Mammalian CLASPs are required for mitotic spindle organization and kinetochore alignment

Yuko Mimori-Kiyosue1,*, Ilya Grigoriev2,3, Hiroyuki Sasaki1,4, Chiyuki Matsui1, Anna Akhmanova3, Shoichiro Tsukita5,6 and Ivan Vorobjev7,∗

1KAN Research Institute, Kyoto Research Park, Shimogyo-ku, Kyoto 600-8815, Japan
2Department of Cell Biology and Histology, Moscow State University, Vorobjevi Gory, Moscow, 119992, Russia
3MGC Department of Cell Biology and Genetics, Erasmus Medical Center, 3000 DR Rotterdam, The Netherlands
4Institute of DNA Medicine, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan
5Department of Cell Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8315, Japan
6Solution Oriented Research for Science and Technology, Japan Science and Technology Corporation, Sakyo-ku, Kyoto 606-8501, Japan
7Laboratory of Cell Motility, A. N. Belozersky Institute, Moscow State University, Vorobjevi Gory, Moscow, 119992, Russia

CLASP1 and CLASP2 are homologous mammalian proteins, which associate with the ends of growing microtubules, as well as the cell cortex and the kinetochores of mitotic chromosomes. Previous studies have shown that in interphase cells CLASPs can attach microtubule plus ends to the cortex and stabilize them by repeatedly rescuing them from depolymerization. Here we show that CLASP1 and 2 play similar and redundant roles in organizing the mitotic apparatus in HeLa cells. Simultaneous depletion of both CLASPs causes mitotic spindle defects and a significant metaphase delay, which often results in abnormal exit from mitosis. Metaphase delay is associated with decreased kinetochore tension, increased kinetochore oscillations and more rapid microtubule growth. We show that the association of CLASP2 with the kinetochores relies on its C-terminal domain, but is independent of microtubules or association with CLIP-170. We propose that CLASPs exhibit at the kinetochores an activity similar to that at the cortex, providing apparent stabilization of microtubules by locally reducing the amplitude of growth/shortening episodes at the microtubule ends. This local stabilization of microtubules is essential for the formation of normal metaphase spindle, completion of anaphase and cytokinesis.

Introduction

The mitotic spindle is a highly dynamic specialized microtubule array required for chromosome separation. Segregation of sister chromatids depends on the interaction of their kinetochores with microtubules. A growing body of evidence suggests that the chromosome–microtubule interaction during mitosis involves numerous proteins associated with microtubules and/or with kinetochores (Cleveland et al. 2003). Since the kinetochores interact with microtubule plus ends, it is not surprising that many proteins acting at the kinetochore–microtubule interface belong to microtubule plus-end-tracking proteins (+TIPs), a group of factors specifically associated with ends of growing microtubules (Schuyler & Pellman 2001; Maiato et al. 2004; Akhmanova & Hoogenraad 2005). These proteins include EB1, CLIP-170, dynein and dynactin as well as CLASPs, which are the focus of the current study.

CLASPs are evolutionary conserved proteins, which are represented in mammals by two homologs, CLASP1 and 2 (Inoue et al. 2000; Lemos et al. 2000). They are involved in spatial organization of microtubule networks both in interphase and in mitosis. In mammalian fibroblasts, CLASPs stabilize microtubules directed to the leading edge (Akhmanova et al. 2001); this function probably depends on the capacity of these proteins to rescue microtubules in the vicinity of certain cortical regions (Mimori-Kiyosue et al. 2005).

The budding yeast CLASP counterpart, Stu1p, participates in the formation of the mitotic spindle (Yin et al. 2002). The human CLASP1, its Xenopus homolog Xorbit/CLASP, the Drosophila homolog, MAST/Orbit, and the C. elegans homolog, CLASP R106.7 all play a role in the proper mitotic progression (Gonczy et al. 2004; Shimamura et al. 2002; Lemos et al. 2000). These proteins include EB1, CLIP-170, dynein and dynactin as well as CLASPs, which are the focus of the current study.
2000; Maiato et al. 2002, 2003; Hannak & Heald 2006). Using *Drosophila* mutants and RNA interference (RNAi) approach, MAST/Orbit was shown to be necessary for maintaining bipolarity of the mitotic spindle, for the microtubule-kinetochore attachment and chromosome congression (Maiato et al. 2002). CLASPs are components of the outer kinetochore layer; however, CLASP depletion does not disrupt the targeting of other proteins that might affect kinetochore-microtubule interactions, such as CLIP-170 or dynein (Maiato et al. 2002, 2003). In insect cells, MAST/Orbit is an essential component of the spindle flux machinery: it is required for the polymerization of the microtubules attached to kinetochores (Maiato et al. 2005). In addition, analysis of the hypomorphic MAST/Orbit mutants revealed a function for this protein in cytokinesis (Inoue et al. 2004). In *C. elegans*, CLASP<sup>h,b</sup> is targeted to the kinetochores by CENP-F-like proteins HCP-1/2 and is required for sister chromatid biorientation (Cheeseman et al. 2005). In *Xenopus* meiotic egg extracts, Xorbit/CLASP is required for normal spindle formation and chromosome congression, as well as microtubule stabilization during anaphase (Hannak & Heald 2006).

The precise role of CLASPs, especially of CLASP2, in mammalian mitosis remains to be elucidated. In this study we used an RNAi approach combined with live cell imaging and immunostaining to examine the spatial distribution and function of both CLASP homologs in mitotic human cells. We show that CLASP1 and 2 have similar and redundant roles in maintaining mitotic spindle. CLASP2 has the same mitotic localization as CLASP1. Its binding to kinetochores occurs through the C-terminal CLIP-170-binding domain, while during cytokinesis it was present in the midbody. Previous analysis of CLASP1 protein has established that it binds to mitotic kinetochores through its C-terminus (Maiato et al. 2003; Hannak & Heald 2006). We have obtained similar results with the CLASP2 deletion mutants (Fig. 2A,B). GFP-CLASP2-C, but not GFP-CLASP2ΔC associated with kinetochores, also when the microtubules were depolymerized with nocodazole (Fig. 2B and data not shown). The C-terminal domain of CLASPs is responsible for their association with CLIP-170 (Akmanova et al. 2001). CLIP-170 also displays kinetochore localization, which depends on the presence of dynactin complex and can be disrupted by over-expression of the dynactin subunit dynamitin (Dujardin et al. 1998). GFP-CLASP2γ could still bind to kinetochores when p50/dynamitin was over-expressed and endogenous CLIP-170 was removed from the kinetochores (Fig. 2C), while GFP-CLASP2γ was displaced from kinetochores by over-expression of DsRed2-CLASP2-C fusion without affecting the kinetochore localization of CLIP-170 (Fig. 2D). Therefore, we conclude that although CLASP2 associates with kinetochores through its CLIP-binding C-terminal domain, this association is independent of CLIP-170.

**CLASP1 and 2 are required for mitotic progression**

To deplete CLASPs from human cultured cells we used siRNA-mediated knockdown. Two pairs of siRNAs, specific for CLASP1 and CLASP2 (CLASP1#A or #B and CLASP2#A or #B) were characterized in detail previously (Mimori-Kiyosue et al. 2005). Three days after transfection with these siRNAs, the levels of CLASP1 and CLASP2 were reduced by ∼70% (Mimori-Kiyosue et al. 2005). In agreement with these results, all CLASP1/2-specific signals in mitotic cells were highly reduced after both CLASPs were knocked down (Fig. 2E,F), confirming the efficiency of CLASP1/2-directed siRNAs.

Down-regulation of a single CLASP with CLASP1 or CLASP2-specific siRNAs had no visible effect on the mitotic progression (Fig. 3A). However simultaneous down-regulation of both CLASPs caused a considerable increase in the proportion of mitotic cells (Fig. 3A), suggesting that CLASP1 and CLASP2 act in mitosis as a common pool. When the progression of mitosis was observed by phase contrast microscopy (Fig. 3B–E, Supplemental Movie S1), the majority of CLASP-depleted cells was unable to exit mitosis in time (within 60 min after mitosis onset detected by the rounding up of the spindle poles, microtubule plus ends within the spindle and kinetochores. In anaphase and telophase, CLASP2 accumulated at the spindle midzone, while during cytokinesis it was present in the midbody.

**Results**

**Mitotic localization of CLASP2**

The localization of CLASP1 during mitosis has been described previously (Maiato et al. 2003). We have analyzed the distribution of CLASP2 during mitosis, by antibody staining and by expressing low levels of GFP-CLASP2α in HeLa cells (Fig. 1 and data not shown). We found that CLASP2 localization was very similar to that of CLASP1: starting from prophase to anaphase, it

cell). Instead, CLASP-depleted cells were delayed in mitosis while forming different abnormal figures (Fig. 3F and see below). The duration of the rounded-up stage in mitotic cells with lowered CLASP levels increased nearly tenfold (from 30 min to up to 5 h). Yet some cells were able to undergo cytokinesis, exit from mitosis and proceed into the interphase.

Analysis of the mitotic progression after CLASP knockdown

Staining of cells for microtubules revealed bipolar spindles as well as several mitotic defects. When bipolar spindles were formed in CLASP-depleted cells, in many cases their pole-to-pole distance was significantly shorter

Figure 1 Localization of GFP-CLASP2 in different phases of mitosis. HeLa cells, transiently expressing low levels of GFP-CLASP2α, were stained for α-tubulin, CENP-A and the DNA. GFP-CLASP2α was localized at the spindle and kinetochores, and during late mitosis, transferred to the central spindle (arrows in D) and the midbody (box in E). Bar 5 µm.
than that in control cells (Fig. 4A,B). Besides, multipolar and severely disorganized spindles were observed (Fig. 4C–I). Staining of cells for DNA showed that after knockdown of both CLASPs a larger number of cells were carrying scattered chromosomes in a prometaphase-like manner (Fig. 4J).

To observe directly the formation of mitotic spindles and centromere movements, we have followed mitotic division in live HeLa cells, which were stably expressing GFP-tubulin or GFP-Aurora B, a centromere protein, three days after transfection with either control siRNA or the CLASP1+2#B siRNA combination (Fig. 5, Figure 2 CLASP association with the kinetochores. (A) Schematic representation of CLASP2 and the relevant deletion mutants. (B–D) Kinetochore labeling in nocodazole-treated Xenopus A6 cells. B. Cells were transiently transfected with GFP-CLASP2-C and stained with antibodies against p150Glued. (C,D) Cells, stably expressing low levels of GFP-CLASP2γ, were transiently transfected with DsRed-p50/dynamitin (C) or DsRed-CLASP2-C (D), and stained for CLIP-170. Cells expressing high levels of DsRed fusion proteins are shown. Note that the kinetochore localizations of DsRed-CLASP2-C and DsRed-p50/dynamitin were undetectable due to their high-level expression in the cytoplasm. Bars, 5 μm. (E,F) HeLa cells, transfected either with the control siRNA or the CLASP1+2#B siRNAs, were treated with 10 μM nocodazole for 1 h, and stained with Hoechst 33342, anti-centromere antibodies (CREST), anti-p150 and antibody #402 against CLASP1. Bar (E) 5 μm and (F) 1 μm.
Supplemental Movies S2, S3). In agreement with previously reported observations (Maiato et al. 2002) some spindles had collapsed after initial formation and lost bipolarity, while others remained bipolar, yet were shorter than metaphase spindles in control cells (Supplemental Movie S2). Some spindles underwent a transition from an initially bipolar configuration to a multipolar state (Fig. 5C, Supplemental Movie S2). Consistently, in many cells with reduced CLASPs, the centromeres visualized with GFP-AuroraB were almost aligned around the equatorial region, but later disorganized again during a prolonged arrest (Fig. 5D, Supplemental Movie S3).

Based on these observations, the mitotic progression in CLASP-depleted cells was classified into the following types: (i) hypomorphic phenotype; characterized by bipolar orientation of chromosomes, prolonged metaphase, and division into two normal daughter cells, (ii) altered mitosis; bipolar orientation of chromosomes was apparently lost, however, after a significant delay the cell entered cytokinesis. These cells occasionally failed to divide normally, (iii) strongly altered mitosis; metaphase spindle had collapsed and chromosomes were gathered in the center of the cell or scattered. These failures were often preceded by rearrangement of the spindle into a
multipolar configuration with gamma-tubulin located in numerous mitotic poles (Fig. 4G,H). Cells with strongly altered phenotype never exit mitosis in a normal way.

The cells of type (ii) and (iii) often underwent asymmetric divisions or died during or soon after the exit from mitosis (Supplemental Movie S1, Fig. 3F). Besides, telophase cells often failed to cleave or fused back immediately after an incomplete division (Fig. 3E–F), possibly due to the presence of lagging chromosomes. Alternatively, since CLASPs localize to the spindle midzone...
CLASPs in mitosis

(Fig. 1D, Maiato et al. 2003), completion of cytokinesis may be compromised due to defects in formation of stable microtubule bundles at the central spindle, as observed in MAST/Orbit-mutated Drosophila spermatocytes (Inoue et al. 2004).

CLASP depletion affects the mitotic microtubule dynamics and tension at the bi-oriented metaphase kinetochores

To understand the reason for metaphase delay after knockdown of CLASPs we further analyzed cells displaying hypomorphic phenotype. Electron microscopy analysis of the siRNA-treated mitotic cells showed normal bipolar orientation of the chromosomes after CLASP knockdown (Fig. 6A,B and data not shown). Kinetochores kept their trilaminar structure and were associated with microtubule ends in the normal way (Fig. 6C–F). The number of microtubules attached to a kinetochore observed within a ~0.1 µm section, was almost the same in control cells (4.5 ± 1.3 per kinetochore), 68 kinetochores in 24 cells) and CLASP-depleted cells (3.7 ± 1.0 per kinetochore, 55 kinetochores, 20 cells). Thus the spatial organization of the kinetochore-microtubule interaction appeared to be unchanged. However, in such CLASP-depleted cells chromosomes failed to align properly; instead they were scattered around the equatorial plane (Fig. 6B, arrow).

To get an insight into the temporal organization of the spindle we have analyzed the dynamics of microtubule plus ends and centromeres using HeLa cells expressing monomeric red fluorescent protein (mRFP)-tagged Aurora B and EB3-GFP, a +TIP protein (Stepanova et al. 2003). High time resolution two color videos have shown that after the knockdown of both CLASPs microtubules and centromeres remained highly dynamic (Supplemental Movie S4, Fig. 7A,B). Transient accumulation of EB3-GFP at the kinetochores, moving away from the spindle poles, similar to that described for EB1 in PtK1 cells (Tirnauer et al. 2002), could still be observed after CLASP knockdown (data not shown).

The rate of microtubule growth in mitotic cells (measured mainly from the astral microtubules) increased from 21.0 ± 7.1 µm/min in control cells (451 microtubule ends in 5 cells) to 26.5 ± 6.7 µm/min (217 microtubule ends in 4 cells) after the knockdown with CLASP1+2#A siRNAs. This difference was statistically significant ($P < 0.001$, Kolmogorov–Smirnov two sample test). The increase in microtubule growth rate during mitosis was fully in line with our observations in

© 2006 The Authors
Journal compilation © 2006 by the Molecular Biology Society of Japan/Blackwell Publishing Ltd.

Figure 5  Analysis of mitotic progression in live HeLa cells. Time-lapse images of HeLa cells, stably expressing GFP-α-tubulin or GFP-Aurora B, 72 h after transfection with control (A,B) or CLASP1+2#A siRNA oligos (C,D). Cell cycle phase is indicated on the lower right of the images (i, interphase; pm, prometaphase; m, metaphase; ml, metaphase-like; t, telophase; ck, cytokinesis; ar, arrested). Lines indicate the outlines of cells. Time (min) is indicated on the upper right of each image. Bar 10 µm.
interphase cells, where the diminished microtubule stability, caused by the lack of CLASPs, resulted in an increased pool of soluble tubulin and a more rapid microtubule polymerization (Mimori-Kiyosue et al. 2005).

The amplitude and the speed of centromere oscillations around the equatorial plate along the pole-to-pole axis after CLASP knockdown significantly increased (Fig. 7C,D). Since kinetochore movements within the spindle are coupled to microtubule polymerization and depolymerization (for review see Inoue & Salmon 1995), these results point to a change in the microtubule dynamics at the tips of the kinetochore fibers. Immunofluorescent staining of kinetochores in CLASP knockdown cells indicated that after a metaphase-like arrangement of chromosomes was achieved, the distances between bi-oriented kinetochores of the two sister chromatids were shorter than in control metaphase cells (Fig. 7E–H), indicating a decreased tension on the kinetochores (Waters et al. 1996). BubR1, a spindle checkpoint protein (for reviews see Cleveland et al. 2003; Lew & Burke 2003), remained accumulated at the kinetochores of metaphase-like cells after CLASP knockdown (Fig. 7G). This observation is in agreement with the data obtained in insect cells (Maiato et al. 2002), indicating that the lack of CLASPs does not affect the maintenance of the spindle checkpoint.

**Discussion**

In this study we have used RNAi approach to reduce the levels of CLASP1 and CLASP2 in HeLa cells. Significant mitotic defects were only present when combinations of siRNAs, specific for the two CLASPs, were transfected simultaneously, and not when cells were treated separately with CLASP1 or CLASP2-directed or control duplexes. The two CLASP isoforms, expressed in HeLa cells (CLASP1α and CLASP2α), are approximately 77% similar and the two proteins display a virtually identical
mitotic localization (Fig. 1 and Maiato et al. 2003). Our previous study in interphase cells pointed to a strong functional similarity and redundancy of these two proteins (Mimori-Kiyosue et al. 2005). All our data indicate that in HeLa cells the two CLASPs display very similar localizations and functions both in interphase and in mitosis. Our observations suggest that in HeLa cells the two CLASPs act as a common pool, the depletion of which below a certain level has severe consequences for the cell division. In agreement with this idea, a genetic
knockout of CLASP2 in mouse fibroblasts does not arrest cell proliferation, indicating that CLASP1 alone can support cell division (H. Maiato, personal communication). However, CLASP2 knockout fibroblasts display multiple mitotic abnormalities (H. Maiato, personal communication), suggesting that mammalian mitosis is exquisitely sensitive to CLASP dosage.

Similar to their invertebrate homologs, CLASPs are needed for proper mitotic progression. CLASPs, as well as their Drosophila homolog MAST/Orbit, localize to the spindle poles, the mitotic spindle and the kinetochores and are necessary for maintenance of spindle bipolarity and the metaphase chromosome alignment (Maiato et al. 2002). In agreement with the results of CLASP depletion in insect cells and in Xenopus extracts (Maiato et al. 2002, 2005; Goshima et al. 2005; Hannak & Heald 2006), bipolar spindles observed after simultaneous CLASP1/CLASP2 knock down in HeLa cells were short and displayed unstable chromosome attachment. In interphase cells, mammalian CLASPs act as rescue factors, which promote microtubule stability (Mimori-Kiyosue et al. 2005). Therefore, after CLASP knock down microtubule density is decreased, while the pool of soluble tubulin and microtubule growth rate are increased. We also observed an increase in microtubule growth rate in CLASP-depleted mitotic cells, suggesting that also in mitosis CLASPs stabilize microtubules. This conclusion is supported by the short pole-to-pole distance in bipolar spindles of CLASP-depleted cells, since microtubule-stabilizing factors have a positive effect on the spindle length (Goshima et al. 2005).

In contrast to the observations in insect cells (Maiato et al. 2002), reduction of CLASP levels in HeLa cells more often lead to the formation of multipolar spindles with chromosomes attached to the microtubules, rather than to monopolar spindles with unattached chromosomes (although it is possible that in insect cells chromosomes were actually attached to very short microtubules). In Drosophila cells, MAST/Orbit is required for maintaining fluxing kinetochore fibers by stimulating subunit incorporation at their plus ends (Maiato et al. 2005). The absence of CLASPs therefore causes gradual shortening and collapse of the bipolar spindle. The situation may be somewhat different in mammalian cells, where flux may play a less important role for metaphase chromosome dynamics (Mitchison & Salmon 1992), explaining the difference in the results obtained in the two systems. Alternatively, CLASP depletion achieved in HeLa cells is less profound than that obtained by RNAi in Drosophila cells, which might explain the divergent phenotypes. Multiple spindle poles were also observed in hypomorphic MAST/Orbit mutants, where they were associated with polyploidy (Inoue et al. 2000). It is likely that some of the multipolar spindles, which we observed, are also due to a cytokinesis failure in the previous round of cell division. However, in addition we frequently monitored an initial formation of a bipolar spindle, which later collapsed into a multipolar configuration (Fig. 5C), suggesting that CLASPs may be involved in maintaining spindle pole integrity.

Partial knockdown of the two CLASPs, achieved by us in HeLa cells, may be phenotypically compared to a hypomorphic mutant. In accord with this idea, some cells do proceed beyond metaphase and complete cell division. In agreement with the observations on effects of a hypomorphic MAST/Orbit allele in fly testis (Inoue et al. 2004), reduced CLASP levels also lead to cytokinesis defects, as cells often failed to undergo cleavage or fused back after an incomplete division (Fig. 3).

Reduced levels of CLASPs did not inhibit spindle dynamics or kinetochore movements, in contrast to the previous observations in monopolar spindles, obtained after the microinjection of CLASP1 antibodies in HeLa cells (Maiato et al. 2003). On the contrary, the rate of microtubule growth was slightly increased, and centromere oscillations were enhanced. This difference might have several explanations. First, it is possible that the CLASP1 antibodies, used in that study, bound to other proteins in addition to CLASP1, since the authors show that the antibody preferentially reacted with an endogenous HeLa cell protein of ∼212 kDa, which significantly exceeded in size the largest known CLASP1 isoform (∼160 kDa). In our hands, the CLASP1 antibodies raised by Maiato et al. (2003) did recognize endogenous CLASP1 of ∼160 kDa (A. Akhmanova, unpublished observations). This raises the possibility that antibody injection caused a dominant-negative effect on the spindle dynamics. Our data on increased kinetochore movements are in line with those obtained in C. elegans embryos, showing dramatic chromosome oscillations after CLASP<sup>de-2</sup> depletion (Cheeseman et al. 2005) and with the chromosome behavior in Drosophila cells after RNAi-mediated CLASP knockdown (Maiato et al. 2002).

In agreement with previous data (Maiato et al. 2003) mammalian CLASPs are not critical for the kinetochore-microtubule end-on attachment. Partial depletion of CLASPs causes metaphase-like arrangement of chromosomes, which is maintained for prolonged time. Bipolar spindles arrested in metaphase were also observed after treatment with colcemid or nocodazole at low concentration (Kleinfeld & Siskin 1966; Alieva & Vorobjev 1991). However, in contrast to these pharmacological
treatments, CLASP depletion enhances, rather than inhibits microtubule dynamics. Increased oscillations of centromeres and reduced kinetochore tension, in the presence of an intact spindle checkpoint, indicate that CLASPs probably help maintain proper interactions between the tips of dynamic microtubules and the kinetochores. It is likely that CLASPs locally regulate microtubule dynamics in the vicinity of kinetochores, promoting their growth. Microtubule growth-promoting activity probably depends on microtubule tip recognition through the middle part of the protein (Mimori-Kiyosue et al. 2005) and on the microtubule-independent association of CLASPs with kinetochores through their C-terminal domains. Therefore, the action of CLASP at the kinetochore is probably similar to that at the cell cortex, to which CLASPs also bind in a microtubule-independent fashion (Mimori-Kiyosue et al. 2005). It appears that CLASPs can be regarded as local regulators of microtubule plus end dynamics at certain cellular structures, which can form a boundary for microtubule growth.

Experimental procedures

Cell lines and transfection of plasmids and siRNAs

HeLa cells were grown at 37 °C, in 5% CO2, atmosphere, in D-MEM (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. The Xenopus kidney epithelial cell line A6 was grown at 23 °C in 50% Leibovitz's L-15 medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. Effectene or Superfect transfection reagents (Qiagen) were used for the transfection of plasmids into HeLa or A6 cells, according to the manufacturer's protocols. Stable clones were selected in the presence of 0.6–0.8 mg/mL or 0.3–0.4 mg/mL G418 sulfate for A6 or HeLa cells, respectively (Calbiochem), by detecting GFP fluorescence.

Synthetic siRNAs (Proligo) were previously described (Mimori-Kiyosue et al. 2005). Ten percent confluent HeLa cells were transfected, using Oligofectamine (Invitrogen) with siRNAs at the minimal effective concentration (10 nM for CLASP1#A and CLASP1#B, 200 nM for CLASP2#A and CLASP2#B), while the control siRNA was used at 200 nM concentration.

Expression constructs

We used the previously described expression vectors for GFP-CLASP2γ (Akhanova et al. 2001), GFP-CLASP2α, GFP-CLASP2ΔC, GFP-CLASP2-C (Mimori-Kiyosue et al. 2005), EB3-GFP (Stepanova et al. 2003), GFP-α-tubulin (Clontech), GFP-dynamitin/p50 was a kind gift of Dr T. Schroer. AuroraB cDNA was amplified from HeLa cell cDNA library (HeLa large-Insert, Clontech) and inserted into pEGFP-C2 vector. GFP was substituted for DsRed2 (Clontech) or mRFP (gift of Dr R. Tsien, UCSD, La Jolla, CA USA (Campbell et al. 2002)) to generate red fluorescent fusions.

Antibodies and immunofluorescent staining

We used mouse monoclonal antibodies (mAbs) against EB1, p150(γlens) and BubR1 (BD Biosciences), CENP-A (MBL, Japan), α- and γ-tubulin and acetylated tubulin (Sigma); rat mAb against α-tubulin (YL1/2, Abcam); rabbit polyclonal antibodies (pAbs) against CLASP1 (Mimori-Kiyosue et al. 2005), CLASP2 (Akhanova et al. 2001), CLIP-170 (Coquelle et al. 2002) and human CREST serum (kindly provided by Dr Y. Muro, Nagoya University); chicken pAb against GFP (Chemicon). For secondary antibodies, Cy2-conjugated anti-mouse IgG and anti-rabbit IgG pAbs, FITC-conjugated anti-human IgG pAb, TexasRed-conjugated anti-mouse IgG, anti-rat IgG and anti-rabbit IgG pAb, Cy5-conjugated anti-mouse IgG, anti-rat IgG and anti-rabbit IgG pAbs were purchased from Jackson. Cell fixation and staining were performed as previously described (Mimori-Kiyosue et al. 2005).

Fluorescence microscopy and image analysis

The cells were observed with a DeltaVision optical sectioning system (Ver. 2.5, Applied Precision Inc.) equipped with an Olympus IX70 inverted microscope and a cooled CCD camera (Series300 CH350, Photometrics), an Aquacosmos system (Hamamatsu photonics) an Olympus IX70 inverted microscope and a cooled CCD camera (ORCA ER, Hamamatsu Photonics), or a LSM510 confocal laser scanning microscope (Ver. 2.3, Carl Zeiss). Quantitative analysis of fluorescent confocal images was performed using MetaMorph software (Universal imaging). To evaluate mitotic abnormalities (Fig. 4I,J), > 200 images of mitotic cells fixed and stained for DNA and tubulin were automatically collected by scanning the specimens using the panel collection function of DeltaVision (UPlanApo 20× objective), and the mitotic phase and spindle morphology were identified on the basis of their chromosome and spindle configurations, respectively.

For live imaging, cells were cultured on glass-bottomed dishes with No.1S coverslips (Iwaki, Japan). Images of cells were collected with a DeltaVision optical sectioning system using PlanApo 100×/1.40 NA oil, PlanApo 60×/1.40 NA oil p3h or UPlanApo 20×/0.70 NA dry objectives (Olympus). Images were acquired with a cooled CCD camera (Series300 CH350, Photometrics) with an appropriate ND filter, binning of pixels, exposure time, and time intervals. Fluorescence signals were visualized using a quad filter set (86000, Chroma) for multiple-color imaging, or Endow GFP bandpass emission filter set (41017, Chroma) for GFP imaging. The out-of-focus signals in the set of optical sections were then removed using the deconvolution technique with the DeltaVision system.

The quantification of the microtubule dynamics was performed, using MetaMorph software (Universal Imaging Corp.). Statistical analysis was performed using SigmaPlot (SPSS Inc.) and Statistica for Windows (StatSoft Inc.). Unless stated differently, the statistical significance of the observed differences was evaluated using Kolmogorov-Smirnov two-sample test.
Electron microscopy

After 72 h from siRNA transfection, mitotic HeLa cells were selectively removed from culture dishes by pipetting and collected by centrifugation. Cells were doubly fixed with 1.2% glutaraldehyde in 0.1 m phosphate buffer and 1% osmium tetroxide in 0.1 m phosphate buffer, and dehydrated with a graded series of ethanol. Cells were then embedded in epoxy resin. One hundred nanometer thin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Hitachi, H-7500).

Acknowledgements

We thank Dr H. Maiato for comments on this manuscript and for communicating results prior to publication. We are grateful to Dr Y. Muro and Dr R. Tsien for providing materials. This study was supported by Russian Foundation for Basic Research, grant no. 05-04-49847 to IAV and by the Netherlands Organization for Scientific Research grants to AA.

References


Received: 1 March 2006
Accepted: 16 April 2006

Supplementary material
The following supplementary material is available for this article online:

**Movie S1** Cell cycle progression of control and CLASP-depleted cells.

**Movie S2** Microtubule dynamics in control and CLASP-depleted cells during mitosis.

**Movie S3** GFP-AuroraB dynamics in control and CLASP-depleted cells during mitosis.

**Movie S4** Simultaneous imaging of kinetochores and growing microtubule ends in metaphase-like control and CLASP-depleted cells.