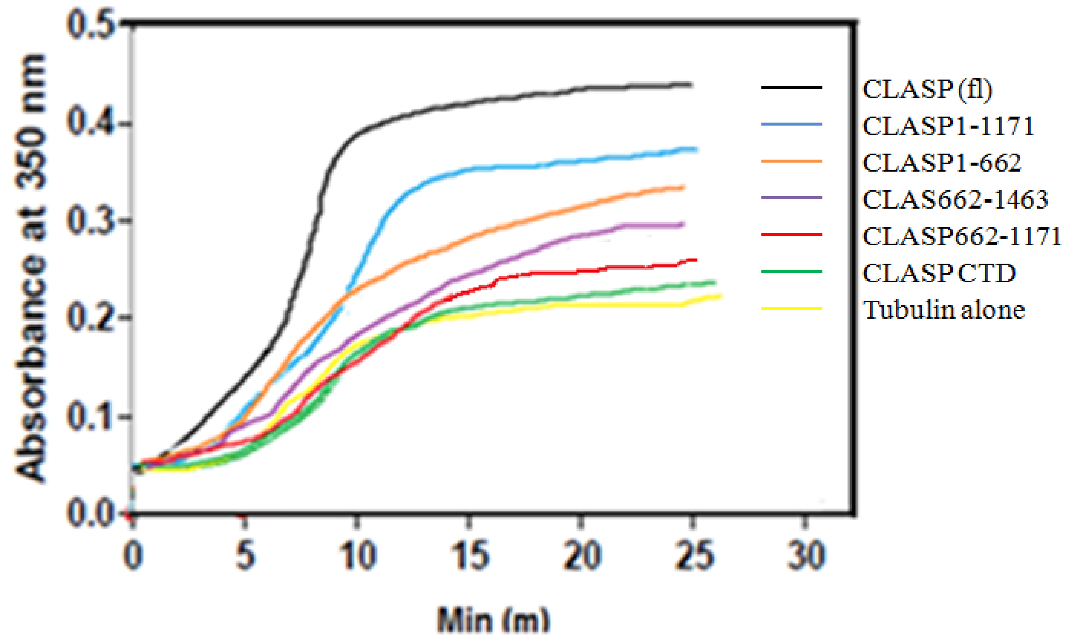


Figure 4.3: 90˚ light scattering curves of tubulin polymerization with various CLASP proteins. Polymerization of tubulin was observed with and without CLASP fragments at 350 nm absorbance. Data was collected at 30 sec intervals and plotted.

Electron Microscopy reveals CLASP as a potent MT bundling protein *in vitro*

While light scattering assays show the effects of CLASP proteins on overall MT polymerization, CLASP has also been seen to bundle MTs, especially in egg extract experiments. In order to determine if CLASP bundles MTs in vitro, samples of CLASP-MTs from the polymerization reactions, previously described, were prepared for EM. The MT-CLASP solutions were diluted 200 fold, and solutions were deposited on grids for staining and microscopy. From EM micrographs, it is apparent that CLASP proteins containing TOG1 and crTOG2 domains have potent bundling activity. For CLASP1-1171 and CLASP1-662, massive bundles were seen throughout the samples. In contrast, CLASP fragments without these domains were found to have dispersed MTs. For fragment CLASP662-1463, there were a few bundles but the activity seen was still dramatically lower than TOG1 and crTOG2 containing proteins (Fig. 4.4).

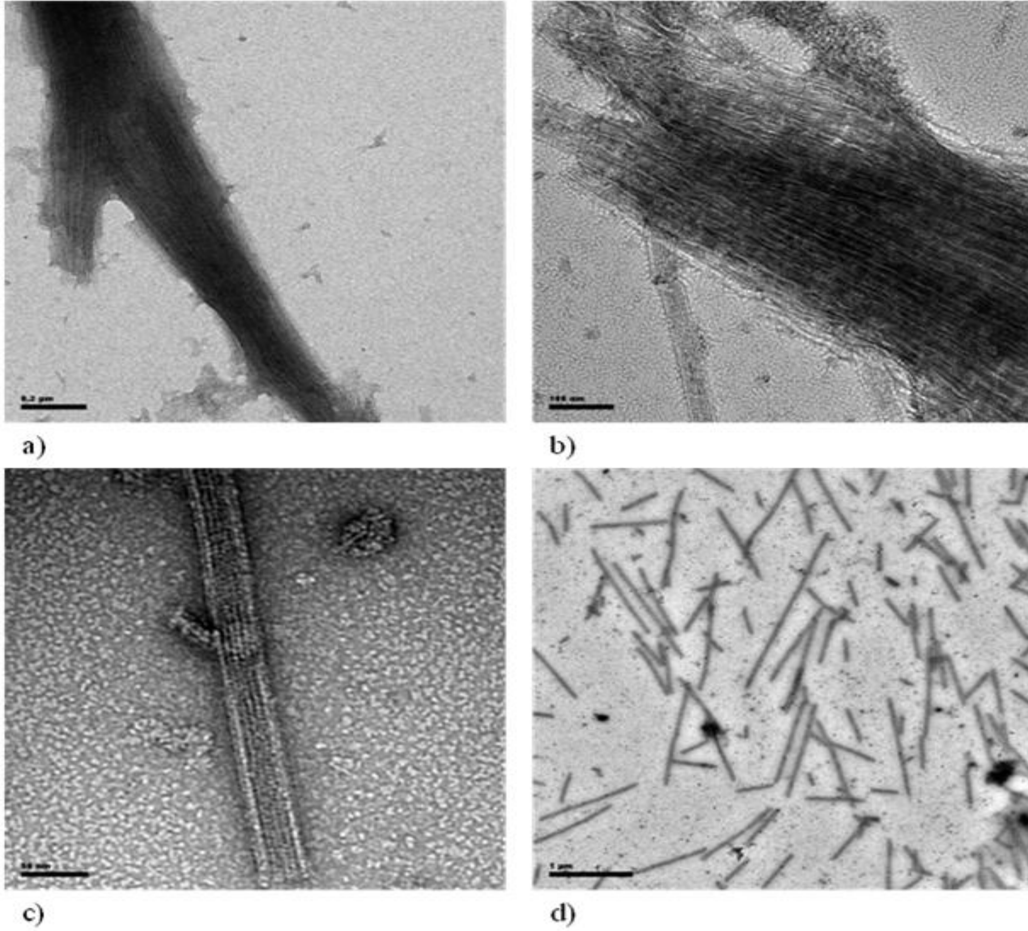


Figure 4.4: Negative stain EM micrographs of bundling effects of CLASP fragments on MTs. Microtubules co-polymerized with a) CLASP1-1171 b) CLASP full length c) CLASP662-1171 d) CLASP662-1463

CLASP stabilized MT bundles are resistant to cold induced de-polymerization

It appears that the bundling activity of CLASP may contribute significantly to MT regulation by this protein. We utilized a cold pelleting assay to provide additional evidence for the stabilizing effect of CLASP on MTs. It is thought that CLASP is essential not only in formation of MT bundles but also in their maintenance for a variety of different cellular processes. In this assay, MTs polymerized in the presence of CLASP constructs are placed on ice. Non-stabilized MTs will naturally de-polymerize at this temperature. However, certain proteins that stabilize MT assembly can slow or prevent this process (Bartolini, 2008; Sousa, 2007; Wu, 2008).

Cold-pelleting of CLASP1-662 bound MTs reveals the presence of highly stable MTs bound to approximately stoichiometric amounts of the construct. Nearly half of the tubulin in the sample remains assembled and found in the pellet after cold treatment (Fig. 4.5) EM visualization of the tubulin-CLASP fragment solution after cold treatment revealed that the pelleted fraction did consist of MT bundles and not simply aggregated tubulin. On the other hand, more than 95% of MTs without CLASP depolymerize and were found in the supernatant (Experiment 2, S). CLASP1-662 remains soluble and does not self pellet , indicating that the co-pelleting of CLASP with MTs remaining in bundles is specific and not due to aggregation. For experiments 2, half the amount of tubulin was used for this tubulin control alone as compared to experiment 3, reflected in the difference intensity of the tubulin bands.

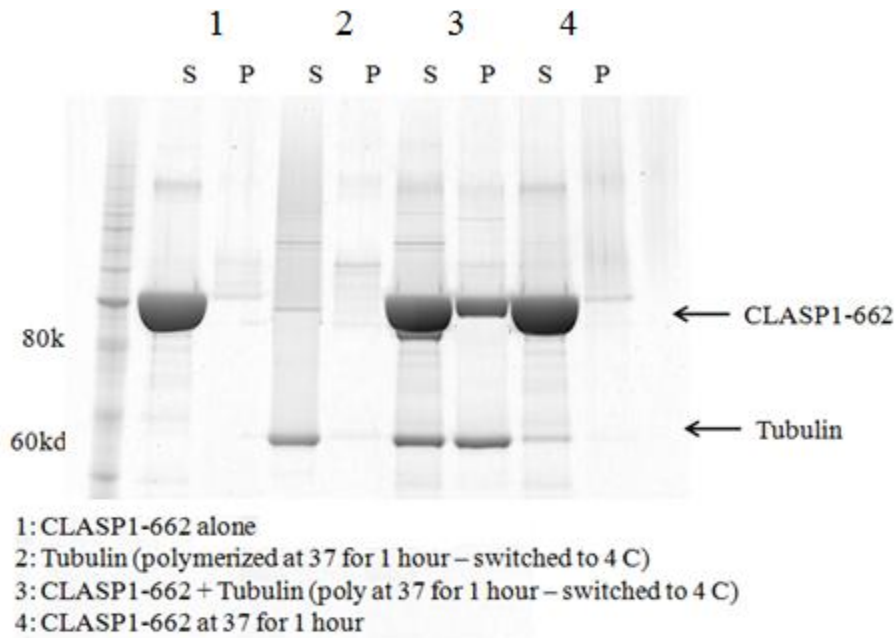


Figure 4.5: Cold-pelleting assay with CLASP1-662. (S) are supernatant fractions and (P) are pellet fractions of assay. For experiments 2, half the amount of tubulin was used for this tubulin control alone as compared to experiment 3, reflected in the difference intensity of the tubulin bands. Samples were pelleted and analyzed via SDS-PAGED (10% Bis Tris) and stained with Sypro-Ruby).

Specific domains in CLASP confer high affinity for the MT lattice

With the ability to observe CLASP in an *in vitro* context, another experimental goal was to observe CLASP bound to the MT using EM. EM could provide a useful tool in producing structural insights into how CLASP is mechanistically regulating MT assembly. However, due to a technical limitation of how the protein appears to binds, or the conditions in which this was tested, this became very difficult to accomplish. Thus, more in depth biochemical experiments were performed in order to gain some insight into how CLASP interacts with both tubulin and the MT lattice. This included a combination of techniques including pelleting assays, affinity pull downs and chemical cross-linking.

To examine CLASP interaction with MTs, CLASP was bound in 2 different manners. Due to high bundling activity, visualization of a single MT with CLASP was difficult when the reaction is setup in solution. Thus, to get around this, MTs were preassembled in the presence of taxol.

In the micrograph there are patches of density seen long the edge of the MT, mostly likely bound CLASP molecules. However, we also observe unbound CLASP in the background (yellow arrows). Despite some evidence of binding, there doesn't appear to an organized pattern of binding that would be amenable to cryo-EM reconstruction methods (Fig. 4.6).

Construction of binding curves for each of the CLASP proteins reveals differences in affinity for each of the constructs. Both full length CLASP appears to bind the MT lattice with a very high affinity. Surprisingly, proteins are also able decorate at a high fraction (0.8) despite the seemingly low decoration as observed by EM. As expected, CLASP662-1463 and CLASP662-1171 also appear to have weaker binding affinities for the MT lattice. From this data it appears that the pattern of binding affinities correlates with the level of polymerization activity, suggesting a possible explanation for the difference in activities.

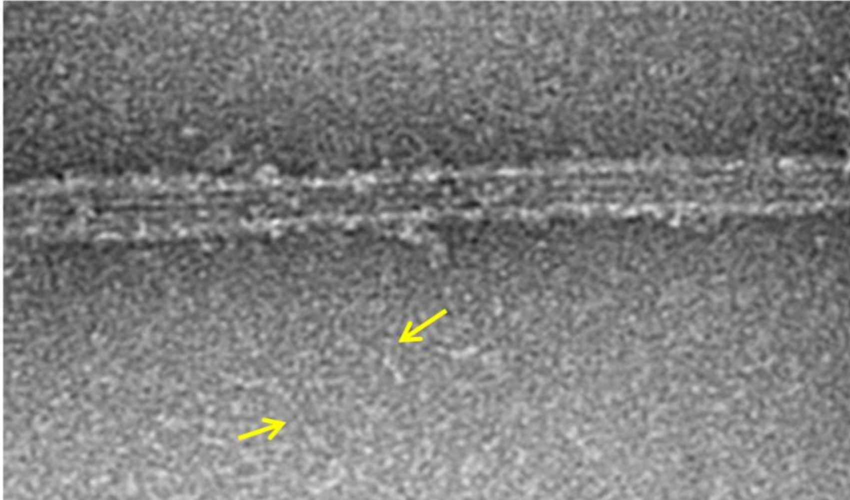


Figure 4.6: EM micrograph of MT decoration with CLASP (fl). Yellow arrows indicate unbound CLASP proteins

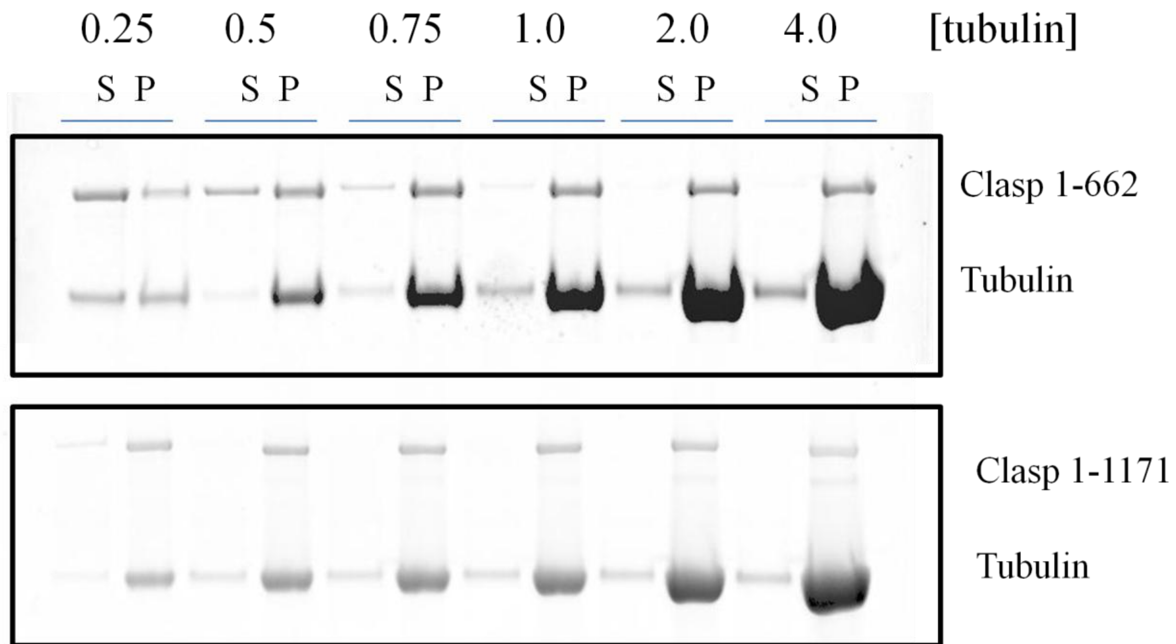


Figure 4.7: Representative gel images of MT pelleting assays for CLASP1-662 and CLASP 1-1171. (S) reflects supernatant, (P) reflects pellet. A fixed amount of CLASP protein (0.5 uM) was added to varying concentrations of MTs pre-polymerized and stabilized with taxol. Binding reaction were centrifuged, pelleted and both supernatant (S) and pellet (p) were recovered and analyzed by SDS-PAGE (10% Bis-Tris) and visualized with Sypro Ruby stain.

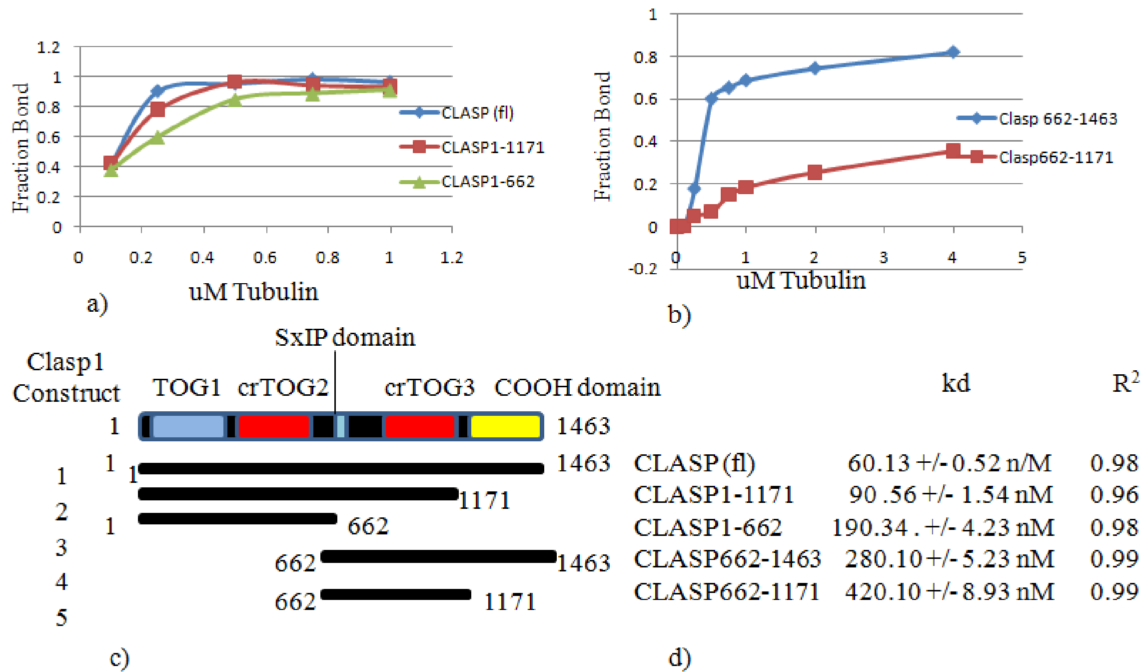


Figure 4.8: Calculations of binding affinity (kd) of CLASP proteins for the MT lattice. a) Affinity curves for CLASP proteins containing TOG1 and crTOG2. b) Affinity curves for CLASP proteins containing putative crTOG3 domain. To examine the binding affinity of individual CLASP fragments, concentrations of taxol stabilized MTs ranging from 0.0 µM to 4 µM were incubated with a fixed concentration of CLASP (0.5 µM) in buffer. After pelleting and SDS-PAGE, band intensities were quantified using ImageJ (NIH) and plotted to construct an affinity curve from which the kd was calculated using a bimolecular binding equation $Y = B_{max}X/(K_d + X)$, where Y is the fraction of CLASP protein in the pellet, X is the concentration of MTs, and B_{max} is the maximal achievable binding.

CLASP is able to bind free tubulin as well as short tubulin oligomers

While CLASP appears to have a high affinity for the MT lattice, previous studies have shown that CLASP and the TOG domain containing protein XMAP215 have affinity for free tubulin. To investigate this, 1 μM of CLASP proteins were incubated with 5 μM of tubulin solution, with either 1 mM GTP (Sigma) or 1 mM GMPCPP (Jena Biosciences) nucleotide in BRB80 solution for 15 minutes on ice. CLASP proteins were then affinity purified via the StrepII tag as previously described. Eluted samples were also observed by negative-stain EM.

The affinity pull down indicates that CLASP and CLASP1-1171 are able to bind free tubulin (1-662 also has some affinity, data not shown). Interestingly CLASP proteins without the TOG1 and crTOG2 domains do not show this activity. By EM, small globular complexes were observed in the sample with GTP tubulin, in which CLASP may be wrapping around a tubulin dimer. These globular particles were only observed in the presence of tubulin and not in preparations of CLASP alone. A similar type of complex has been proposed to form by XMAP215 and the *S. pombe* homolog of CLASP in the presence of unassembled tubulin (Al-Bassam, 2010; Brouhard, 2008). Affinity purification of CLASP also shows its binding to tubulin in the presence of the slowly hydrolyzing nucleotide analog GMPCPP (data not shown). Interestingly, in samples with GMPCPP, long, slightly curved particles were observed in greater abundance than in the sample with GTP. These particles, which are much larger than single tubulin dimers, resemble previously observed oligomers of GMPCPP-tubulin. However, these particles appear thicker than GMPCPP tubulin alone, suggesting that CLASP may be bound along their length.

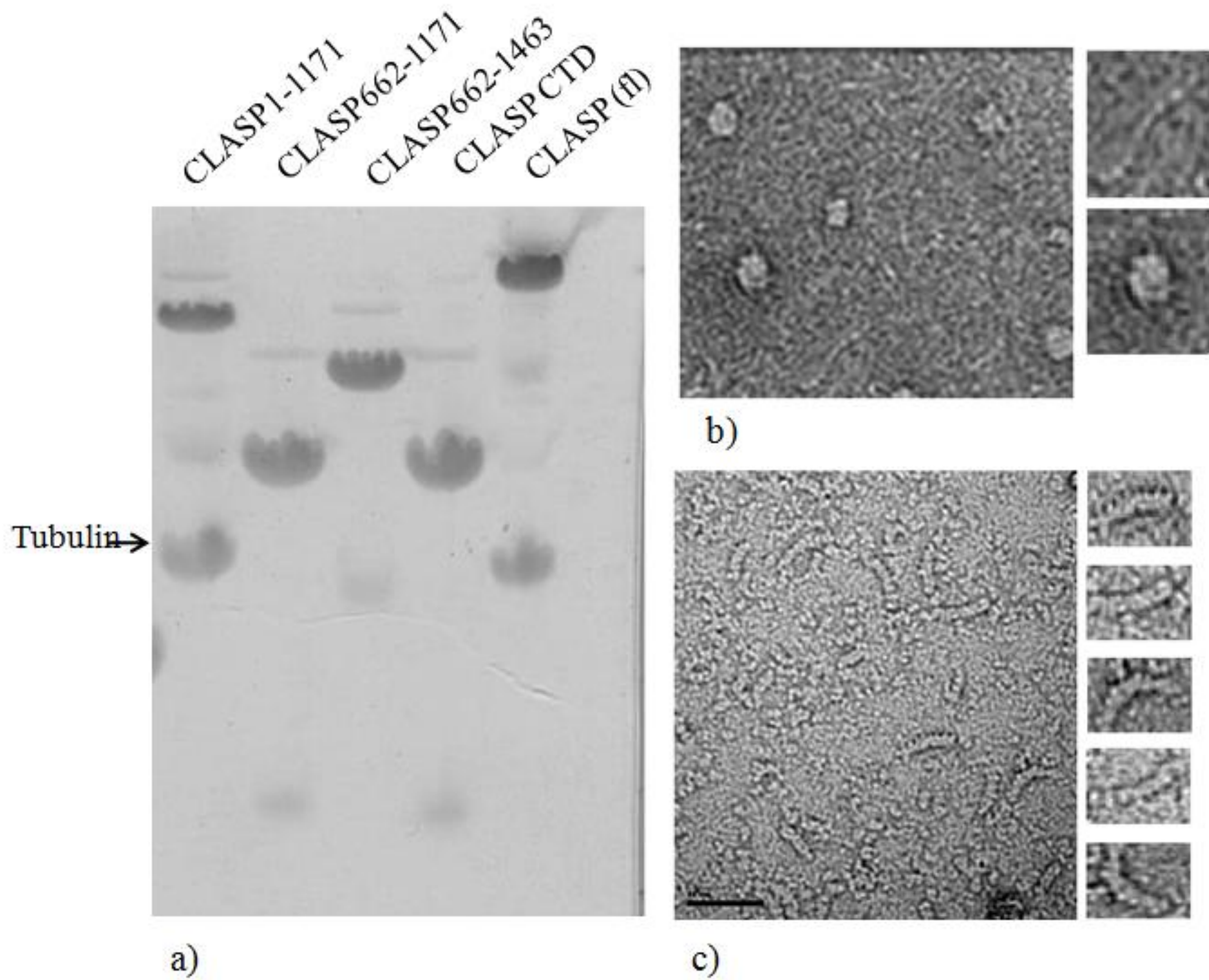


Figure 4.9: Affinity assay and EM of CLASP proteins binding to free tubulin. a) gel products of affinity pull down of free GTP-tubulin. b) Representative EM micrograph of CLASP with GTP-tubulin. Top right outset shows free CLASP, bottom shows CLASP-tubulin complex. c) Representative EM micrograph of CLASP with GMPCPP-tubulin oligomers.

CLASP interacts with MTs primarily through electrostatic interactions with the E-hook of tubulin

Various biochemical experiments were used to probe the nature of the interaction between CLASP and MTs. Cross-linking was performed in order to determine if CLASP is able to bind to both *α* and *b* tubulin. EDC is a zero-length cross linker that can be used to test if there is a specific interaction between lysine residues in CLASP and glutamate or aspartate residues in tubulin. To explore if this interaction was driven by electrostatic forces, sensitivity to salt was tested using pelleting assays. Lastly, there is increasing evidence that the unstructured C-terminal tail of tubulin, or E-hook, is important for the binding of various MAPs. To investigate if this feature of tubulin is important for CLASP binding, MTs were treated with the protease subtilisin which, under controlled conditions, will cleave the C-terminal tail off of tubulin. CLASP bound to both native MTs and MTs treated with this protease. Our data indicates that CLASP is capable of binding both *α* and *b* tubulin, likely through electrostatic interactions, with an important contribution of the C-terminal domain of tubulin. A small amount of CLASP can still bind after subtilisin cleavage of MTs, suggesting an additional interaction between this protein and the globular domain of tubulin within the MT lattice.

CLASP interacts with multiple binding partners through several domains

Previous studies have shown CLASP to a highly versatile protein, capable of binding many different proteins. Using an *in vitro* biochemical approach we sought to use the recombinant CLASP fragments to probe for novel interacting partners as well provide a rough map for possible domains responsible for these interactions. Using a simple pull down assay, CLASP and associated fragments were added to samples of Xenopus egg extract, affinity purified through the StrepII tag, washed and prepared using SDS-PAGE. Potential interacting proteins were probed using various antibodies available in the lab. Interestingly, the chromokinesin XKID was found as a CLASP binding partner, interacting through the C-terminal tail of CLASP. This was interesting, as this domain has been previously found to bind other proteins such as the kinetochore motor XCENP-E and the +TIP Clip-170 which were also confirmed by our results. Additionally, the MT stabilizing and bundling protein PRC1 was also found to bind CLASP, but in a middle region of the sequence, flanked by the S/R region and the putative crTOG3 domain. It appears that it is distinct from the SxIP region that is responsible for binding EB1 (Fig. 4.12)

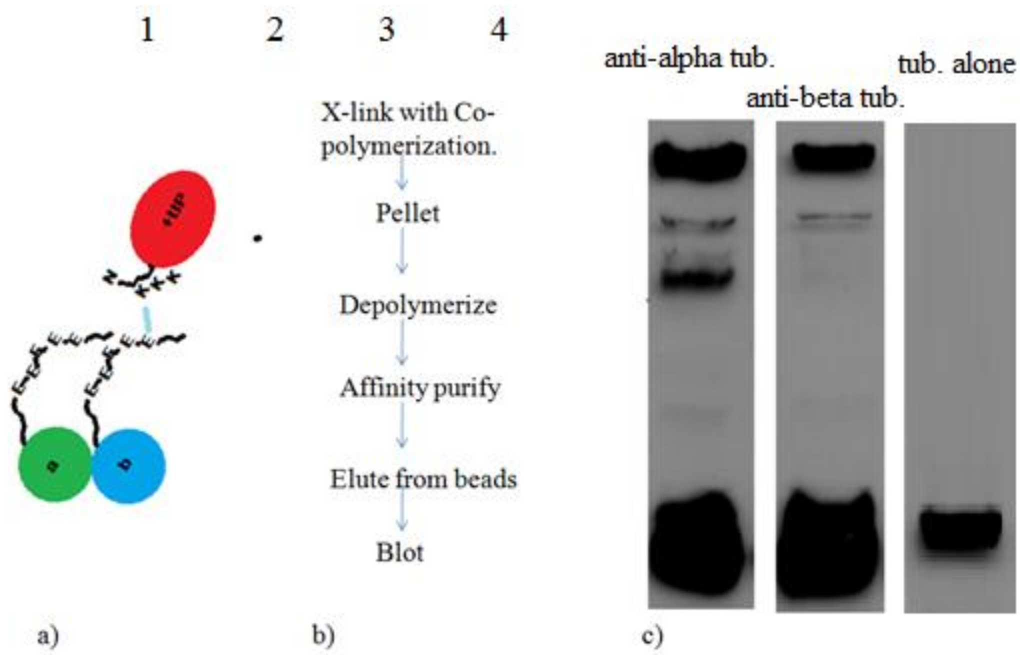


Figure 4.10: EDC cross-linking of CLASP to free tubulin. a) Diagram of possible interaction with E-hook of tubulin. b) Experimental scheme. c) Western blot of elution from Streptactin affinity beads with α-tubulin antibody. The blot reveals that after crosslinking, multiple higher molecular weight complexes form in the presence of CLASP protein. Tubulin alone does not appear to crosslink to itself suggesting specificity between CLASP protein and tubulin. At concentrations of EDC, only a fraction of tubulin is crosslinked, as the majority of tubulin appears not to be higher molecular weight complexes (lower bands). Presence of less intense higher molecular weight bands also appear but their origin is unclear.

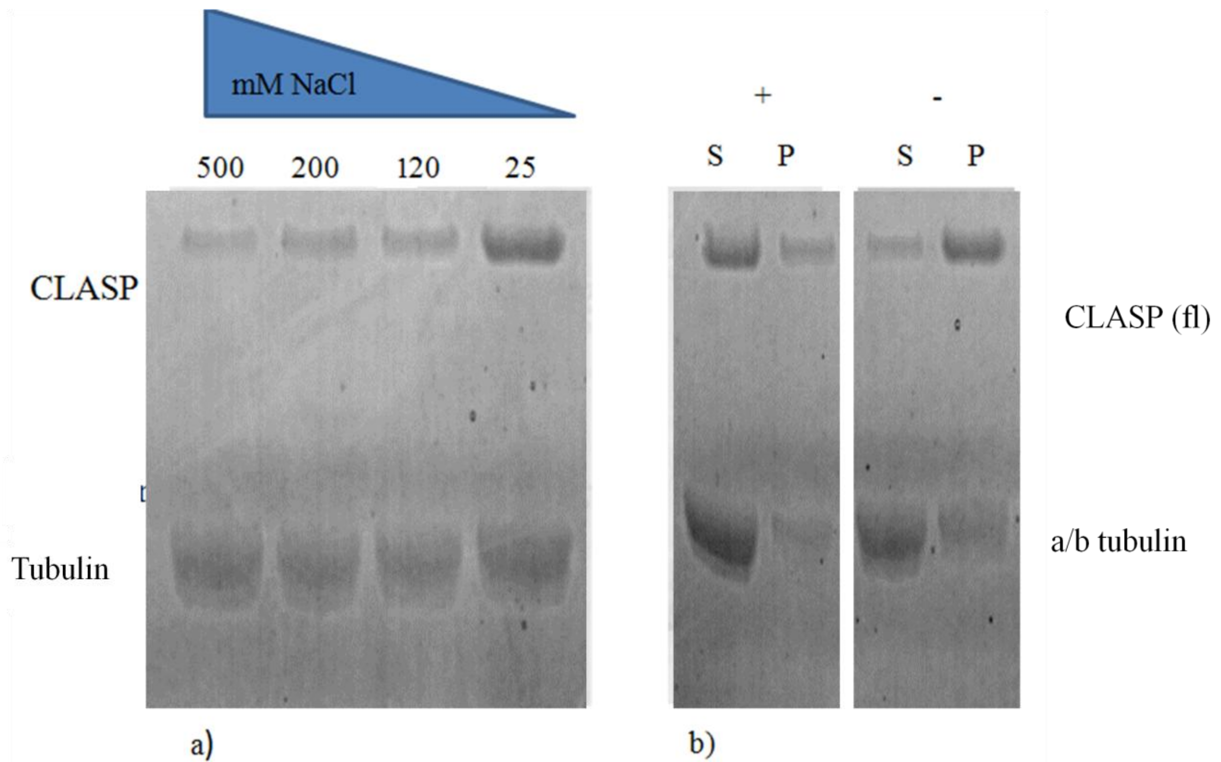


Figure 4.11: Pelleting assays of CLASP (fl) with MTs in the presence of increasing salt concentration or subtilisin-treated MTs. a) Pellet fractions of CLASP and MTs with increasing NaCl concentrations analyzed by SDS-PAGE (10% Bis-Tris) and stained with Sypro Ruby. b) CLASP binding to subtilisin treated (+) and non treated MTs (-) analyzed by SDS-PAGE (12% Bis-Tris) and stained with Sypro Ruby. It is likely that α and b tubulins are seen as separate bands due to greater subtilisin cleavage of C-terminal tail of b tubulin under conditions used.

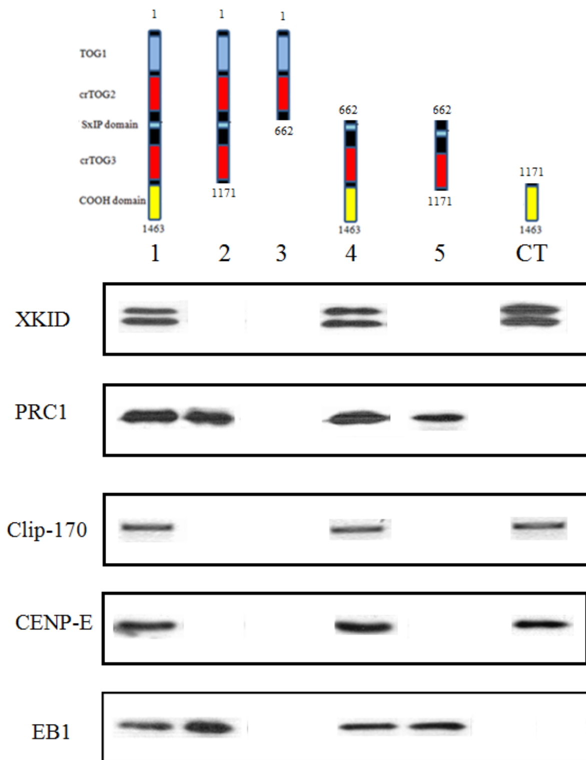


Figure 4.12: Identification of CLASP interacting partners and interacting domains. Full length CLASP and corresponding fragments were used to pull down various interacting proteins from Xenopus extract. For each protein fragment, potential binding partners were separated by SDS-PAGE and blotted for using available antibodies. Blots reveal chromokinesin XKID as a novel partner that interacts with CLASP most likely through the C-terminal domain, similar to XCENP-E and Clip-170. PRC1 was also found to interact with CLASP within a region flanked by the S/R region and the putative crTO3 domain. EB1 binds to all fragments containing the conserved SxIP motif.

Discussion

CLASP is a large (180 kD) and flexible elongated protein that appears to be able to form homo-dimers. Our data is consistent to what has been reported for the *S. pombe* CLASP, which appears as a *10* nm long filamentous molecule that compacts in the presence of tubulin. Thus, it appears that CLASP is similar in overall structure to *S. pombe* protein (they contain less than 50% sequence similarity). It is also interesting that CLASP homo-dimerization is mediated by the C-terminal domain. CLASP has been observed to interact with various other partners, such as Clip-170 and XCENP-E and it is unclear whether such interactions involves coiled-coil domains or if there is an interplay between CLASP dimerization and interaction with other binding partners. It is likely that CLASP is capable of multiple interactions through this modular yet promiscuous C-terminal binding region and that heavy regulation, either through localization or post translational modification helps to determine how different binding partners are capable of interacting through a common domain.

Our in vitro experiments demonstrate that CLASP is a potent regulator of MTs, promoting both polymerization and bundling. These activities, which we also observed in *Xenopus* egg extract experiments, are driven primarily by the N-terminal TOG1 and crTOG2 domains. These domains are responsible for increasing the rate of bulk MT polymerization, as well as overall assembly of MT polymer. Previous studies have shown that the CLASP homolog in *S. pombe* does not increase the polymerization rate of individual MTs, but rather increases the numbers of rescue events. While light scattering assays do not inform us on individual MT behavior, it is possible that CLASP activity might drive assembly of more MTs over time.

The protein promotes stable bundles, which are dramatically resistant to cold-induced de-polymerization (cold resistant) and could contribute or result in an overall increase in bulk assembly. The effect of CLASP on MT polymerization and bundling is more significant in the presence of the C-terminal domain, suggesting that dimerization increases its activity, maybe by coordinating the simultaneous binding of the N-terminal crTOG3 domains.

Additionally, there appears to be MT binding activity in a region nearer the C-terminal domain, as illustrated by experiments with the CLASP662-1463 construct, although not as strong as that found for the N-terminal TOG domains. This binding also appears to be affected by the C-terminal domain, with the absence of this region (e.g. CLASP662-1171), lowering affinity. The likely explanation for this observation is the presence of an additonal MT binding region, such as the crTOG3 domain. From previous experiments, it is known that at least 2 TOG domains must be arrayed for tubulin binding activity. We suggest that dimerization via the C-terminal domain generates an array of two crTOG3 domains, resulting in increased binding and polymerization activity. It is unclear if this domain binds differently from the canonical N-terminal TOG domains. Structural information is required to determine if this region does in fact contain a third TOG domain.

Taken together, our biochemical data supports a developing model for the mechanisms of CLASP interaction with MTs. Activity and affinity assays suggest the presence of an additional MT binding region of CLASP in addition to the N-terminal TOG1, crTOG2 and S/R region. It is possible that a third cryptic TOG domain, which is arrayed in trans via dimerization with another cryptic TOG domain confers another tubulin binding site in CLASP. Perhaps this region is able to recognize the tubulin lattice, while TOG1 and crTOG2 are able to bind free tubulin, although this is a speculative model. Studies with *S. pombe* CLASP have shown that the protein is able to bind both free tubulin and the lattice simultaneously suggesting that CLASP can act as

a tubulin shuttle, delivering free tubulin to the plus end of the MT, although it is not clear what domains of CLASP interact specifically with the MT lattice (Al-Bassam, 2010). Based on tracking experiments, EB1 might help localize or enrich CLASP towards the end of the MT. A key question is how CLASP binds differently to the MT lattice than to free tubulin. It is possible that the protein recognizes certain structural features in polymerized tubulin, perhaps through the putative crTOG3 domain (Fig 4.12a). There are certain limitations to this proposed mechanism, including questions arising as to how CLASP releases tubulin and promotes incorporation into the MT lattice.

An alternative model proposes that CLASP can act to promote protofilament stabilization perhaps similar to a model proposed for XMAP215. In this model, the different domains along the length of the protein bind along protofilaments, promoting tubulin-tubulin interactions (Asbury, 2008; Brouhard, 2008; Widlund, 2011). Both models are not totally incompatible and the mode of binding of CLASP may be modulated by other +TIPs such as EB1 and Clip-170.

Interestingly, CLASP has not been found to increase the rate of polymerization as the other major TOG domain protein XMAP215 does (Brouhard, 2008). Rather, it promotes rescues perhaps by interacting with curved filaments to help stop peeling, yet another potential mechanism of action (Al-Bassam, 2010). It appears that while both proteins share similar domains, the detailed architecture of the protein might account for the distinct mechanism of interaction with tubulin. If cryo-EM studies of MTs decorated with CLASP protein were possible in the future, in would be interesting to see how these TOG domains interact with tubulin and will greatly add to the understanding of its mechanism of action in the regulation fo tubulin assembly in the MT.

In addition to MT polymerization, another major CLASP function appears to be MT bundling. It is unclear how CLASP achieves this precisely, but TOG1 and crTOG2 domains appear to be the primarily responsible for this activity. Based on crystal structures, TOG domains contain 2 discrete 'faces' that are proposed to interact with tubulin. It is unclear if only one face can interact with tubulin at a time. One model proposed in the field suggests that only one face is able to bind tubulin. While TOG1 and crTOG2 bind one side of the MT, perhaps the S/R region can bind another (Slep, 2009; Slep, 2010; Slep, 2007). This would help explain why bundling was observed even for the protein CLASP1-662. Another model suggests that either face of the TOG domains can bind tubulin (Fig 4.12b). Structural information and further work with single molecule systems should help elucidate how CLASP functions on the MT.

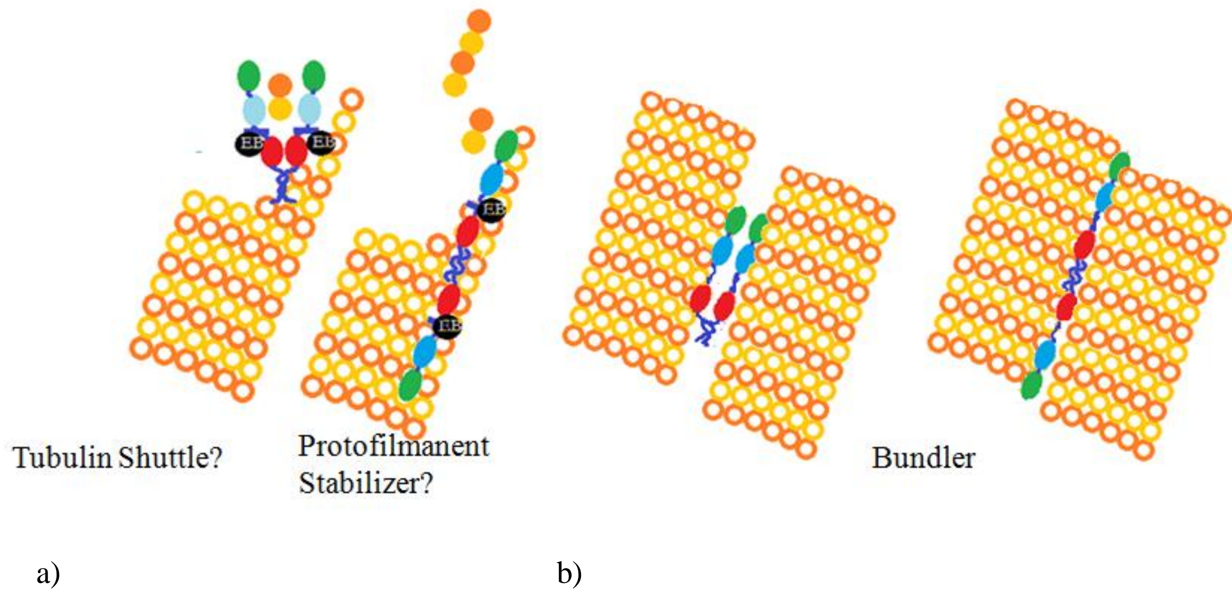


Figure 4.13: Models of CLASP regulation of MTs. a) potential mechanism for MT polymerization. b) potential mechanisms for MT bundling.

Materials and Methods

Negative Stain EM

In order to gain information about the structure and molecular nature of CLASP, full length protein was visualized using electron microscopy of negatively stained samples. Purified protein (3.2 mg/ml) was diluted in elution buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, 0.1% Tween-20, 10 mM d-biotin) to a concentration of 9 µg/ml. A 400-mesh copper grid (Ted Pella) overlaid with a very thin continuous carbon layer was gently glow discharged and 5 ul of the diluted protein was applied to the grid. After 5 minutes adsorption, the protein was blotted away from the grid with filter paper (Whatman, No.1) leaving a thin layer of solution on the grid. The grid was then picked-up and gently floated on 5ul drop of 4% uranyl-acetate solution for 30 seconds on a parafilm support. Stain was blotted from the grid and the sample was floated again on 5ul drop of fresh stain for 2 minutes. After blotting, the grid was left to dry for 2 minutes (Fig. 4.1). The negative stain sample of CLASP was imaged using a Tecnai12 TEM (FEI, C_s mm) and recorded on a Gatan CCD camera at 1.5 µM defocus.

Gel Filtration of CLASP full length protein

Protein samples were diluted in low salt solution similar to elution buffer ((50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM DTT, 0.1% Tween-20, 10 mM d-biotin). Since the protein begins to aggregate at lower salt concentrations, the samples were ultra centrifuged for 20 min at 90K rpm (TLA100.1 rotor; Beckman) to clarify the sample before loading on the column. A Superdex 200 HR/30 was equilibrated with the same dilution buffer and 100 ul of each sample was analyzed. Traces were compared to standards also run on the column to allow estimation of the molecular weight.

90˚ light scattering assays of CLASP and tubulin

In these assays, a fluorimeter (Hitachi XW-100 model) was used with excitation and emission wavelengths set at 350 nm and a slit width of 1.5 nm. After clarification by centrifugation at 90K for 20 min (rotor TLA100.1; Beckman) to sediment protein aggregates before assay, CLASP protein (50 µg/ml) in MT polymerization buffer (80 mM PIPES, 1 mM MgCl2, 0.5 mM EGTA) supplemented with 1 mM GTP, 1 mM DTT and 80 mM NaCl was placed in a glass cuvette. 15 µM concentration of bovine tubulin (Cytoskeleton) was then added to the cuvette and then the cuvette placed in a heated chamber (~32 C) within the fluorimeter. Data was collected for 25 min, with time points taken every 10 sec.

Tubulin (cytoskeleton) was polymerized at 37 degrees for1 hour in the presence of 1 mM GTP (Sigma), BRB80, 1 mM DTT and 2 mM Taxol (Cytoskeleton). The solution of MTs was then diluted 1:100 buffer with the same components, deposited on an EM grid and adsorbed. After 2 minutes, the MT solution was blotted off the grid. CLASP proteins, diluted from stock to 50 ug/ml were then pipette onto the grid. CLASP was allowed to adsorb for 2 minutes and this step was repeated at least twice. The grid was then stained and imaged with uranyl acetate as previously described.

Cold Pelleting Assay

CLASP1-662 protein (selected based on quantity in stock) was incubated with tubulin and polymerized at 37 C in a similar reaction as described previously. After polymerization, the reaction was placed in ice for 30 minutes. The samples were then sedimented at 90K rpm for 20 min (rotor TLA100.1; Beckman). The supernatant was removed and 4X SDS sample buffer added. Sample buffer was also added to the pellet, vortexed for 2 min and incubated on ice for 30 min before loading on gel.

CLASP-MT Pelleting Assays and kD determination

To examine the binding affinity of individual CLASP fragments, concentrations of taxol stabilized MTs ranging from 0.0 µM to 4 µM were incubated with a fixed concentration of CLASP (0.5 µM) in a buffer consisting of BRB80, 80 mM NaCl, 1 mM DTT. Samples were incubated for 15 minutes and then sedimented by centrifugation at 90K rpm (rotor TLA100.1; Beckman). After the supernatant was removed, the pellet was resuspended on ice for 30 minutes with sample buffer. The pellet and supernatant fractions were analyzed by SDS PAGE , stained with Sypro Ruby stain (Bio-rad) and imaged with an *A* Imager (Biorad). Band intensities were quantified using ImageJ (NIH) and plotted to construct an affinity curve from which the kd was calculated. Data were fitted to a bimolecular binding equation $Y = B_{max}X/(K_d + X)$, where Y is the fraction of CLASP protein in the pellet, X is the concentration of MTs, and B_{max} is the maximal achievable binding.

Chemical Cross-linking using EDC

A cross linking experiment was performed with the zero-length cross linker EDC to determine if CLASP interacts with either *α* or *b* tubulin. To generate cross-linked products, CLASP (Grallert et al.) (2.0 µM) was incubated with 5.0 µM of GTP-MTs in BRB80 buffer for 30 min at 37 C. EDC (2 mM) and *N*-hydroxysulfosuccinimide (5 mM) were added, and the mixture was incubated for another 30 min at 25 °C. The reaction was terminated by the addition of 10 mM hydroxylamine. CLASP proteins were purified as previously described and analyzed by western blot, using antibodies specific for *α* tubulin (Santa Cruz Biosciences) and *b* tubulin (Santa Cruz Biosciences).

Pelleting Assays with subtilisin and salt treated MTs

To further investigate the nature of interaction, sensitivity of CLASP binding was tested with both salt and subtilisin treated MTs. Taxol MTs were prepared as previously described. For salt sensitivity tests, 5.0 µM of full length CLASP was incubated with 1 µM MTs and sedimented. Bound CLASP was then re-suspended and incubated with 25 – 500 mM NaCl in BRB80 buffer. Microtubules were sedimented and analyzed as previously described. For subtilisin treatment, Taxol MTs (2 mg/mL) with subtilisin (80 µg/mL) at 37 °C for 10 min. Microtubules missing both tails were produced by 120 min of digestion under similar conditions. In both cases, subtilisin cleavage was halted by the addition of 4 mM PMSF (pimindimethyl sulfoxide) for 20 min at 25 °C, and the MTs were then pelleted at 100,000*g* for 15 min. The pellet was washed once and re-suspended to 1 µM tubulin concentration in BRB80 containing 1

mM GTP and 20 μM taxol. 5 μM CLASP (Grallert et al.) was added and incubate for 15 minutes, after which the sample was sedimented and analyzed as previously described.

Chapter 5

Future Perspectives

Dissecting the various roles of CLASP in the mitotic spindle

Various studies of CLASP, including this work, show this protein to be highly versatile and involved in a number different functions. The ability of CLASP to bind multiple partners and localize to highly specific regions in the spindle illustrate its functional complexity in spindle assembly. Perhaps one of the most interesting aspects of the protein is its dual nature concerning its effect on MTs – the ability to bind and track dynamic MTs as well as bind and stabilize robust MT bundles. Key questions remain as to how these functions are achieved in the cell and what possible mechanisms of regulation exist to ensure that CLASP activity is either appropriately localized or tuned to the appropriate levels. Our data shows the dramatic effects that CLASP activity can have on spindle assembly and on MT organization in general when its regulation is altered.

There are several important questions regarding CLASP activity at the kinetochores and at the spindle mid-zone. It appears that CLASP promotes MT growth, but how is this activity regulated to maintain spindle bipolarity and normal spindle length? This could be achieved through a balance of activities with other MT de-polymerizing proteins such as MCAK. Alternatively, CLASP may be regulated via re-localization to other regions of the spindle, perhaps by flux from the plus-end to the minus-end. It would be interesting to study the levels of these MT regulators, perhaps in *Xenopus* egg extracts, and conduct experiments in which these levels of activity are manipulated, either through addition or depletion of factors such as XMAP215, MCAK and CLASP. It is likely that a specific balance is required for these difference pathways, and that there exists some redundancy for various proteins.

Other important remaining questions include the specific nature of CLASP-MT bundles. While there are other MAPs known to promote MT growth, CLASP appears to be specific it its ability to form highly robust bundles. These bundles appear to be important in a variety of different contexts. At the kinetochore, k-fibers appear to be essential for attachment in mammalian cells. What is the contribution of CLASP to this bundling in the cellular context? How does a bundle contribute to attachment? How is the number of MTs in a bundle determined? Perhaps the number of MTs and hence the size of bundles can be explained by mechanisms that finely tune rates of nucleation, stabilization and catastrophes, via regulation of expression, localization or activity of CLASP. Quantitative analysis and highly sensitive fluorescent experiments will be needed to help address these questions.

Another potential functional relevance of the MT bundling may relate to do with the role of CLASP and the central spindle. In both *C. elegans* and *X. laevis*, it has been shown that CLASP plays a critically important role in producing stabilized bundles of MTs in late anaphase. In worms, it has been proposed that these bundles are a major driving force in separating chromosomes during this stage of mitosis. While the kinetochore plays a crucial role in chromosome alignment and forces generation in the early stages of anaphase, it is the central spindle that provides a “push” zone” for chromosomes. It is unclear, however, how or if CLASP might be involved in the generation of these forces. The spindle mid-zone is the site of localization of numerous mitotic factors, including known MT bundlers such as PRC1 and KLP-19, but it is unknown how individual components, including CLASP, play specific roles in the process. Biophysical experiments and perhaps single molecule studies with one or more of these components should shed some light on how these bundles help generate these important chromosome separating forces.

Generating structural data for mechanism of CLASP regulation of tubulin

At the molecular level, there are many important questions to be answered regarding CLASP mechanism of action on MTs and how it may differ from other MT regulators. It appears that both CLASP and XMAP215 are TOG containing proteins, and yet, their behavior concerning MT regulation is surprisingly different. XMAP215 appears to act as a monomer, with 5 arrayed TOG domains that promote accelerated growth of the MT plus-end, even in the absence of EB1 (Brouhard, 2008; Kronja, 2009). On the other hand, while CLASP contains highly similar TOG domains, sufficient number may only be obtained upon dimerization of the protein via its coiled-coil. Furthermore, these domains may confer rescue activity by preventing de-polymerization although this proposed mechanism of action needs to be explored further *in vivo*. Amazingly, these two proteins still appear to bind un-polymerized tubulin in a similar manner. It is likely that other domains in these proteins, such as the S/R region in CLASP, or the differences in the C-terminal domains between both proteins, help to regulate TOG activity for specific purposes. Biochemical experiments involving domain swapping between the CLASP and XMAP215 might yield clues to the specific functions of either protein and how these domains do regulate TOG-tubulin interactions.

Other interesting questions remain about the nature of the TOG-tubulin interaction itself, and how these interactions produce such activity. What types of tubulin interactions do TOG domains promote – lateral contacts between protofilaments? Or longitundinal contact with an incoming free tubulin? Or both? Despite the present inability to generate structural data with the reagents I generated, more work needs to be done to investigate the nature of these interactions using a structural approach. If samples are not amenable to cryo-electron microscopy, perhaps cryo-tomography will be useful, at least to help visualize the protein bound to the MT. In addition to this approach, CLASP bound to MTs with other +TIPs such as EB1 and Clip-170 might also provide interesting insights into MT binding. My data has shown that EB1 can affect CLASP activity and further work needs to be done to investigate how other binding partners in complex with CLASP can affect its activity.

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