Hsp90 is required to localise cyclin B and Msps/ch-TOG to the mitotic spindle in *Drosophila* and humans

Renata Basto[1,*], Fanni Gergely[1,‡], Viji M. Draviam[1,§], Hiroyuki Ohkura[2], Kathryn Liley[3] and Jordan W. Raff[1,*]

[1]The Wellcome Trust/Cancer Research UK Gurdon Institute of Cancer and Developmental Biology. University of Cambridge, Tennis Court Road, Cambridge, CB2 1QN, UK
[2]Wellcome Trust Centre for Cell Biology, Institute of Cell Biology, School of Biological Sciences, The University of Edinburgh, Edinburgh, EH9 3JR, UK
[3]University of Cambridge, Department of Biochemistry Building O, Downing site, Cambridge, CB2 1QW, UK
*Authors for correspondence (e-mail: r.basto@gurdon.cam.ac.uk; j.raff@gurdon.cam.ac.uk)
‡Present address: Department of Oncology, Cancer Research UK, Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge, CB2 0RE, UK
§Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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Summary

During mitosis, cyclin B is extremely dynamic and although it is concentrated at the centrosomes and spindle microtubules (MTs) in organisms ranging from yeast to humans, the mechanisms that determine its localisation are poorly understood. To understand how cyclin B is targeted to different locations in the cell we have isolated proteins that interact with cyclin B in *Drosophila* embryo extracts. Here we show that cyclin B interacts with the molecular chaperone Hsp90 and with the MT-associated protein (MAP) Mini spindles (Msps; the *Drosophila* orthologue of XMAP215/ch-TOG). Both Hsp90 and Msps are concentrated at centrosomes and spindles, and we show that Hsp90, but not Msps, is required for the efficient localisation of cyclin B to these structures. We find that, unlike what happens with other cell cycle proteins, Hsp90 is not required to stabilise cyclin B or Msps during mitosis. Thus, we propose that Hsp90 plays a novel role in regulating the localisation of cyclin B and Msps during mitosis.

Key words: Cyclin B, Hsp90, Mitotic spindle, centrosome, Msps/ch-TOG

Introduction

Cdc2-cyclin B complexes are key regulators of the entry into mitosis, and cyclin B has an extremely dynamic localisation during the cell cycle. In human cells in G2, cyclin B1 is concentrated at centrosomes and shuttles between the nucleus and cytoplasm. It rapidly accumulates in the nucleus just prior to the entry into mitosis, and then binds to the centrosome, spindle and to the mitotic chromosomes from prophase to metaphase (Clute and Pines, 1999). In late metaphase the protein is rapidly degraded prior to the exit from mitosis. In *Drosophila* cells, cyclin B exhibits a similar pattern of localisation, although it does not accumulate on mitotic chromosomes, and, in addition, is strongly concentrated in the middle of the spindle where the MTs from each pole overlap with one another (Huang and Raff, 1999). In yeast cells, the B-type cyclin clb2 is also concentrated on SPBs (the yeast equivalent of the centrosome) and on spindle MTs. Thus, the association of cyclin B with MT-organising centres (MTOCs) and MTs appears to be a highly conserved feature of cyclin B behaviour during mitosis (Decottignies et al., 2001). Indeed, cyclin B is one of the proteins that binds most strongly to MTs in cell extracts (e.g. Huang and Raff, 1999).

Although highly conserved, the mechanisms by which cyclin B associates with centrosomes and MTs remain poorly understood. It has been shown that cyclin B complexes can interact with MT associated proteins (MAPs) such as MAP4 and XMAP215 (in *Xenopus* and its human orthologue ch-TOG), and it has been proposed that these associations contribute to the localisation of cyclin B on MTs (Ookata et al., 1993; Ookata et al., 1995; Charrasse et al., 2000). It has not been shown, however, that cyclin B requires these proteins to interact with MTs in vivo.

In order to identify proteins that might be involved in localising cyclin B to centrosomes or MTs, we isolated proteins that associated with an MBP-cyclin B full-length (MBP-CBFL) fusion protein in *Drosophila* embryo extracts. Using MALDI mass spectrometry, we identified two proteins that specifically co-purified with MBP-CBFL: the molecular chaperone Hsp90, and the MT-associated protein Mini spindles (Msps – the *Drosophila* homologue of ch-TOG and XMAP215). It has previously been shown that, like cyclin B, Hsp90 and Msps are concentrated at centrosomes and on MTs (Lange et al., 2000; Cullen et al., 1999; Charrasse et al., 1998). We show that endogenous cyclin B interacts with endogenous Hsp90 and Msps, and that Hsp90, but not Msps, is required for the efficient localisation of cyclin B to centrosomes and MTs in both *Drosophila* and human cells. Surprisingly, however, Hsp90 is not required to stabilise cyclin B. Thus, Hsp90 appears to play a novel and unexpected role in targeting cyclin B to centrosomes and MTs.

Results

Cyclin B interacts with Hsp90

In order to identify proteins that interact with cyclin B we added bacterially expressed MBP-cyclin B full-length protein (MBP-CBFL) to *Drosophila* embryo extracts. The MBP-

CBFL, together with any interacting proteins, was then isolated on an amylose column. We could distinguish several bands on a Coomassie-stained gel that appeared to specifically co-purify with MBP-CBFL (Fig. 1A). In four separate experiments, two proteins, of ~220 kDa and ~80 kDa, co-purified with cyclin B (Fig. 1A, arrowheads), and these were identified by mass spectroscopy as the products of the *Drosophila* genes *Hsp83* and *mini spindles* (*msps*), respectively.

Hsp83 is the *Drosophila* orthologue of the mammalian molecular chaperone Hsp90 (Yue et al., 1999; Lange et al., 2000) and we hereafter refer to it as Hsp90. This chaperone is one of the most abundant proteins in the cell, and it interacts with several proteins and is required for their conformational maturation (Pratt, 1998; Walter and Buchner, 2002; Young et al., 2004). Hsp90 has several functions within a cell (Pratt, 1998; Grandin and Charbonneau, 2001; Lange et al., 2000). Mini spindles is a member of the XMAP215/ch-TOG family of microtubule-associated-proteins that can bind directly to MTs and are essential for mitosis in many systems (Cullen et al., 1999; Tournebize et al., 2000; Popov et al., 2001; Charrasse et al., 1998) (for a review, see Kinoshita et al., 2002). Interestingly, like cyclin B, both Hsp90 and Msps are concentrated at centrosomes and on spindle MTs.

To test whether endogenous cyclin B can interact with the endogenous Hsp90 and/or Msps we performed co-immunoprecipitation experiments with wild-type (WT) embryo extracts (Fig. 1B). Anti-cyclin B antibodies immunoprecipitated both Hsp90 and Msps, and anti-Hsp90 and anti-Msps antibodies both immunoprecipitated cyclin B; a random rabbit IgG was unable to immunoprecipitate any of these proteins. Thus, cyclin B specifically interacts with both Hsp90 and Msps in embryo extracts. In addition, we noticed that anti-Msps antibodies immunoprecipitated Hsp90, and vice versa, demonstrating that Hsp90 and Msps also interact with one another in embryo extracts.

As several groups have shown an interaction between Cdc2 and Hsp90 (Rubin et al., 1993; Munoz and Jimenez, 1999), we wondered whether the interaction between cyclin B and Hsp90 might be indirect, via Cdc2. To test this possibility, we constructed several MBP-cyclin B truncations and tested whether they could interact with Hsp90 or Msps in WT *Drosophila* embryo extracts. The MBP fusion proteins and associated interactors were immunoprecipitated from embryo extracts with affinity-purified anti-MBP antibodies. We tested three truncated forms of cyclin B in this way (Fig. 1C): MBP-CBNT contains the N-terminal domain of cyclin B that includes the destruction box (D-box) but excludes the two Cdc2-binding cyclin boxes; MBP-CBCT1 contains the C-terminal domain of cyclin B and includes the two cyclin boxes and the 'helix' domain that is thought to promote the specific interaction with cell division protein kinases (CDKs) (Goda et al., 2001); MBP-CBCT2 is a truncated version of MBP-CBC1 that is missing the helix domain. A western-blot analysis of this pull down experiment (Fig. 1D) revealed that Hsp90 bound to both MBP-CBFL and MBP-CBNT but not to MBP-CBCT1 or MBP-CBCT2. As this N-terminal domain cannot interact with Cdc2 we conclude that cyclin B can bind to Hsp90 independently of Cdc2. The N-terminal region also seems to be sufficient for the interaction with Msps, although CBCT1 also interacts with Msps, suggesting that the N-terminal helix domain might promote this interaction. As we wanted to exclude the possibility that Hsp90-cyclin B interaction might be happening through the Cdc2 kinase subunit we used Cdc2 antibodies in our experiments. Unfortunately, in our hands, commercially available antibodies cannot recognise a 34 kDa band (Cdc2 predicted size) in a western blot from *Drosophila* embryo extract or even in immunofluorescence experiments.

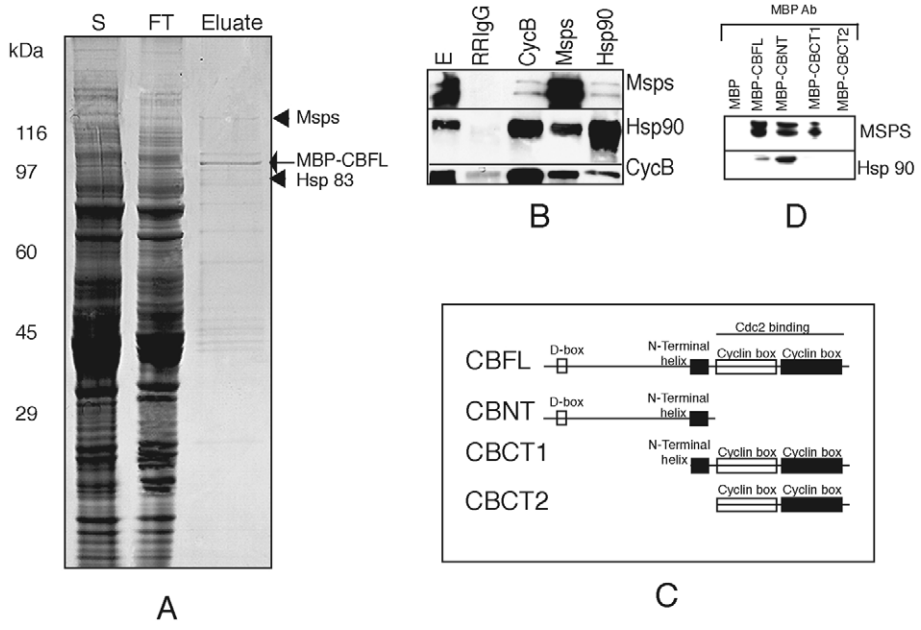

Fig. 1. Cyclin B interacts with Hsp90 and Msps. (A) A Coomassie-Blue-stained gel of the proteins that bind to MBP-CBFL after incubation with embryo extract. Lane 1, the high speed embryo extract that was loaded onto the amylose column; lane 2, the proteins that flowed through the amylose column; lane 3, the proteins that were eluted from the amylose column. The MBP-CBFL protein is highlighted with a black arrow. Hsp90 (Hsp83) and Msps are indicated with black arrowheads. (B) A western blot analysis of an immunoprecipitation experiment performed with *Drosophila* embryo extracts (lane 1), random rabbit IgG (lane 2), affinity-purified anti-cyclin B antibody (lane 3), affinity-purified anti-Msps antibody (lane 4) and anti-Hsp90 antibody (lane 5). The membrane was probed with anti-Msps, anti-Hsp90 and anti-cyclin B antibodies as indicated. (C) A schematic diagram of the MBP cyclin B fusion proteins used in the MBP pull-down experiment shown in D: MBP-cyclin B full length (MBP-CBFL); MBP-cyclin B N-terminal (MBP-CBNT; contains the D-box and N-terminal helix, but no cyclin boxes); MBP-cyclin B C-terminal 1 (MBP-CBC1; contains the N-terminal helix and the two cyclin boxes); MBP-cyclin B C terminal 2 (MBP-CBC2; contains only the two cyclin boxes). (D) A western blot of an MBP pull-down experiment performed with affinity-purified anti-MBP antibodies and probed with anti-Hsp90 and anti-Msps antibodies as indicated.

Cyclin B localisation is impaired when Hsp90 activity is perturbed in both *Drosophila* and human cells

As Hsp90 is concentrated at centrosomes (Lange et al., 2000; de Carcer et al., 2001) and can interact directly with tubulin (Garnier et al., 1998) we wanted to test whether it was required to localise cyclin B to centrosomes or spindles. We examined the distribution of cyclin B in larval neuroblasts that were trans-heterozygous for a combination of hypomorphic *Hsp90* mutant alleles (Lange et al., 2000) (see Materials and Methods).

In WT neuroblasts, cyclin B was strongly concentrated at centrosomes during prophase (Fig. 2A). As chromosomes congressed and aligned on the metaphase plate, cyclin B was detected on centrosomes, spindle microtubules and very strongly at the spindle mid-zone (Fig. 2C), as previously described in *Drosophila* embryos (Huang and Raff, 1999). In *Hsp90* mutant neuroblasts there is a high frequency of mitotic abnormalities, such as monopolar spindles, bipolar spindles of reduced size, and a prometaphase arrest (see Lange et al., 2000). Numerous mitotic *Hsp90* mutant cells display MT abnormalities and, therefore, analysis of spindle morphology or localisation of proteins to the MTs could be problematic. To overcome this problem we decided to analyse cyclin B localisation exclusively in mitotic cells displaying a normal-looking bipolar spindle (see Materials and Methods). In prophase cells, however, cyclin B could still be detected at centrosomes, although this localisation consistently appeared to be weaker and more diffuse than that seen in WT cells (compare Fig. 2A and B; Fig. 2E). During prometaphase and metaphase, the mislocalisation of cyclin B in *Hsp90* mutant cells was even more pronounced: cyclin B was undetectable at the centrosomes or on spindle MTs in the majority of prometaphase cells and metaphase cells (compare Fig. 2C and D; Fig. 2F and G). The localisation of cyclin B to the spindle mid-zone, however, appeared unperturbed in *Hsp90* mutants (Fig. 2D). Similar results were obtained when WT brains were incubated for 1 hour with the Hsp90 inhibitor, geldanamycin (GA; data not shown), a drug that interacts with the ATP site of Hsp90, preventing its interaction with client proteins (for a review, see Isaacs et al., 2003). These observations suggest that the efficient localisation of cyclin B to the centrosome and spindle MTs, but not to the spindle mid-zone, requires Hsp90.

To test if the centrosomal and microtubule localisation of cyclin B is dependent of Hsp90 in vertebrates, we treated HeLa cells with GA for 24 hours and examined the localisation of cyclin B and the centrosome- and MT-associated protein TACC3 (Gergely et al., 2000b). In control cells, cyclin B was detected at the centrosome during prophase (Fig. 3A, arrows) and it stained spindle MTs until the end of metaphase (Fig. 3C). After GA incubation, however, the localisation of cyclin B on prophase centrosomes appeared relatively normal (Fig. 3B), but the levels of cyclin B on centrosomes and spindle MTs was dramatically reduced in prometaphase and metaphase cells (Fig. 3C-F,G). Thus, in both flies and humans, Hsp90 is required for the efficient localisation of cyclin B to centrosomes and spindle MTs during mitosis.

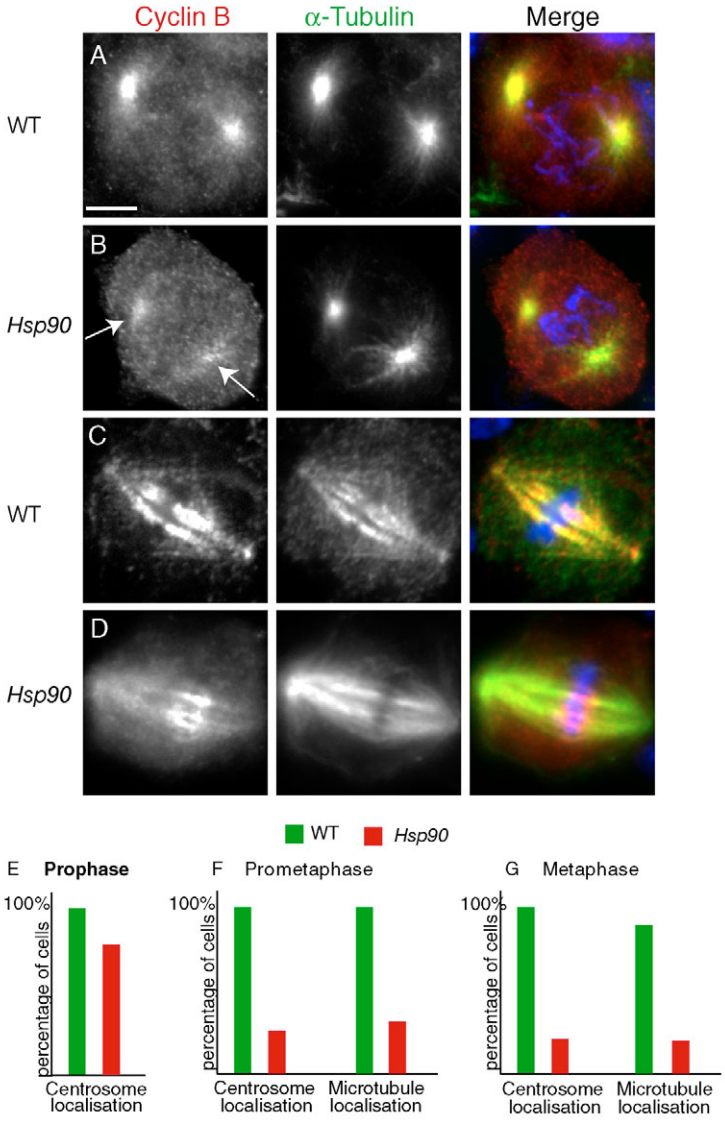

Fig. 2. Cyclin B is mislocalised in *Hsp90* mutants. (A-D) Immunostaining of *Drosophila* third instar larvae neuroblasts. Cyclin B is shown in red (left panel), α-tubulin in green (middle panel), and DNA in blue. (A) In WT prophase cells (A) cyclin B accumulates on centrosomes. (B) In *Hsp90* mutant prophase cells cyclin B can be detected on centrosomes (white arrows) but this localisation is weaker and more diffuse than normal. (C) In WT metaphase cells cyclin B stains the centrosomes, the spindle MTs and the spindle mid-zone. (D) In *Hsp90* mutant metaphase cells cyclin B strongly stains the spindle mid-zone, but staining on the spindle and centrosomes is much weaker than normal. (E-G) Bar graphs showing the quantification of cyclin B staining on centrosomes or on MTs in WT (green bars) and *Hsp90* mutant (red bars) *Drosophila* neuroblasts (see Materials and Methods) in prophase (E), prometaphase (F) and metaphase (G). Bar, 5 μm (A-D).

Cyclin B localisation to centrosomes and MTs does not appear to require Msps

Cyclin B and XMAP215 have previously been shown to interact in *Xenopus* extracts (Charrasse et al., 2000), and CDK1 complexes can phosphorylate XMAP215 in vitro (Vasquez et al., 1999). It has been proposed that cyclin B requires XMAP215 for its localisation to centrosomes and MTs

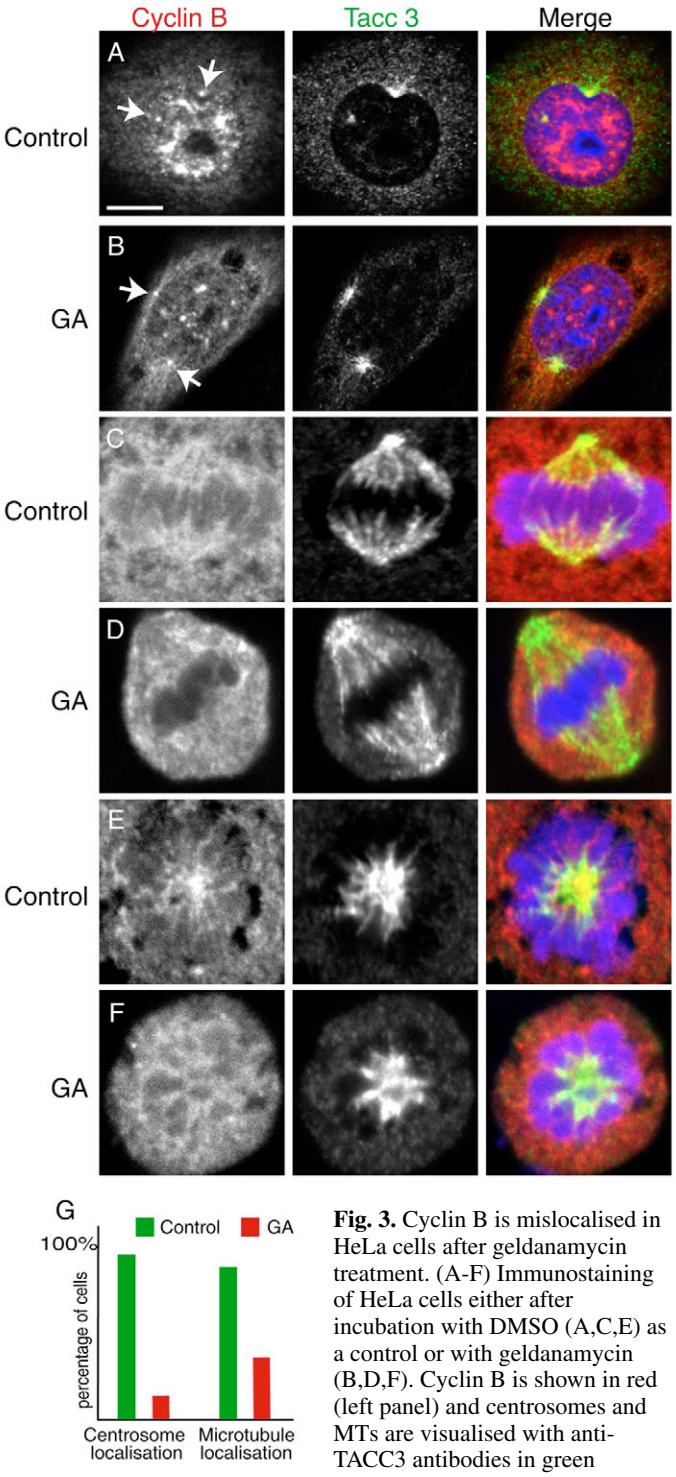

Fig. 3. Cyclin B is mislocalised in HeLa cells after geldanamycin treatment. (A-F) Immunostaining of HeLa cells either after incubation with DMSO (A,C,E) as a control or with geldanamycin (B,D,F). Cyclin B is shown in red (left panel) and centrosomes and MTs are visualised with anti-TACC3 antibodies in green (middle panel); DNA is shown in blue in the merged images (right panel). Before NEB, cyclin B can be detected at centrosomes in both control cells and in GA-treated cells (A and B, white arrows). In metaphase control cells, cyclin B decorates the mitotic spindle (C) whereas after GA incubation (D) cyclin B cannot be detected on the MTs. (E,F) Metaphase cells viewed from one end of the spindle. In control cells (E) cyclin B is seen on the centrosomes and MTs whereas after GA treatment (F) no clear centrosomal or MT staining is observed. (G) Bar graphs showing the quantification of cyclin B staining on centrosomes or on MTs in control (green bars) and GA-treated cells (red bars). Bar, 10 μm (A-F).

(Charrasse et al., 2000). To test whether cyclin B localisation requires Msps, we examined the distribution of cyclin B in homozygous *msp*^{s51} mutant larval neuroblasts, which contain essentially undetectable levels of Msps protein. As previously described, *msps* mutant cells have a variety of mitotic defects, but the localisation of cyclin B to centrosomes and spindles was essentially indistinguishable from that seen in control cells, suggesting that Msps is not required for the localisation of cyclin B to centrosomes or MTs in *Drosophila* cells (Fig. 4A,B compare with Fig. 2A,C).

To test whether ch-TOG, the human homologue of Msps, was required to localise cyclin B in human cells, we examined the localisation of cyclin B in a population of HeLa cells where ch-TOG levels had been reduced by siRNA. An overall reduction of ~60% of ch-TOG levels was obtained (Gergely et al., 2003). The levels of depletion in individual cells were examined by immunofluorescence and we concentrated our analysis in mitotic bipolar cells where a significant reduction of ch-TOG could be seen. In ch-TOG-depleted cells, cyclin B was recruited to centrosomes and microtubules just as in control cells (compare Fig. 4E with F). Thus, although cyclin B and Msps/XMAP215 appear to interact in both flies (this work) and frogs (see also Charrasse et al., 2000) we conclude that Msps is not required to localise cyclin B to centrosomes or spindles in *Drosophila* neuroblasts or human tissue culture cells.

Hsp90 is required to efficiently localise Msps/ch-TOG to centrosomes and spindle MTs

As Msps interacts with Hsp90 (Fig. 1B), we wondered whether Hsp90 was required for Msps localisation to centrosomes or spindles. In WT *Drosophila* neuroblasts, Msps associates with centrosomes and spindle MTs during prometaphase and metaphase (Fig. 5A). In *Hsp90* mutant neuroblasts (Fig. 5B), or after incubation of WT neuroblasts with GA (not shown), the centrosomal localisation of Msps was somewhat reduced, whereas the spindle localisation of Msps was strongly reduced (Fig. 5F).

In HeLa cells, ch-TOG localises to the centrosome and spindle MTs throughout mitosis (Fig. 5C) (Gergely et al., 2003). In HeLa cells treated with GA (Fig. 5D), the centrosomal localisation of ch-TOG appeared largely unperturbed, but the amount of Msps associated with the spindle MTs was significantly reduced. Thus, perturbing Hsp90 function in both *Drosophila* and human cells disrupts the localisation of cyclin B to the mitotic spindle apparatus.

Hsp90 is not required for the recruitment of all centrosome and/or spindle components to the centrosome and/or mitotic spindle

Our data suggest that Hsp90 is required for the efficient recruitment of cyclin B to centrosomes and MTs. Human and fly cells in which Hsp90 function has been perturbed by either mutation or drug inhibition, however, exhibit a large range of relatively pleiotropic mitotic defects (Lange et al., 2000; de Carcer, 2004) and we wondered whether the defects in cyclin B and Msps localisation to centrosomes and spindles might be non-specific. We therefore tested the localisation of several other centrosomal and/or spindle components in *Hsp90* mutant larval neuroblasts. We found that the localisation of D-TACC, CP190, CP60 (not shown), CNN and γ-tubulin (Fig. 6A-C)

appeared to be unperturbed in mutant cells. Thus, Hsp90 is not required to efficiently recruit all proteins to the centrosome and/or spindle.

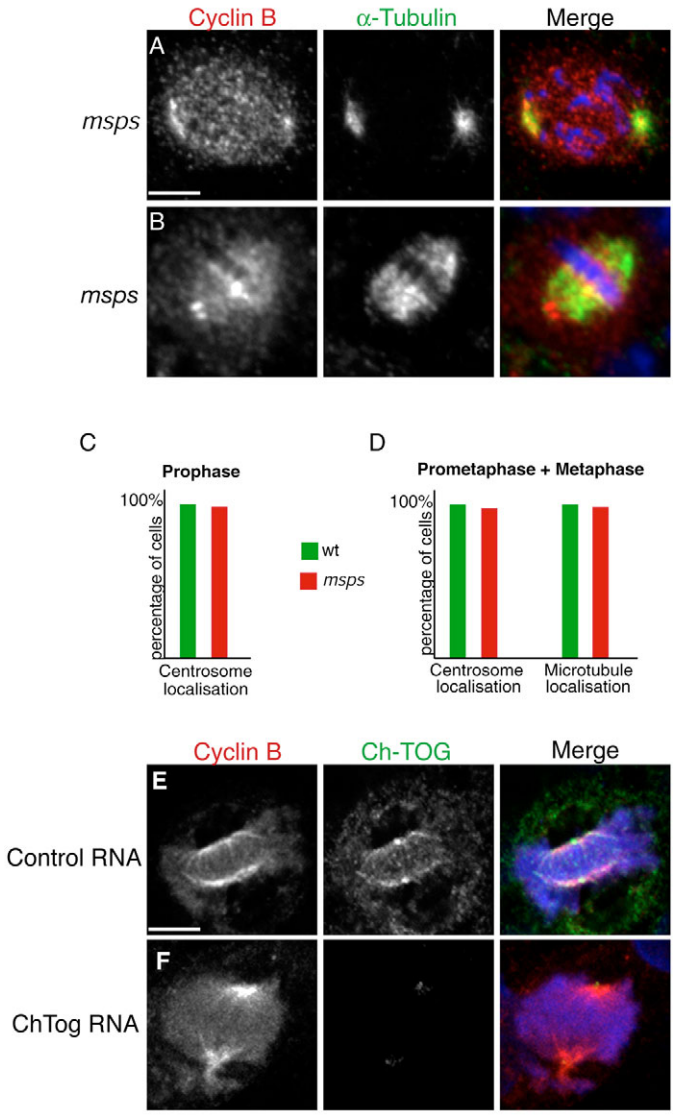

Fig. 4. Msps/ch-TOG is not required for the centrosomal or MT localisation of cyclin B. (A,B) Immunostaining of *Drosophila* neuroblasts cells showing cyclin B (red, left panel), tubulin (green, middle panel) and DNA (blue in merged panel). (A) *msps* mutant neuroblast cell in prophase. Although the poles are somewhat disorganised in this cell, cyclin B is associated with the centrosomes. (B) *msps* mutant neuroblast cell in metaphase. Cyclin B is present at the poles and is associated with the mitotic spindle. (C,D) Bar graphs showing the quantification of cyclin B staining on centrosomes or on MTs in WT (green bars) and *msps* mutant (red bars) neuroblasts in prophase and prometaphase + metaphase. Bar, 5 μm (A,B). (E,F) Immunostaining of control and ch-TOG-depleted HeLa cells with cyclin B (red, left panel) and ch-TOG (green, middle panel); DNA is shown in blue in the merged panel. (E) In mock-depleted HeLa cells, both cyclin B and ch-TOG can be detected on centrosomes and MTs. Note that cyclin B has already disappeared from the centrosome in this cell that has completely aligned its chromosomes at the metaphase plate (Clute and Pines, 1999). (F) In ch-TOG partially depleted cells cyclin B associates with the spindle. Bar, 10 μm (C,D).

The requirement for Hsp90 to recruit cyclin B and Msps to the spindle is not an indirect consequence of the inactivation of Polo kinase

It has previously been shown that Hsp90 is required to stabilise Polo kinase, (de Carcer et al., 2001), a key mitotic regulator that influences many aspects of mitosis (for a review, see Glover, 2005). We therefore tested whether the mislocalisation of cyclin B and Msps that we observe in *Hsp90* mutant cells could be an indirect consequence of the inactivation of Polo kinase. In *polo*^{10} mutant neuroblasts, that have virtually undetectable levels of Polo kinase (Donaldson et al., 2001), the localisation of cyclin B and Msps on centrosomes and MTs was indistinguishable from that seen in WT cells (Fig. 7A,B). Thus, the defects in cyclin B and Msps localisation seen in *Hsp90* mutant neuroblasts are unlikely to be simply a consequence of a lack of Polo function.

Hsp90 is not required to stabilise cyclin B or Msps protein

Several studies have shown that one of the major functions of Hsp90 chaperones is to correctly fold and stabilise proteins such as Polo (de Carcer et al., 2001) or LKB1 (Boudeau et al., 2003). It seemed possible, therefore, that Hsp90 might be required to stabilise Msps/ch-TOG and cyclin B. To test this possibility, we dissected both WT and *Hsp90* mutant brains and subjected half of the brains to a 2-hour heat shock at 37°C. The other half was kept at 25°C for the same period of time. We then performed a western blot with these brains using antibodies against Polo, Msps, cyclin B and also CP190 as a loading control (Fig. 8A). Polo kinase is slightly destabilised in non-heat shocked *Hsp90* mutant brains compared to WT controls, and it is dramatically destabilised in heat shocked *Hsp90* mutant brains [as previously described in *Drosophila* cultured cells (de Carcer et al., 2001)]. By contrast, the levels of cyclin B and, to a lesser extent, Msps were slightly increased in *Hsp90* mutant brains, even after heat shocking (Fig. 7A). This increase in cyclin B and Msps protein levels may be explained by a general increase in the number of cells in mitosis in Hsp90 mutant cells, or in WT cells treated with GA (Lange et al., 2000; de Carcer et al., 2001; de Carcer, 2004). Thus, unlike the case for Polo kinase, Hsp90 does not appear to be required to stabilise cyclin B or Msps protein levels.

Cyclin B can bind directly to MTs in vitro

The data described above suggests that Hsp90 is required for the efficient targeting of cyclin B to MTs. It seems unlikely, however, that Hsp90 directly mediates the interaction between cyclin B and MTs, as we have previously shown that virtually all of the cyclin B in an embryo extract can interact with MTs in co-pelleting assays (Huang and Raff, 1999) whereas only a small fraction of the cyclin B in an embryo extract appears to be bound to Hsp90 (R.B., unpublished observations; see Discussion). We therefore tested whether any of the MBP-cyclin B fusion proteins (Fig. 1C) could directly interact with purified MTs in vitro. To our surprise, the full-length (MBP-CBFL) and N-terminal (MBP-CBNT) fusions both strongly co-pelleted with MTs (Fig. 8B), whereas MBP alone did not. A portion of the C-terminal fusion (MBP-CBCT1) also seems to co-pellet with MTs, suggesting that the N-terminal domain of cyclin B is required for MT binding.

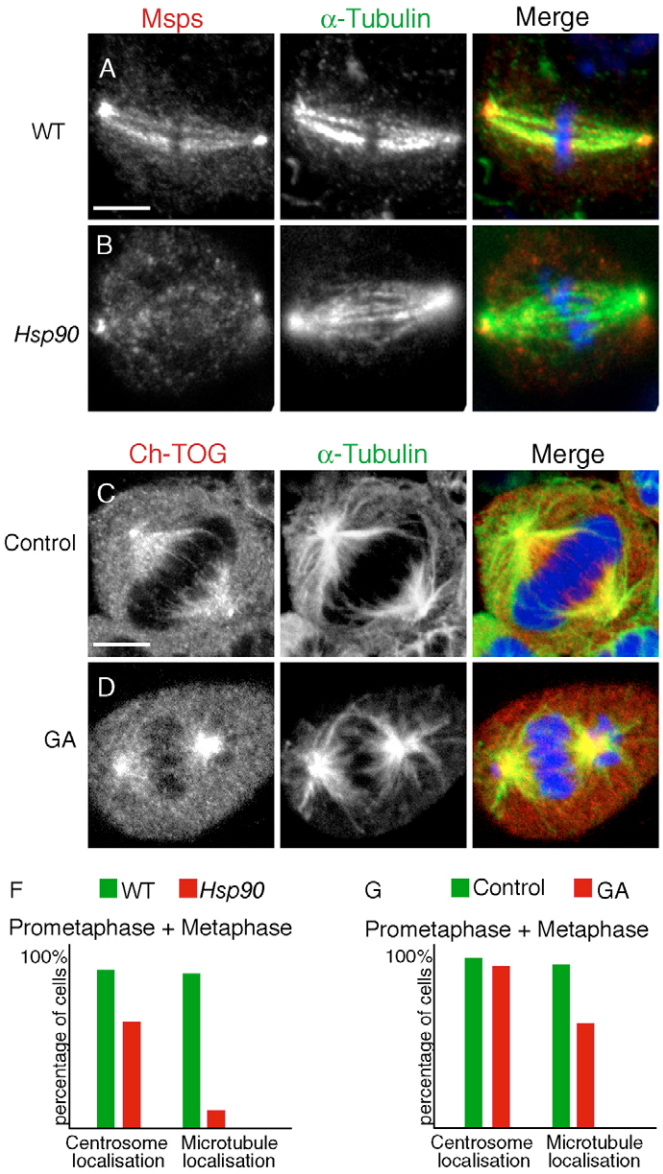

Fig. 5. Hsp90 activity is required for Msps/ch-TOG localisation to spindle MTs. (A,B) Immunostaining of *Drosophila* third instar larvae neuroblasts with Msps (red, left panels), and α-tubulin (green, middle panels); DNA is shown in blue in the merged panels. (A) In WT metaphase cells Msps stains the centrosomes and MTs. (B) In *Hsp90* mutant cells Msps can be detected on the centrosomes but not on the spindle MTs. (C,D) Immunostaining of HeLa cells either after incubation with DMSO (C) or with geldanamycin (D). In control cells, ch-TOG (red, left panels) strongly decorates the MTs (green, middle panels) of the mitotic spindles but in GA-treated cells there is a decrease in MT staining. (F) Bar graphs showing the quantification of Msps staining in WT (green bars) and *Hsp90* mutant (red bars) *Drosophila* neuroblasts in prometaphase and metaphase. (G) Bar graphs showing the quantification of CH-TOG staining in control (green bars) and GA-treated (red bars) HeLa cells in prometaphase and metaphase. Bars, 5 μm (A,B); 10 μm (C,D).

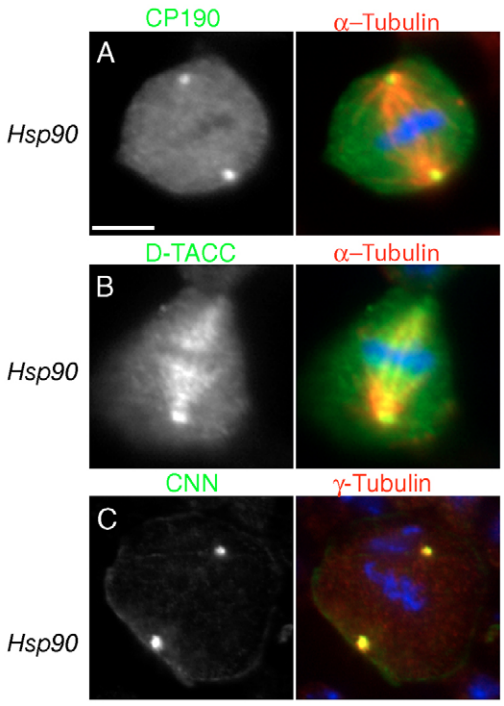

Fig. 6. Hsp90 is not required for the recruitment of CP190, D-TACC, CNN and γ-tubulin. Immunostaining of *Drosophila hs90p* mutant neuroblasts cells (A-C). (A,B) CP190 and D-TACC are shown in the left panel and in green in the merged images. MTs are shown in red and DNA in blue in the merged images. (C) CNN is shown separately in the left panel and in green in the right panel. γ-tubulin is shown in red and DNA in blue in the merged images (right panel). Bar, 5 μm.

Discussion

In an attempt to understand how cyclin B is targeted to centrosomes and MTs we searched for proteins that interacted with a full-length MBP-cyclin B fusion protein in *Drosophila* embryo extracts. We found two proteins, Hsp90 and Msps, that specifically interacted with MBP-CBFL, and we showed that endogenous Hsp90 and Msps interacted with endogenous cyclin B. Msps is a centrosomal MAP that had previously been shown to interact with cyclin B in human cells (Charrasse et al., 2000) whereas Hsp90 is a centrosomal protein that can also interact with tubulin (Garnier et al., 1998), making both proteins plausible candidates for factors that target cyclin B to centrosomes and MTs. In *Drosophila msps* mutant larval neuroblasts, or in human cells in which ch-TOG levels have been reduced by RNAi, cyclin B appeared to localise to centrosomes and MTs normally. In *Drosophila Hsp90* mutant larval neuroblasts, however, the levels of cyclin B at centrosomes and MTs were strongly reduced, and the same was true in larval neuroblasts or human tissue culture cells treated with the Hsp90 inhibitor geldanamycin (GA). Thus, we conclude that Hsp90, but not Msps, is required for the efficient localisation of cyclin B to centrosomes and MTs.

In unperturbed *Drosophila* and human cells, cyclin B is initially recruited to centrosomes during G2, and it binds to the centrosomes and the mitotic spindle from prophase to metaphase. In *Drosophila* cells, cyclin B is also strongly recruited to the middle region of the spindle, where the MTs from opposite poles overlap (Huang and Raff, 1999). The initial recruitment of cyclin B to centrosomes during G2 is not perturbed in human cells treated with GA, and it is only moderately perturbed in *Hsp90* mutant cells in flies, suggesting

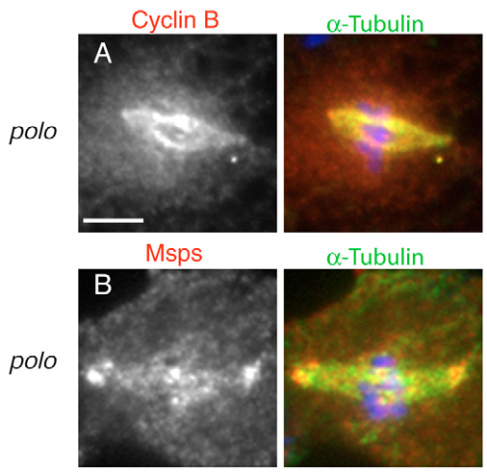

Fig. 7. Polo kinase is not required for the centrosomal and MT localisation of cyclin B and Msps. Immunostaining of *Drosophila polo* mutant neuroblasts cells. (A,B) Cyclin B and Msps are shown in the left panel, and in red in the merged images. MTs are sown in green and DNA in blue in the merged images. In *polo* mutants both cyclin B and Msps can localise to the mitotic apparatus just as in control cells. Compare Fig. 7A with Fig. 2C, and Fig. 7B with Fig. 5A. Bar, 5 μm.

that the initial recruitment of cyclin B to centrosomes does not require Hsp90. As cells enter mitosis, however, cyclin B is strongly mislocalised from centrosomes and spindles in *Hsp90* mutant cells and cells treated with GA. Intriguingly, the localisation of cyclin B to the spindle mid-zone occurs normally in *Drosophila Hsp90* mutant cells. Thus, the recruitment of cyclin B to centrosomes and spindles appears to occur through multiple mechanisms, only some of which require Hsp90.

Hsp90 is one of the best-studied molecular chaperones, and it plays an important part in the proper folding of many proteins in both normal and stressed cells (Pratt et al., 1996; Pratt, 1998; Pratt and Toft, 2003). It has recently been shown that Hsp90 is required for the proper stabilisation of another centrosomal cell cycle-regulator, Polo kinase (de Carcer et al., 2001). If Hsp90 function is perturbed, Polo is degraded, and many of the mitotic defects associated with Hsp90 perturbation have been proposed to be caused by defects in Polo function (de Carcer et al., 2001; Glover, 2005). The defects in cyclin B localisation that we observe after Hsp90 perturbation do not seem to be a consequence of Polo inactivation, however, as cyclin B is still concentrated on centrosomes and spindles in *polo* mutant cells. Moreover, our data suggests that Hsp90 does not influence cyclin B function simply by stabilising it. Cyclin B is not destabilised in *Hsp90* mutant cells, even after they have been heat shocked, and cells are capable of entering mitosis in *Hsp90* mutants and after GA treatment; many of these cells appear to arrest in mitosis with high levels of cyclin B (R.B., unpublished observations) (see also Lange et al., 2000; de Carcer, 2004). Finally, cyclin B is still capable of binding to the spindle mid-zone in *Drosophila* cells when Hsp90 function is perturbed. Taken together, these observations suggest that Hsp90 is not generally required for cyclin B function, but is specifically required for the efficient localisation of cyclin B to centrosomes and spindles.

How might Hsp90 function in recruiting cyclin B to centrosomes and spindles? As Hsp90 is itself located at centrosomes and can bind to tubulin, it is possible that Hsp90 binds cyclin B and directly targets it to these locations. We suspect that this is not how Hsp90 targets cyclin B to MTs, as, in our hands, only a small fraction of the total cyclin B is bound to Hsp90 in embryo extracts. We previously showed that virtually all of the cyclin B in an embryo extract was capable of binding to MTs in MT spin-down experiments (Huang and Raff, 1999). Thus, it seems unlikely that Hsp90 could act as an essential co-factor that directly mediates the interaction between cyclin B and MTs. Similarly, we suspect that Hsp90 does not directly target cyclin B to centrosomes, as the initial recruitment of cyclin B to centrosomes during prophase is only mildly disrupted in *Drosophila* cells (in HeLa prophase recruitment is not disrupted) when Hsp90 has been perturbed. Rather, Hsp90 seems to be involved in maintaining the centrosomal localisation of cyclin B during prometaphase and metaphase. Perhaps Hsp90 is essential for the proper folding or function of a specific domain of cyclin B that is required for the localisation of cyclin B on centrosomes and spindles. In such a scenario Hsp90 could even act indirectly to allow cyclin B to associate with other proteins that target it to centrosomes and MTs.

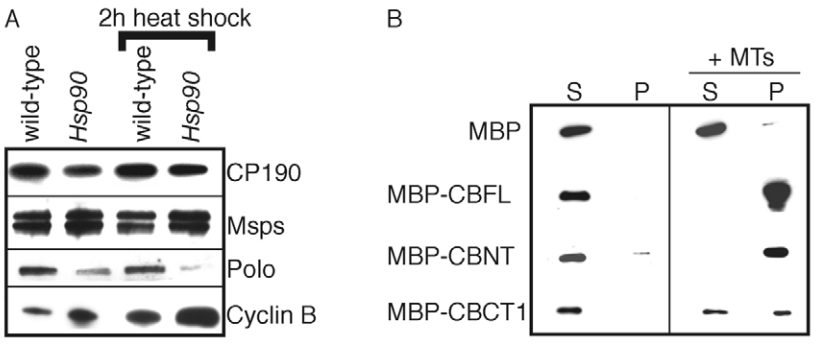

Fig. 8. Cyclin B and Msps are not destabilised in the absence of Hsp90, and cyclin B can interact directly with MTs in vitro. (A) Western blot of wild-type and *Hsp90* neuroblasts. The brains were dissected and either incubated at 25°C for 2 hours or heat shocked at 37°C for the same amount of time. The filters were probed with anti-Msps, anti-cyclin B and anti-Polo antibodies. CP190 was used as loading control. In *Hsp90* mutants, Polo protein is not stabilised (see de Carcer et al., 2001) but levels of Msps and cyclin B are comparable to, or even higher than, levels in WT. (B) Cyclin B can associate directly with MTs in vitro. Western-blot of a MT co-pelleting assay developed with affinity purified MBP antibodies. Supernatants (S) and pellets (P) from a control sedimentation assay with no MTs (left) or with MTs (right) performed with the same amount of the following proteins (top to bottom) MBP; MBP-CBFL, MBP-CBNT, MBP-CBC2. When MTs are not present all the proteins are detected in the (S) fraction. When incubated with MTs, MBP is still detected in the S fraction but a shift in MBP-CBFL and MBP-CBNT to the P fraction is detected. MBP-CBCT1 seems to be equally distributed into S and P fractions, suggesting that the N-terminal domain of cyclin B is required for MT binding.

If our assumption that Hsp90 does not directly target cyclin B to centrosomes or MTs is correct, it raises the intriguing question of what targets cyclin B to these locations? It has previously been shown that cyclin B can interact with XMAP215 and it has been proposed that this interaction could target cyclin B to centrosomes and MTs. We also found that cyclin B could interact with Msps/XMAP215 in *Drosophila* embryo extracts, suggesting that this interaction is conserved between frogs and flies. In *msps* mutant cells, or in human cells partially depleted of ch-TOG, however, we found that cyclin B was still localised to centrosomes and MTs. Thus, we conclude that Msps is not directly responsible for targeting cyclin B to centrosomes or MTs. Msps family members play an important role in regulating MT dynamics during the cell cycle (Cullen et al., 1999; Tournebize et al., 2000; Brittle and Ohkura, 2005) (for a review, see Kinoshita et al., 2002) so the interaction between cyclin B and Msps may simply reflect the fact that cyclin B/Cdc2 regulates Msps activity during the cell cycle. Indeed, it has previously been shown that Msps/XMAP215 is phosphorylated by cyclin B/Cdc2 in vitro (Vasquez et al., 1999).

If Msps and Hsp90 do not directly target cyclin B to centrosomes and MTs, it remains unclear what does. A priori, we expected that cyclin B in *Drosophila* embryo extracts would exist in a tight complex with any factor that would target it to MTs, as the vast majority of cyclin B binds to MTs in embryo extracts. As cyclin B appears to localise at centrosomes and MTs in virtually all systems, it remains possible that cyclin B can directly bind to centrosomes and MTs. Indeed, we find that bacterially expressed MBP-CBFL interacts strongly with purified MTs in MT-pelleting assays (see Fig. 8B). In light of our results it seems that cyclin B is capable of interacting directly with MTs, although we remain cautious in our interpretation of this *in vitro* experiment as fusion proteins containing cyclin B could have a tendency to aggregate in solution.

Nevertheless, the fact that no one has identified a factor that directly mediates the interaction between cyclin B and MTs or centrosomes, despite many years of effort in identifying cyclin B interacting proteins, suggests that there may be no other protein directly required for these interactions. In our favoured hypothesis, Hsp90 would serve simply to ensure that cyclin B was correctly folded to allow it to directly interact with MTs and with centrosomes. Interestingly it has been proposed that Hsp90 also contributes to increasing the association efficiency of Tau with MTs (Dou et al., 2003). Tau is a MAP with an important role in Alzheimer's disease. In the absence of Hsp90, Tau tends to aggregate and therefore less soluble Tau is available to bind to MTs (Dou et al., 2003). In our study, although we also find that cyclin B and Msps require Hsp90 for their efficient recruitment to the spindle we do not think that their activity, outside the spindle, is compromised.

Finally, we found that Hsp90 was not only required to allow cyclin B to localise efficiently to centrosomes and MTs, it was also required to allow Msps to localise properly, and we showed that the endogenous Hsp90 can interact with the endogenous Msps. Importantly, Hsp90 is not required for the localisation of several other proteins to centrosomes or MTs, demonstrating that its function in localising cyclin B and Msps is specific. Like cyclin B, the levels of Msps protein were not decreased in cells where Hsp90 function had been perturbed, suggesting that Hsp90 is not simply required to stabilise Msps protein. Thus, we propose that Hsp90 may act on several MT-associated proteins to ensure that specific domains of these proteins are in the correct conformation to allow these proteins to be targeted to different locations within the cell.

Materials and Methods

Purification and identification of MBP-cyclin B-associated proteins

A maltose binding protein (MBP; New England Biolabs) fusion to the full-length *Drosophila* cyclin B protein was expressed in *E. coli*, and purified essentially according to the manufacturers instructions, except that cells were grown at 25°C.

The protein was desalted on a P6 column (Bio-Rad) into C-buffer (50 mM Hepes pH 7.6, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA) using a Bio-Logic FPLC system (Bio-Rad). Glycerol was added to 50% of the total volume, and the protein was stored at –20°C. High speed, 0- to 4-hour-old embryo extracts were prepared in C-buffer as described previously (Gergely et al., 2000a) and 5 μg of either MBP or MBP-CBFL protein was added to 2 ml of embryo extract. The mixture was incubated at room temperature for 30 minutes, at 4°C for 15 minutes, and then loaded onto a 2 ml amylose-resin column at a flow rate of 0.1 ml/minute using a Bio-Logic FPLC system. The column was washed with 20 volumes of C-buffer plus 0.1% Tween 20, and then eluted with five volumes of C-buffer plus 10 mM maltose. The peak protein fractions eluting from the column were pooled, precipitated with 10% trichloroacetic acid and resuspended in protein sample buffer. The mixture was neutralized with ammonium hydroxide and loaded onto 4-12% gradient pre-cast Novex gels (Invitrogen).

To identify the proteins that specifically co-purified with MBP-CBFL, protein bands of interest were excised from a Coomassie-Blue-stained gel, washed, in-gel digested to peptides using trypsin on a MassPrepStation (Micromass UK Ltd, Elstree, UK). The resulting peptides were analysed using liquid chromatography-mass spectroscopy (LC-MS/MS; QTof with capLC, Micromass UK Ltd) in conjunction with a PepMap C18 180 mm internal diameter 15 cm capillary column LC-MS/MS (LC Packings). Fragmentation data was used to search the National Centre for Biotechnology Information (NCBI) Drosophila database using the MASCOT search engine (Matrix Science). Manual sequence assignment was assisted using the peptide sequencing feature of BioLynx (Micromass).

Antibodies

The following antibodies (all affinity-purified rabbit antibodies unless otherwise stated) were used in this study: anti-cyclin B (Huang and Raff, 1999); anti-Msps (Lee et al., 2001); anti-TACC3 (Gergely et al., 2000a); anti-ch-TOG (Tournebize et al., 2000); anti-MBP (AbCam, Cambridge, UK); anti-Hsp90 rabbit polyclonal (SP-771, StressGene, Hamburg, Germany); mouse monoclonal anti-α-tubulin (DM1α, Sigma); mouse monoclonal anti-cyclin B (GNS1, Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies were used at 1-2 μg/ml or at a dilution of 1/200-1/500 for both immunofluorescence and western blotting. All secondary antibodies, FITC- or Cy3-conjugated were obtained from Jackson Immunoresearch (Baltimore, PA), whereas horseradish peroxidase-conjugated antibodies were obtain from Amersham Biosciences (Amersham, UK).

Immunoprecipitation experiments

Immunoprecipitation experiments (IP) were performed essentially as described previously (Huang and Raff, 2002) Briefly, after coupling and cross linking IgG to magnetisable polystyrene beads coated with protein A (Dynabeads, from Dynal Biotech, Trondheim, Norway), the beads were incubated at 4°C for 2 hours with a *Drosophila* extract from 0- to 4-hour-old embryos. The beads were then washed five times in PBT (PBS + 0.1% Triton X-100), resuspended in sample buffer, and samples were analysed by SDS-PAGE and immunoblotting.

Pull down experiments

The four following fusions proteins were used in this study: MBP-CBFL (described above) MBP-cyclin B N-terminal (MBP-CBNT), containing amino acids 2-304, also described by Huang and Raff (Huang and Raff, 1999). MBP-cyclin B C-terminal 1 (MBP-CBCT1) containing amino acids 247-523 and MBP-cyclin B C-terminal 2 (MBP-CBCT2) containing amino acids 273-523. 1 mg of each fusion protein was incubated, with rotation, with a *Drosophila* embryo extract for 30 minutes at room temperature and then for 15 minutes at 4°C. Immunoprecipitation was then performed using affinity purified anti-MBP antibodies.

Electrophoresis and immunoblotting

For western blotting, proteins were separated in 4-12% pre-cast Novex acrylamide gels (Invitrogen) and then transferred by electroblotting to a nitrocellulose membrane (Millipore). The membrane was pre-blocked with dried milk in TBS + glycerol for 20 minutes, hybridised with primary antibodies (diluted in the milk block) for 2 hours at room temperature (RT), washed in TBST (TBS + 0.05% Tween

20) for 1 hour (3× 20 minutes), incubated with secondary antibody 1:10000 dilution in milk block) for 1 hour at RT, washed in TBST (3× 10 minutes) and developed by ECL (Supersignal, Pierce).

MT binding assay

Pure bovine tubulin was centrifuged at 40,000 ***g*** for 10 minutes at 4°C. The supernatant was recovered and tubulin concentration was adjusted to 10 mg/ml. Tubulin was polymerised with taxol in the presence of 2 mM GTP, and the various MBP fusion proteins (1.5 μg of each) were then incubated with or without the polymerised tubulin for 15 minutes at RT. The samples were then gently layered on a 50% glycerol cushion and centrifuged at 60,000 ***g*** for 12 minutes. Supernatants and pellets were then loaded on a 10% Novex acrylamide gel (Invitrogen) and transferred to nitrocellulose as described above. Western blotting was performed with affinity-purified anti-MBP antibodies.

Fly strains

Flies were maintained on standard corn meal *Drosophila* medium at 25°C. The following strains were used in this study: *w67* was used as a WT, *Hsp83-9J1* and *Hsp83-13F3-80B* (both stocks from the E. Haffen lab, Developmental Genetics Zoologisches Institut, Zürich, Switzerland) were used in a transheterozygous combination. The *msps* mutation we used in this study, MJ51 allele, is a product of P-element imprecise excision from the msps[P] allele.

Immunostaining of third instar neuroblasts

Third instar larvae were dissected in PBS and brains were fixed in 3.7% formaldehyde in PBS for 30 minutes, transferred to 45% acetic acid for 30 seconds and then to 60% acetic acid drops on coverslips for no longer than 3 minutes. Brains were squashed between slide and cover slip followed by liquid nitrogen immersion. After removal of the coverslip the slides were incubated in cold methanol for 10 minutes at –20°C. Slides were washed in PBT three times (10 minutes each) and then incubated with a primary antibody (diluted in PBT) overnight in a humidified chamber at 4°C. Secondary antibodies were added after washing the slides three times for 5 minutes each. Incubation with a secondary antibody was done for 4 hours at RT or overnight at 4°C. The slides were finally washed with PBT four times for 15 minutes each. Chromosomes were counterstained with 0.5 g/ml Hoechst by incubation of slides for 10 minutes. Finally, the slides were mounted on *n*-propyl gallate glycerol mounting medium. The Hsp90 inhibitor geldanamycin (Sigma) was used at 1 μg/ml in PBS and brains were incubated for 1 hour at room temperature prior to fixation.

For heat shock purposes, 10 brains were dissected and immersed in a drop of PBS and placed in a closed container at 37°C for 2 hours. As a control, 10 brains were dissect and prepared in exactly the same way but placed at 25°C. All observations on brains were made in a Zeiss Axioskop 2 microscope using a 63× 1.4NA PlanApo objective. MetaMorph software (Universal Imaging) was used for image acquisition.

To quantify cyclin B and Msps localisation we scored at least 20 brains in each case, i.e. more than 500 mitotic cells from wt, *Hsp83-9J1/Hsp83-13F3-80B* transheterozygous and *msps* mutant were quantified. Immunostaining was always performed with α-tubulin antibodies. To detect cells in mitosis we searched, using the tubulin channel, for mitotic spindles, and together with the DNA staining we ascertained the phase of the mitotic cycle. We then switched to the channel required to detect cyclin B or Msps staining, and determined if we could detect a signal at centrosomes, spindle MTs and spindle mid-zone.

Preparation of brains for western blot analysis has been described previously (Scaerou et al., 1999).

Immunostaining of HeLa Cells

HeLa cells were cultured, fixed and stained with antibodies as described previously (Gergely et al., 2000a). All images were obtained on a Nikon E800 Eclipse microscope using a 60× 1.4 NA Plan Neufluor objective and a Bio-Rad Radiance 2000 confocal microscope system. Quantification of cyclin B and ch-TOG localisation was done in 250 mitotic cells randomly chosen from different experiments. Cells were incubated with geldanamycin for 24 hours. Preparation and transfection of HeLa cells with ch-TOG siRNA is described by Gergely et al. (Gergely et al., 2003).

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