Human CLASP1 mediates kinetochore interactions with the plus ends of dynamic microtubules

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ABSTRACT

One of the most intriguing aspects of mitosis is the ability of kinetochores to hold onto the plus-ends of dynamic microtubules that are actively gaining or losing tubulin subunits. Here we show that the microtubule-associated protein CLASP1 is localized preferentially near the plus-ends of growing microtubules during spindle formation and is also a component of a novel region that we term the outer kinetochore corona. A truncated form of CLASP1 lacking the kinetochore-binding domain behaves as a dominant-negative, leading to the formation of unique single or double asters comprised of radial arrays of microtubule bundles that are highly resistant to depolymerization. Microinjection of cells with antibodies specific to CLASP1 causes bipolar spindles to collapse, forming bipolar or monopolar arrays of microtubules with chromosomes buried in the interior. Suppression of microtubule dynamics in injected cells rescues the kinetochore association with plus ends of microtubules at the periphery of the asters. Our data suggest that CLASP1 is required for kinetochore-associated microtubules to exhibit normal dynamic behaviour.
INTRODUCTION

Kinetochores are the specialized structures that attach chromosomes to the plus ends of spindle microtubules (Brinkley, 1966; Jokelainen, 1967; Euteneuer and McIntosh, 1981). One constraint on this attachment is that microtubules are highly dynamic structures that alternate between states of growth and shrinkage (Mitchison and Kirschner, 1984; Desai and Mitchison, 1997). Furthermore, the bundled kinetochore microtubules exhibit dynamic behaviour whilst remaining attached (Mitchison and Kirschner, 1985; Mitchison et al., 1986; Koshland et al., 1988; Coue et al., 1991; Hyman and Mitchison, 1991). Thus, a captured microtubule is stabilized at a kinetochore not because it becomes less dynamic, but because it cannot detach (Hyman and Karsenti, 1996). The demonstration that kinetochores could hold onto dynamic microtubules identified one of the key questions in chromosome segregation: how do kinetochores remain attached to microtubules that are actively gaining or loosing tubulin subunits at their plus ends?

The dynamic behaviour of microtubules is largely regulated by microtubule-associated proteins (MAPs), and recently it has been possible to reconstitute apparently normal microtubule dynamics in solution from purified components (Kinoshita et al., 2001). However, the situation at the kinetochore is more complex, and alternations between states of microtubule growth and shrinkage are regulated at least in part by kinetochore components (Hyman and Mitchison, 1990). Candidates for this role include MCAK/XKCM1 (Wordeman and Mitchison, 1995; Walczak et al., 1996) and the Kin1 kinesins (Desai et al., 1999), but many other factors are likely to be involved. More recently, extensive studies in budding yeast have pointed to a critical role for a number of non-motor MAPs in kinetochore-microtubule attachment (He et al., 2001; Lin et al., 2001; Cheeseman et al., 2001a;
Cheeseman et al., 2001b; Janke et al., 2002). This is currently a rapidly advancing area, though difficulties in finding metazoan homologues for several of the gene products have restricted further progress (but see Wigge and Kilmartin, 2001; Howe et al., 2001).

The first protein shown to be involved in tethering kinetochores to microtubules was the kinesin-related protein CENP-E (Lombillo et al., 1995), which is now thought to report the status of kinetochore attachment to the metaphase checkpoint (Abrieu et al., 2000). Another protein potentially involved in microtubule plus end binding by the kinetochore is CLIP-170, which was first identified as a factor required for binding of endocytic transport vesicles to microtubules (Pierre et al., 1992). Loss of the S. pombe homolog of CLIP-170, tip1p, results in an increased frequency of microtubule catastrophes (Brunner and Nurse, 2000). The discovery that CLIP-170 localizes at prometaphase (though not metaphase) kinetochores (Dujardin et al., 1998) suggested that the protein might also be important for interactions of kinetochores with microtubules. Subsequent observations appeared to exclude the possibility that CLIP-170 is the prime microtubule-binding factor in the kinetochore (Dujardin et al., 1998), but it remains possible that the protein is important for initial interactions between kinetochores and microtubules.

Other microtubule plus-end binding proteins, collectively referred to as +TIPs (Schuyler and Pellman, 2001) have been studied primarily due to their role in promoting polarized cell growth during interphase (McNally, 2001). The CLASP proteins, isolated through their ability to interact with CLIP-170/CLIP-115, were shown to associate with and stabilize microtubule plus ends at the leading edge during fibroblast motility, thereby promoting polarized growth (Akhmanova et al., 2001). Another family of microtubule plus end-binding proteins, the EB1 proteins
(Su et al., 1995), also appears to be involved in polarized growth of cells apparently through interaction with specific sites on the cell cortex (McNally, 2001).

The role of these plus end-binding proteins in mitotic events has been relatively little studied. However APC is found at kinetochores (Kaplan et al., 2001), thereby suggesting a possible role for EB1 in chromosome segregation. A recent RNAi study of *Drosophila* EB1, confirmed that the protein is required for spindle assembly, particularly stabilization of astral microtubules (Rogers et al., 2002). Kinetochoore fibres were detected in EB1-depleted cells, suggesting that this protein does not have an essential role in microtubule binding.

Prior to the discovery of the CLASPs, genetic screens had identified a conserved non-motor MAP called variously MAST/Orbit in *D. melanogaster* (Lemos et al., 2000; Inoue et al., 2000) and Stu1 in *S. cerevisiae* (Pasqualone and Huffaker, 1994). These proteins are the fly and yeast homologues of the CLASPs. Mitotic phenotypes of MAST/Orbit mutants were complex, but suggested that the protein was essential for spindle assembly and function, possibly by promoting microtubule stability (Kline-Smith and Walczak, 2000). Subsequent RNAi and time-lapse microscopy analysis of MAST alleles revealed that the protein is required for chromosome alignment and maintenance of spindle bipolarity in mitosis (Maiato et al., 2002).

The goal of the present work was to determine the role of the human CLASP1 protein in mitotic events. We have shown that CLASP1 is a component of the outer corona of kinetochore that binds to microtubules near their plus ends during mitosis. Interference with CLASP1 function causes the accumulation of monopolar spindles with chromosomes buried in the interior. Our functional analysis of these structures enables us to propose a single simple hypothesis that can explain the multitude of phenotypes seen after perturbation of CLASP1
function. We propose that CLASP1 is required for the normal regulation of microtubule dynamics at the kinetochore. Thus, CLASP1 is essential for one of the most remarkable and mysterious properties of the kinetochore: the ability to bind and influence the dynamic properties of spindle microtubules.
RESULTS

CLASP1 exhibits dynamic changes in localization during mitosis

Endogenous CLASP1 was localized in mitotic HeLa cells using an antibody directed against a peptide sequence specific to CLASP1 (Experimental Procedures). This antibody stained centrosomes and kinetochores in early mitotic cells (Figure 1A, B). Preimmune or peptide competition with the immune sera failed to give any specific staining in mitotic cells (data not shown). CLASP1 relocalized dramatically following the metaphase-anaphase transition, moving to the spindle midzone (Figure 1C), and ultimately concentrating in the midbody (Figure 1D). Staining of the centrosomes remained strong through anaphase, but declined in telophase. This staining pattern was confirmed by expression of GFP-CLASP1 and examination of fixed cells (Figure 1E-H). The pattern of localization by CLASP1 is reminiscent of the localization of CENP-E, a kinesin-related protein that has been implicated in kinetochore attachment to the mitotic spindle and in checkpoint signalling (Yen et al., 1992; Yao et al., 2000; Abrieu et al., 2000). However, the two proteins are unlikely to function in the same pathway, as the phenotypes produced by interference with CENP-E and MAST/CLASP1 function are distinct (Schaar et al., 1997; Wood et al., 1997; Maiato et al., 2002; see below). Furthermore, we have found that perturbation of CENP-E localization by injection of function-blocking antibody or by overexpression of a dominant-negative construct have no effect on the behaviour of CLASP1 in cells (data not shown).

CLASP1 localises to microtubule plus ends during mitosis

Four-dimensional analysis by restoration microscopy revealed that EGFP-CLASP1 was preferentially associated with the plus-ends of growing microtubules in living mitotic cells (Figure 2). EGFP-CLASP1 also associated with centromeres during prometaphase (Figure 2A and movie 1 in supplementary information). In metaphase, EGFP-CLASP1 appeared to coat the spindle more or less uniformly.
At the metaphase-anaphase transition, EGFP-CLASP1 underwent a dramatic re-
distribution, accumulating in the central spindle and midbody during telophase
(Figure 2B and movie 2 in supplementary information). CLASP1 remained
associated with the plus ends of growing microtubules at the leading edges of the
expanding cytoplasm during the later stages of cytokinesis (Figure 2C and movie 3
in Supplementary information). This confirms the earlier observation that CLASPs
associate with the plus ends of microtubules in interphase (Akhmanova et al.,
2001). These observations that CLASP1 remains associated with the plus ends of
microtubules throughout mitosis raise the possibility that CLASP1 also associates
with one of the most interesting subsets of microtubule plus ends in mitosis – those
associated with the kinetochores.

**CLASP1 defines a new region of the outer kinetochore**

CLASP1 and EGFP-CLASP1 were localized to kinetochores and centrosomes
both in the presence and absence of microtubules (Figure 3). Within the
kinetochore, CLASP1 was located distal (external) to the area stained by anti-
centromere antibodies (ACA) or anti-CENP-C alone (Figure 3A and supplementary
information). ACA staining extends from the centromeric heterochromatin to the
inner kinetochore plate, where CENP-C has been localized (Cooke et al., 1990;
Saitoh et al., 1992). Surprisingly, CLASP1 was also largely distal to CENP-E, a
constituent of the fibrous corona (Cooke et al., 1997; Yao et al., 1997) that lies
outside the outer kinetochore plate (Figure 3B). EGFP-CLASP1 could be clearly
seen to lead the kinetochore to the pole during anaphase (Figure 3C). Thus,
CLASP1 is the outermost kinetochore component that we have identified to date.
We refer to the region containing CLASP1 as the “outer corona”. This localization of
CLASP1 implies that the protein must bind to microtubules (which typically end at the
kinetochore plate) internal to the plus ends. Because CLIP-170, a binding partner of
CLASPs, undergoes a radical relocalization on microtubules in the presence of taxol
Maiato et al. CLASP1 in the kinetochore

(Perez et al., 1999), we next examined whether the localization of CLASP1 is affected by taxol.

EGFP-CLASP1-expressing cells were treated for 30 minutes with taxol to specifically suppress plus end microtubule dynamics (Jordan and Wilson, 1999). This caused a dramatic redistribution of CLASP1 (Figure 3D, E). Traces of kinetochore-associated EGFP-CLASP1 remained following incubation with taxol (green dots seen at the ends of the kinetochore microtubule bundles, Figure 3E, right panel). However, the bulk of the protein was substantially depleted from the kinetochore-proximal microtubule plus ends, and redistributed to regions of microtubules closer to the poles, which appeared to widen upon taxol treatment. CLASP1 could remain associated with minus end-proximal regions of the kinetochore microtubules, or it may bind to newly assembled short astral microtubules, such as have been observed in insect spermatocytes following taxol treatment (LaFountain et al., 2001). This behaviour of CLASP1 is reminiscent of that of CLIP-170 in interphase cells (Perez et al., 1999).

The microtubule-binding domain of CLASP1 stabilizes and bundles microtubules

To identify the domains of CLASP1 responsible for its localization to different compartments of the mitotic apparatus, we constructed a series of vectors expressing different regions of the protein fused to EGFP (Figure 4A). This analysis revealed that the 300 C-terminal amino acids were necessary and sufficient for kinetochore targeting (Figure 4A-C). This required the C-terminal HEAT repeat (Figure 4A, D), which might be involved in interactions with other proteins such as CLIP-170 (Andrade and Bork, 1995; Andrade et al., 2001; Akhmanova et al., 2001).

A large central region, comprising residues 250-943 of CLASP1, was sufficient for association with microtubules in transfected cells (Figure 4A, E-G). This region includes regions of homology to known microtubule-binding domains
Overexpression of this construct during interphase caused the formation of tightly packed microtubule bundles (Figure 4E, G). These bundles were highly resistant to depolymerization induced by long-term exposure to colcemid (Figure 4F). Thus CLASP1 both bundles and stabilizes microtubules.

When overexpressed during mitosis, EGFP-CLASP1<sub>250-943</sub> had a highly potent dominant negative effect (Figures 4H and 5). In over 60% of the cases, cells had unique single or double asters in which the astral rays consisted of bundles of extremely closely packed microtubules (Figures 4H, 5). Within the bundles, the center-to-center spacing between adjacent microtubules was 24.3 ± 4.5 nm (Figure 5F). Since the diameter of a microtubule is 25 nm, this means that EGFP-CLASP<sub>1250-943</sub> can bundle microtubules so that there is little or no free space between them. In images where bundles were visualized in cross section, it was possible to see microtubule profiles that appeared to be close-packed with no space in between (insert in Figure 5D). The distal ends of these microtubule bundles were enriched for EGFP-CLASP1 and EB1 (Figure 5A and B), but lacked detectable adenomatous polyposis coli protein (APC – data not shown).

Although chromosomes were buried deep within the EGFP-CLASP1<sub>250-943</sub>–induced asters, kinetochores, as revealed by anti-CENP-C immunostaining, did not appear to be attached to the ends of microtubule bundles (Figure 5C). However, serial-section electron microscopy revealed that this appearance was misleading, and many kinetochores had normal end-on attachments to microtubules (Figure 5E). Some kinetochores could be seen in close proximity to the centrosome (Figure 5E). Furthermore, examples could be found of chromosomes that had achieved an apparently normal bipolar attachment (Figure 5F). The discrepancy between light and electron microscopy observations likely reflects the enormous differences in the amount of tubulin in the astral bundles as opposed to the kinetochore fibres. The unusual radial distribution of microtubule bundles induced by CLASP1<sub>250-943</sub> suggests that the protein either induced microtubule-microtubule
interactions after radial growth from the poles had started, or that these bundles originated as normal kinetochore fibres, but persisted and grew after detachment of the kinetochores from the distal plus ends.

**Microinjected anti-CLASP1 antibodies perturb the dynamic behaviour of kinetochore microtubules**

To further probe the function of CLASP1 in mitosis, we microinjected cells with anti-CLASP1 or control (pre-immune) antibodies during interphase. Approximately 50% of cells that reached mitosis 12 h after microinjection with anti-CLASP1 antibodies formed monopolar spindles (Figure 6A), a 10-fold increase over control injections (data not shown). Injection of anti-CLASP1 antibodies caused dispersal of the endogenous protein to the cytoplasm (Figures 6A and 7B', C') and the formation of monoasters with chromosomes buried deep within the radiating microtubule bundles. Very few kinetochores were associated with the plus ends of microtubules around the periphery (Figure 6B-B'). Remarkably, this phenotype observed after a 12h exposure to antibody changed dramatically within 30 min after the addition of low doses of taxol. Kinetochores were now seen to occupy their normal positions at the periphery of the aster in association with the plus ends of astral microtubules (Figure 6C-C'). Similar results were obtained when antibody-injected cells were treated with nanomolar concentrations of nocodazole, which suppresses microtubule dynamics rather than promoting microtubule disassembly (Jordan et al., 1992; Vasquez et al., 1997; and data not shown).

When antibody-injected cells were fixed and subjected to serial section electron microscopy, kinetochores in the interior of the asters were found to have normal end-on attachments to microtubules (Figure 6D). Taken together these results indicate that functional CLASP1 is not required for kinetochores to bind to microtubule plus ends *per se* but may instead be essential for the normal dynamic behaviour of kinetochore-associated microtubules.
CLASP1 is essential for chromosome congression and spindle integrity

Microinjection of anti-CLASP1 antibodies caused the formation of monopolar spindles. To determine how this phenotype arises, we used video microscopy to follow cells microinjected during late prophase with either control or anti-CLASP1 antibodies (Figure 7). Cells injected with control (pre-immune) antibodies underwent normal mitotic progression (Figure 7A and movie 4 in supplementary information). However, cells injected with anti-CLASP1 antibodies showed a lengthy delay in prometaphase: chromosomes failed to oscillate normally and some failed to congress to the metaphase plate (Figure 7B-B’ and movie 5 in supplementary information). Furthermore, although these cells initially assembled apparently normal bipolar spindles, these suffered a progressive collapse as mitosis progressed (Figure 7C-C’ and movie 6 in supplementary information). As discussed below, all of these phenotypes are consistent with a role for CLASP1 in the regulation of microtubule dynamics at kinetochores.
DISCUSSION

Genetic analysis in *Drosophila* first identified the product of a conserved gene, MAST/Orbit, which when mutated, resulted in the production of monopolar spindles and highly polyploid cells (Lemos et al., 2000; Inoue et al., 2000). A subsequent study reported that the two human homologues of this family are associated with the microtubule plus-end tracking proteins CLIP-115 and CLIP-170 in interphase cells (Akhmanova et al., 2001). These CLIP-associating proteins, termed CLASPs were proposed to be involved in regulating microtubule dynamics at the leading edge of motile fibroblasts, however no role for CLASPs in mitosis was proposed. The data presented here provide strong support for the hypothesis that these proteins, which we propose to refer to as CLASPs in all species, have an essential role in the regulation of microtubule dynamics at kinetochores.

The central region of CLASP1 directs the association of the protein with microtubules, and causes a remarkable microtubule bundling phenotype when overexpressed in cells. Given the localization of CLASP1 to the central spindle and midbody in normal mitotic cells, this could mean that the protein has a normal role in bundling and stabilizing microtubules in these regions. However, although defects in microtubule bundling could contribute to the spindle collapse seen when CLASP1 function is inhibited, as discussed below, this phenotype could also result from a perturbation of microtubule dynamics.

The characteristic phenotype produced by interference with CLASP1 function is the production of monopolar spindles with chromosomes buried in the interior. This is seen with the *Drosophila* mutants as well as following RNAi in *Drosophila* tissue culture cells, microinjection of anti-CLASP1 antibody into HeLa cells, and overexpression of the CLASP1 microtubule-binding domain in HeLa cells. The phenotype is highly unusual, as multiple redundant mechanisms exist to expel
chromosomes from the interior of asters (the "polar wind") (Rieder and Salmon, 1994). Two hypotheses could explain this phenotype. First, CLASPs could be required for the stable binding of kinetochores to dynamic microtubules. Second, CLASPs could be required for the regulation of the dynamics of kinetochore-associated microtubules.

According to the first hypothesis, disruption of CLASP1 function would cause kinetochores to lose their grip on microtubules exhibiting dynamic behaviour. This would normally cause chromosomes to be expelled from the aster, but if they were able to reattach laterally and move poleward along the sides of microtubules, as in the initial interactions normally seen during prometaphase (Rieder and Alexander, 1990), then they could accumulate in the interior of the aster. Indeed, our previous analysis of mitotic Drosophila cells depleted for MAST supported this hypothesis (Maiato et al., 2002). However, serial section electron microscopy in HeLa cells injected with anti-CLASP1 antibodies or overexpressing the CLASP1 microtubule-binding region has clearly shown kinetochores within the aster to have normal end-on attachments with microtubules (Figure 6). This appears to rule out the first hypothesis.

Overall, our data are more consistent with hypothesis 2, in which CLASP1 is involved in regulation of the dynamics of kinetochore-associated microtubules (Figure 8). Specifically, if CLASP1 were required for regulation of the switching of kinetochore-associated microtubules from a shrinking to a growing phase, then in the absence of CLASP1, any transition to shortening would cause the chromosome involved to be "reeled in" to the centrosome. Under this model, the kinetochores would have almost exclusively end-on attachments with microtubule bundles and the chromosomes would be very close to the centrosomes, as we have observed by electron microscopy. If the dynamics of non-kinetochore microtubules were less...
affected by the absence of CLASP1, then, the overall astral morphology would be normal, and the chromosomes would be "buried" within the aster.

A number of observations are consistent with a role for CLASP proteins in regulating microtubule dynamics. First, the microtubule-binding region of CLASP1 bundles microtubules and renders them non-dynamic. These bundles were highly resistant to depolymerization induced by colcemid. Second, chromosome oscillations appear to be dampened following the microinjection of anti-CLASP1 antibodies. Chromosome oscillations require dynamic instability of the microtubule bundles attached to the kinetochores (Skibbens et al., 1993). *Drosophila* MAST/Orbit mutants are defective in chromosome alignment, with chromosomes oscillating aimlessly on the spindle (Maiato et al., 2002). This could also be explained by defects in the regulation of microtubule dynamics. Third, the distribution of CLASP1 on microtubules during mitosis was radically different following the addition of taxol, a drug classically used to alter microtubule dynamics. Fourth, the abnormal astral structure observed in antibody-injected HeLa cells was largely reversed upon the addition of taxol. Suppression of microtubule dynamics with taxol would be expected to stabilize the kinetochore-attached microtubules, preventing them from further shrinking. Taxol would alter the equilibrium towards net microtubule growth, thereby pushing the chromosomes toward the periphery of the aster, and could account for the observed reorganization of the asters after addition of the drug. This effect was not solely due to any specific property of taxol, as the relocation of chromosomes from the interior of asters was also observed following treatment of injected cells with low dose nocodazole or vinblastine (data not shown).

One of the key phenotypes observed following perturbation of CLASP function in *Drosophila* and human cells is the accumulation of monopolar spindles.
in mitosis (Lemos et al., 2000; Inoue et al., 2000; Maiato et al., 2002). Although it was initially thought that this was likely to represent an inability of cells deficient in CLASP function to separate spindle poles, we now know that these cells can initially assemble what appear to be structurally normal mitotic spindles. These spindles subsequently undergo a slow and progressive collapse, ultimately producing the monopolar structures. Importantly, when crane fly spermatoocytes are treated with taxol, they assemble bipolar spindles, but these spindles progressively collapse as meiotic division progresses. This provides another link between alterations in microtubule dynamics and the phenotypes seen upon perturbation of CLASP function.

Recent EM data have led to a model where the plus ends of growing microtubules are present as open sheets (Arnal et al., 2000) that are postulated to close prior to microtubules entering a shrinking phase. It is possible that the flared ends of kinetochore-associated microtubules seen in cryo-electron microscopy studies correspond to the sheets seen in \textit{in vitro} assembly studies (McEwen et al., 1998; O’Toole et al., 1999; Mastronarde et al., 1997). In any event, the location of CLASP1 in the outer kinetochore corona would place the protein along the sides of the microtubules near the plus ends – possibly near the junctions where the flared tubules adopt a cylindrical profile (Figure 8). In its position near the plus ends at the kinetochore, CLASP1 would be well placed to influence the topology of the microtubules, for example, by promoting the transition of closed tubes to sheets (and thereby favouring growth).

A variety of evidence suggests that the preferential localization of CLASP1 to the kinetochore-proximal microtubule plus ends might at least partly involve recognition of a particular lattice conformation. First, the binding of CLASPs to microtubules is likely to be direct, as the \textit{S. cerevisiae} homologue of CLASP1,
Stu1p, binds directly to β-tubulin (Yin et al., 2002). Second, the distribution of
CLASP1 on microtubules *in vivo* is sensitive to taxol, which alters the structure of
the tubulin lattice (Arnal and Wade, 1995). Third, the Orbit protein (one name for
*Drosophila* CLASP) binds to microtubules in the presence of GTP, but not GTP-γS
(Inoue et al., 2000). This could also reflect a sensitivity of CLASPs to the
microtubule lattice, as shown for the related poorly hydrolyzable GTP analogue
GMPPCP (Hyman et al., 1995). Given the observation that the microtubule lattice
is influenced by whether the tubulin is in the GTP or GDP form (Hyman et al.,
1995), a preference for binding to one state of the lattice could provide a
mechanism by which CLASP1 might influence the structure at the end of the
microtubule (Figure 8).

Although the previous analysis of mutants in MAST/Orbit, the *Drosophila*
homologue of CLASP1, suggested that this protein might have a complex role in
mitosis (Lemos et al., 2000; Inoue et al., 2000), our present work suggests that
CLASP function can be largely understood in terms of regulation of microtubule
dynamics at the kinetochore. Regardless of its detailed mechanism of action,
CLASP1 appears to be a key component in enabling kinetochores to perform one of
their most distinctive behaviours in mitosis – interaction with microtubules that are
constantly adding or loosing subunits at their plus ends.
Experimental Procedures

Antibodies, Immunofluorescence and microscopy

A peptide corresponding to the sequence RIRTRQSSGSATNVASTPDNR present in CLASP1 was synthesised, conjugated to KLH and injected into 2 rabbits (ABCAM). The reactivity of the sera against the peptide was high by ELISA, and the third bleed from Rb1277 was used for immunofluorescence. Briefly, HeLa cells grown in poly-lysine treated coverslips at 37ºC in RPMI (Gibco) supplemented with 10 % FBS and antibiotics in the presence of 5 % CO₂, were fixed with methanol at -20ºC for 20 min, blocked with 0.5 % BSA in PBS containing 0.1 % Tween and then incubated for 40 min with Rb1277 diluted 1:500 in the same buffer. Cells were washed twice with PBS and once with PBS containing 0.1 % Tween between and after incubation with secondary antibodies. All the other stainings that did not involve Rb1277 were performed as described (Maiato et al., 2002). Other antibodies used were against α-tubulin (clone B512, Sigma), CENP-C (rabbit), CENP-E (mouse, gift from Tim Yen), EB1 (mouse, Transduction Labs) and ACA (human). Secondary antibodies conjugated with FITC, Texas Red, rhodamine and Cy5 were purchased from Jackson Immunoresearch. DNA was counterstained with DAPI and the preparations mounted with Vectashield (Vector). For analysis of chromosome spreads, identical results were obtained using HeLa cells treated for 3 h with 0.3 µg/ml colcemid or for 16 h with 0.03 µg/ml colcemid, incubated for 20 min in a buffer containing 75 mM KCl and then processed as before. Three dimensional data sets of representative cells were collected using a DeltaVision microscope (Applied Precision, Issaquah, WA), based on an Olympus IX-70 inverted microscope with a Photometrics CH350L cooled CCD camera, and subsequently deconvolved (except in Figure 4H) and projected onto a single plane using
Constructs and transfections

Full-length CLASP1 was obtained by fusion of the cDNA KIAA0622 (Ishikawa et al., 1998) with a 5’ RACE product that was fully sequenced and cloned altogether into a pEGFP-C1 vector (Clontech) using convenient restriction sites. All the other constructs are derivatives of the full-length CLASP1 that was digested, sub-cloned or re-ligated into the same vector using convenient restriction sites (full details can be provided upon request). Transfections were performed as described (Lemos et al., 2000).

Four-dimensional analysis by restoration microscopy

HeLa cells were grown in 40 mm coverslips and transfected with EGFP-CLASP1 (full-length) as before. 24 h after transfection, cells were transferred into FCS2 chambers (Bioptechs) and kept at 37°C in the presence of RPMI without phenol red (Gibco) supplemented with 10 mM Hepes. Three-dimensional data sets of representative cells were collected every 30 s and movie frames processed as before using SoftWorx (Applied Precision).

Microinjection and time-lapse video microscopy

Rb1277 serum was incubated with protein A sepharose beads (BioRad) for 2 h, washed with PBS, and bound IgGs were eluted with 100 mM Glycine (pH 2.5) then neutralized with 1 M Tris-HCl pH 8.0. Antibodies were concentrated to 20 mg/ml and changed to microinjection buffer containing 100 mM KCl and 10 mM KH₂PO₄ (pH 7.4) using 0.5 ml concentration columns (Millipore). For injections, HeLa or CF-
PAC cells were grown in glass coverslips and kept at 37°C in an inverted Nikon Diaphot microscope with a Narishige microinjector within a heated chamber. Interphase cells were microinjected in the cytoplasm (anti-CLASP1 injected = 433 cells; control IgG injected = 499 cells), fixed after 12 h and processed for immunofluorescence analysis as before. Injected cells were detected using anti-rabbit conjugated secondary antibodies. For time-lapse video microscopy, prophase cells were injected in the cytoplasm prior to nuclear envelope breakdown and observed in an inverted Nikon Diaphot DIC light microscope. Frames were collected immediately after NEB every 30 s and fixed immediately at the end of the movie for immunofluorescence analysis. The results were consistent between independent experiments and representative movies were compiled using Adobe Premiere 5.0 and ImageJ 1.28u.

**Drug treatments**

Taxol was used at 10 µM in HeLa cells transfected with EGFP-CLASP1 full length or at 100 nM in injected cells for 30 min prior to fixation as described previously (Jordan and Wilson, 1999). Injected cells were incubated with Nocodazole used at 100 nM for 30 min prior to fixation as described (Vasquez et al., 1997). For the microtubule depolymerization assay, HeLa cells transfected with EGFP-CLASP\textsubscript{250-943} were incubated for 16 h with 0.03 µg/ml colcemid prior to fixation.

**Electron microscopy**

HeLa cells overexpressing EGFP-CLASP\textsubscript{1,250-943} were grown in 6 well plates and processed for EM 36 h after transfection as described previously (Maiato et al., 2002). For ultrastructural analysis after antibody injection, HeLa cells growing in
glass coverslips were injected during interphase as described before either with anti-CLASP1 antibodies or control pre-immune IgGs together with rhodamine-labelled dextran (0.25 mg/ml) and kept in culture for 12 h. Drug experiments were performed at this stage (30 min incubation). Injected cells in mitosis were then detected under the fluorescence microscope, scribed with a diamond, fixed in 2.5% glutaraldehyde in PHEM buffer, and processed flat for ultra-thin serial sectioning (Rieder and Cassels, 1999).
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REFERENCES


O'Toole, E.T., Winey, M., and McIntosh, J.R. (1999). High-voltage electron tomography of spindle pole bodies and early mitotic spindles in the yeast


FIGURE LEGENDS

FIGURE 1. Cellular localization of CLASP1 and GFP-CLASP1 during mitosis. The left two columns show endogenous CLASP1, the right two columns show GFP-CLASP1. (A-C’ and E-G’) CLASP1 localizes at centromeres and spindle poles in prophase through late anaphase. (D, D’ and H, H’) After anaphase, CLASP1 accumulates at the spindle midzone and in the midbody at telophase. Insertions in the anaphase pictures correspond to 3X amplifications of the corresponding centrosome. In all merged images, CLASP1/EGFP-CLASP1 is shown in green, microtubules in red, and DNA in blue. Bar is 10 µm.

FIGURE 2. Localization of CLASP1 at microtubule plus-ends in living mitotic cells. (A) Images from living prometaphase HeLa cell showing EGFP-CLASP1 associated with growing microtubule plus-ends (arrows) and at the centromeres of congressed chromosomes (parenthesis). (B) During metaphase, EGFP-CLASP1 spreads all over the spindle and after anaphase onset it concentrates in the central spindle and midbody during telophase. (C) The re-localization with microtubule plus ends (arrows) occurs at later stages of cytokinesis specially near the leading edge of the expanding cytoplasm (arrowheads). Bar is 10 µm and time between frames is shown in minutes.

FIGURE 3. Mapping of CLASP1 within the kinetochore and its relation with microtubule dynamics. (A, B) Co-immunolocalization of endogenous CLASP1 or EGFP-CLASP1 with anti-centromere antibodies (ACA) and CENP-E in colcemid treated chromosome spreads. Asterisks indicate the EGFP-CLASP1 localization at the centromeres in the absence of microtubules. (C) EGFP-CLASP1 leads the kinetochore during poleward movement in anaphase, as shown by co-
immunolocalization with CENP-C (red). (D-E’) Suppression of microtubule dynamics by a short incubation with taxol causes significant redistribution of EGFP-CLASP1 from the kinetochores to the poles, although some EGFP-CLASP1 remains at kinetochores upon more careful examination. CLASP1/EGFP-CLASP1 is shown in green, microtubules in red and DNA in blue. Bar is 10 µm.

FIGURE 4. Functional analysis of CLASP1 and identification of a dominant-negative mutant. (A) Mapping of the microtubule- and kinetochore-binding domains of CLASP1 by transient expression of deletion constructs tagged with EGFP at the N-terminal. Red and blue boxes represent HEAT repeats and a region shared with Tau, respectively. Putative phosphorylation sites “P” by CDK1 correspond to residues S\textsuperscript{688}, S\textsuperscript{731}, S\textsuperscript{757}, T\textsuperscript{1099} and S\textsuperscript{1123}. Localization in the several mitotic compartments was classified with “+” or “-”, corresponding to presence or absence respectively, and with “+/−” or “+/+”, corresponding to less or more than normal respectively. (B) The C-terminal region of CLASP1 comprised of amino acids 1256-1538 is sufficient for kinetochore targeting. (C) The central portion of CLASP1 is sufficient for centrosome localization during mitosis, but is unable to target to kinetochores. (D) Removal of the HEAT repeat by deletion of amino acids 1465-1538 from the C-terminal of CLASP1 also abolishes normal kinetochore localization. The HEAT repeat may mediate interaction with other proteins responsible for CLASP1 targeting to kinetochores, namely CLIP-170. (E) A large central domain of CLASP1 comprising amino acids 250-943 is sufficient for microtubule binding and induces the formation of bundles in interphase cells. (F) Cells transfected with EGFP-CLASP\textsubscript{1250-943} (+) shows microtubule bundles mostly resistant to depolymerization by colcemid treatment while microtubules in non transfected cells (-) are completely depolymerized. (G) Electron micrograph of
microtubule bundles in an EGFP-CLASP1_{250-943} transfected cell during interphase show a highly compact structure. (H) Overexpression of the EGFP-CLASP1_{250-943} construct during mitosis causes formation of single or double monopolar asters. A control non-transfected cells is also shown. In all merged images EGFP-CLASP1 is shown in green, microtubules in red and DNA in blue. Bar is 0.2 µm in G. In all other figs bar is 10 µm.

**FIGURE 5.** Effect of overexpression of dominant negative CLASP1 during mitosis. (A-A'') Characterization of a mitotic cell transfected and over expressing EGFP-CLASP1_{250-943}, which can be seen associated with the microtubule-plus-ends (green), showing a monopolar spindle formed by bundles of microtubules (red) organized by a single aster with 2 centrosomes. (B-B'') Co-localization of EGFP-CLASP1_{250-943} (green) with EB1 (red) at the microtubule-plus-ends in a monopolar spindle. (C-C') HeLa cell overexpressing EGFP-CLASP1_{250-943} (green) showing the kinetochores, as determined by CENP-C staining, not associated with bundled microtubule plus ends. Nevertheless, the chromosomes (blue) can be seen in the interior of the asters. (D) Electron micrograph from a serially sectioned mitotic cell overexpressing EGFP-CLASP1_{250-943} showing the ultrastructure of the monopolar spindle formed by two astral arrays (asterisks) of microtubule bundles (arrows). Insert the upper right corner shows a cross section of microtubule bundles. (E) Deeper section of the same cell showing the ultrastructure of the centrosome (Ct) with a bundle of microtubules (arrow) and a mono-oriented kinetochore (Kt) where some microtubules can be found. (F) Another section from the same cell showing a microtubule bundle (arrow) and a bi-oriented kinetochore (Kts) with few microtubules attached. Bar is 10 µm except in figures E,F and G that is 1 µm.
FIGURE 6. Analysis of the microtubule kinetochore attachment after injection of antibodies specific for CLASP1. (A) Effect of inhibition of CLASP1 12 h after the injection during interphase HeLa cells (anti-CLASP1 injected cells n = 433; control IgG injected cells n = 499). (B-B’) Detailed analysis of the microtubule attachment (green) with the kinetochores (red), as determined by ACA staining in a cell injected with anti-CLASP1 antibodies. Insertions show that most kinetochores are not associated with the microtubules at the periphery of the aster. (C-C’) The inhibition of microtubule dynamics by a short incubation with ultra-low dose of taxol rescues the association of the kinetochores (red) with the microtubule-plus ends (green) at the periphery of the aster in a cell injected with anti-CLASP1 antibodies. (D) Thin section through a monopolar spindle formed after injecting a HeLa cell during G2 with CLASP-antibody. Two kinetochore fibres are visible near the middle of the image. In these cells many of the kinetochore fibre microtubules terminate in the outer kinetochore plate (insert). Bar is 10 µm except in figure D that corresponds to 0.5 µm.

FIGURE 7. Mitotic progression of cells injected with control or anti-CLASP1 antibodies. (A) Time-lapse series of a CF-PAC cell injected with pre-immune or (B, C) with anti-CLASP1 antibodies analysed by DIC microscopy, respectively. (B) Time-lapse series of a CF-PAC cell injected with anti-CLASP1 antibodies showing deficient chromosome congression. (B’) Same cell as in B that was fixed and processed for immunofluorescence immediately after time lapse imaging, showing the spindle (green) and the chromosomes (blue). The cell was identified using anti-rabbit antibodies (red). (C) Time-lapse series of a CF-PAC cell injected with anti-
CLASP1 antibodies showing the progressive collapse of the spindle. The position and size of the spindle is marked with a yellow scale bar. (C') Same cell as in C that was fixed and processed for immunofluorescence reveal anti-CLASP1 antibodies (red), the spindle (green) and chromosomes (blue). The initial size of the spindle is shown for comparison as a yellow scale bar. Arrows in B, B’ and C’ show some of the chromosomes that did not aligned at the metaphase plate. Time was counted from the moment after breakage of the nuclear envelope in prophase. Bar is 10 µm.

**FIGURE 8.** Proposed model for the role of CLASP1 at microtubule-plus ends and kinetochores. CLASP1 may assemble with the polymerising microtubules either by recognition of a particular conformation of the lattice or by co-assembly with tubulin heterodimers. The first possibility might involve preferential binding to open sheets at the plus ends of growing microtubules. The second might involve specific binding to β-tubulin monomers (Yin et al., 2002). If CLASP1 undergoes the treadmilling characteristic of +TIPs (Schuyler and Pellman, 2001), this implies that it must detach behind the region of growth. This could be a consequence of GTP-hydrolysis and result in tube closure, and could correlate with a tendency of the microtubules to depolymerize and shrink. The microtubule-independent anchoring of CLASP1 at kinetochores may promote the addition and loss of tubulin subunits on microtubules that remain attached. Under this model, CLASP1 assumes a key role in the regulation of microtubule dynamics at the kinetochore, essential for proper chromosome positioning and spindle stability.
**LEGENDS FOR SUPPLEMENTARY MATERIAL**

**Movie 1** – Localization of EGFP-CLASP1 to the microtubule plus-ends during prometaphase in living HeLa cells. Time-lapse sequences were collected by four-dimensional restoration microscopy every 30 s using a DeltaVision microscope. EGFP-CLASP1 dots seen near the metaphase plate most likely represent the kinetochores.

**Movie 2** – Microtubule localization of EGFP-CLASP1 at the metaphase-anaphase transition and during late mitotic events. Living HeLa cells expressing EGFP-CLASP1 were analysed by four-dimensional restoration microscopy and time-lapse series collected every 30 s. In metaphase, EGFP-CLASP1 was weakly associated with spindle microtubules overall. After anaphase onset, EGFP-CLASP1 remains associated with the shorting spindle and preferentially accumulates at the central spindle. During telophase and cytokinesis EGFP-CLASP1 concentrates with astral microtubules and ultimately accumulates in the midbody.

**Movie 3** – Localization of EGFP-CLASP1 to the microtubule plus-ends at late stages of cytokinesis in living HeLa cells. Time-lapse sequences were collected by four-dimensional restoration microscopy every 30 s using a DeltaVision microscope. EGFP-CLASP1 dots can be seen at the leading edges of the expanding cytoplasm.

**Movie 4** – Time-lapse video microscopy analysis by DIC of a human CF-PAC cell injected with pre-immune antibodies during late prophase. Chromosomes can be seen to fully congress to the metaphase plate within the first 15 min upon NEB. Metaphase lasts for ~25 min and the cell enters anaphase.
telophase/cytokinesis chromosomes in the two daughter cells start to decondense after ~1 hour from NEB.

**Movie 5** - Time-lapse video microscopy analysis by DIC of a human CF-PAC cell injected with anti-CLASP1 antibodies during late prophase. During prometaphase full congression of all chromosomes is not accomplished even after more than 2 h 30 m. At this time, the cell was fixed and processed for immunofluorescence to reveal the details of the spindle and chromosomes (see Figure 4b').

**Movie 6** - Time-lapse video microscopy analysis by DIC of another human CF-PAC cell injected with anti-CLASP1 antibodies during late prophase. Chromosome oscillations are abnormal and the chromosomes accumulate as a disorganized mass in the centre of the cell. The spindle can be seen to progressively collapse. Immunofluorescence analysis immediately after the collapse, ~100 min after NEB, revealed the presence of a short bipolar spindle very close to the chromosomal mass (see Figure 4c).

**Supplementary Figure 1** – Mapping the kinetochore localization of CLASP1. EGFP-CLASP1 (green) localizes outside the inner-kinetochore plate defined by CENP-C (red) in a chromosome spread from a colcemid-treated HeLa cell. DNA is shown in blue. Asterisks indicate EGFP-CLASP1 localization at the centrosomes in the absence of microtubules. Bar is 10 µm.
Prophase/Prometaphase

Metaphase

Anaphase

Telophase

Figure 1 - Maiato et al, 2002
Figure 2 - Maiato et al., 2002
**Figure 3 - Maiato et al., 2002**
Amino acids

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Figure 4 - Maiato et al., 2002
Figure 5 - Maiato et al., 2002
Figure 6 - Maiato et al., 2002
Figure 7 - Maiato et al., 2002
Figure 8 - Maiato et al., 2002