Hsp90 is a core centrosomal component and is required at different stages of the centrosome cycle in *Drosophila* and vertebrates

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To determine the molecular composition of the centrosome of a higher eukaryote, we carried out a systematic nano-electrospray tandem or MALDI mass spectrometry analysis of the polypeptides present in highly enriched preparations of immunisolated *Drosophila* centrosomes. One of the proteins identified is Hsp83, a member of the highly conserved Hsp90 family including chaperones known to maintain the activity of many proteins but suspected to have other essential, unidentified functions. We have found that a fraction of the total Hsp90 pool is localized at the centrosome throughout the cell cycle at different stages of development in *Drosophila* and vertebrates. This association between Hsp90 and the centrosome can be observed in purified centrosomes and after treatment with microtubule depolymerizing drugs, two criteria normally used to define core centrosomal components. Disruption of Hsp90 function by mutations in the *Drosophila* gene or treatment of mammalian cells with the Hsp90 inhibitor geldanamycin, results in abnormal centrosome separation and maturation, aberrant spindles and impaired chromosome segregation. This suggests that another role of Hsp90 might be to ensure proper centrosome function.

Keywords: centrosome/*Drosophila*/*Homo sapiens*/Hsp90/MALDI

Introduction

The centrosome is the main microtubule organizing center (MTOC) in most animal cells (Kellogg *et al.*, 1994). Despite its major role in a large number of essential processes, such as cell division and the organization of the cytoskeleton, the molecular characterization of the centrosome is still preliminary (reviewed in Kalt and Schliwa, 1993; Kellogg *et al.*, 1994; Moritz and Alberts, 1999; Zimmerman *et al.*, 1999). In most systems, the few centrosomal components known have been identified through rather indirect approaches. In *Drosophila*, for instance, CP190 was cloned as the antigen recognized by a monoclonal antibody raised against a purified nuclear fraction obtained from *Drosophila* embryos (Whitfield *et al.*, 1988) and, together with CP60 and LK6, through its interaction with microtubules (Kellogg *et al.*, 1989; Kidd and Raff, 1997). Cnn (Li and Kaufman, 1996), Polo

(Llamazares *et al.*, 1991), Aurora (Glover *et al.*, 1995) and Asp (Saunders *et al.*, 1997; do Carmo Avides and Glover, 1999) were originally identified by genetic analysis. Finally, the identification of the two γ -tubulin isoforms, γ TUB23C and γ TUB37C (Zheng *et al.*, 1991; Sunkel *et al.*, 1995; Tavosanis *et al.*, 1997), and PP4 (Helps *et al.*, 1998), was made possible by their high degree of sequence conservation.

The same applies to other model systems whose centrosomal components have been identified by various approaches including molecular biology, biochemistry, immunochemistry and genetics. One exception to this rule is the spindle pole of *Saccharomyces cerevisiae*, the budding yeast counterpart of the centrosome of higher eukaryotes. The components of this organelle have been identified by systematic matrix-assisted laser desorption/ionization (MALDI) peptide mapping of the bands resolved by SDS-PAGE of highly enriched spindle pole preparations (Wigge *et al.*, 1998). We are using a similar approach to identify the polypeptides present in the centrosome of a higher eukaryote. To this end, we are taking advantage of previously developed protocols for the isolation of a centrosome-enriched fraction prepared from *Drosophila* embryos (Moritz *et al.*, 1995), which we use as the starting point to produce highly pure preparations of immunisolated centrosomes. The yield and enrichment of these preparations are such that they allow accurate peptide mass mapping of individual SDS-PAGE bands by MALDI mass spectrometry.

One of the proteins identified following this approach is heat-shock protein 83 (Hsp83; Cutforth and Rubin, 1994; van der Straten *et al.*, 1997), the *Drosophila* member of the Hsp90 family, which includes highly conserved, abundant proteins that are expressed in all eukaryotic cells (reviewed in Parsell and Lindquist, 1993; Pratt, 1997; Pratt and Toft, 1997; Buchner, 1999). The Hsp90 proteins are chaperones known to maintain the activity of a large number of proteins, including members of signal transduction pathways and the cell cycle control machinery, like Raf, steroid hormone receptors and Wee (Cutforth and Rubin, 1994; Nathan and Lindquist, 1995; Nathan *et al.*, 1997; Pratt and Toft, 1997), but suspected to also have other essential, unidentified functions (Nathan *et al.*, 1997; Yue *et al.*, 1999).

To determine more precisely the association of Hsp90 to the centrosome and the possible centrosomal role of this protein, we have carried out a detailed study of this protein in *Drosophila* and in cell lines derived from different species of vertebrates. This study has included immunolocalization by fluorescence and electron microscopy as well as functional assays based on the phenotypic characterization of mutant alleles of this gene in *Drosophila* and of the effects brought about by the Hsp90 inhibitor geldanamycin (Prodromou *et al.*, 1997;

Fig. 1. Immunoadsorption of centrosomes on magnetic beads and protein profile of purified centrosomes. **(A)** Projection of a series of laser scanning confocal microscopy images of centrosomes (yellow) immunoadsorbed to magnetic beads (red). Some beads carry up to four centrosomes. Scale bar, 5 μ m. **(B)** Immunoelectron microscopy image of a thin section through a centrosome (arrow) immunoadsorbed between two magnetic beads. This section was labeled with the monoclonal antibody GTU-88 anti- γ -tubulin, revealed with a secondary 10 nm gold-conjugated antibody. Scale bar, 1.4 μ m. **(C)** Coomassie-stained 10% SDS-PAGE of three stages of the centrosome purification procedure. Left: *Drosophila* embryo homogenate (EH). Center: centrosome-enriched fraction (CEF) from sucrose density gradient. Right: immunopurified centrosome (IPC) preparation. BSA and IgG were added during the purification procedure. MALDI protein sequencing revealed that the major 140 kDa band corresponds to the previously characterized centrosomal protein Centrosomin (Cnn). The position of Hsp90 is also shown. **(D)** Western blot of the different purification stages probed with the anti-CNN antibody for a centrosomal marker and with the anti-DNA polymerase II antibody as a probe for a contaminating protein. The level of Cnn protein in the purified and immunopurified samples increases by several orders of magnitude while the DNA polymerase II in the final IPC sample is not detected.

Stebbins *et al.*, 1997). We have found that a fraction of the total Hsp90 pool is a core centrosomal component in *Drosophila* as well as in vertebrates. We have also found that disruption of Hsp90 results in abnormal centrosome separation and maturation, aberrant cell division spindles and impaired chromosome segregation in *Drosophila* and mammalian cells. From these observations we conclude that one of the previously unknown roles of Hsp90 is to ensure proper centrosome function.

Results

*Hsp90 is an abundant component of a subcellular fraction highly enriched in immunopurified *Drosophila* centrosomes*

Recent developments in mass spectrometry allow the identification of individual proteins from polyacrylamide gels with high sensitivity and throughput (Shevchenko *et al.*, 1996). Based on this approach, we are carrying out a systematic study of *Drosophila* centrosomal components using immunopurified centrosome (IPC) fractions prepared by immunoadsorption of centrosomes to magnetic beads. Figure 1A shows a confocal view of a sample of IPC where a clump of magnetic beads (red) can be seen carrying immunoadsorbed centrosomes (yellow) isolated

with an anti- γ -tubulin antibody. Immunoelectron microscopy of the IPC fraction with antibodies against several centrosomal markers (Figure 1B) confirms that these particles are centrosomes. It also reveals the absence of non-centrosomal particles, which are frequent in the centrosome-enriched cell fraction (CEF; Moritz *et al.*, 1995) used as the starting material for our purification procedure. SDS-PAGE analysis of the IPC fraction shows a complex protein pattern with ~20 major bands visible in Coomassie-stained gels (Figure 1C) and ~50 distinct bands in silver-stained gels (not shown). This protein profile is considerably different from the SDS-PAGE pattern of the total embryo homogenate (EH) and the intermediate CEF (Figure 1C). Western blot analysis of these fractions with several antibodies raised against well characterized centrosomal and non-centrosomal proteins shows that the IPC fraction is highly enriched in centrosomal components. One of these markers is the centrosomal protein Centrosomin (Cnn; Li and Kaufman, 1996), whose concentration is enriched several orders of magnitude in IPC (Figure 1D). The enrichment of Cnn in this fraction is such that, apart from the light and heavy immunoglobulin chains and bovine serum albumin (BSA), which were added during the purification procedure, it becomes the major band revealed by Coomassie staining,

as confirmed by nano-electrospray tandem mass spectrometry. Several non-centrosomal proteins, like DNA polymerase II, drop down to undetectable levels in the IPC fraction (Figure 1D), although the highly abundant yolk proteins can still be detected by Western blot analysis. A detailed report of these results will be published elsewhere. The second major band that we have identified by mass spectrometry is Hsp83, the *Drosophila* member of the highly conserved Hsp90 family (Parsell and Lindquist, 1993).

Hsp90 is a core centrosomal component in *Drosophila* and vertebrate cell lines

Hsp90 is an abundant protein that in some cells may account for up to 2% of the total protein content (Pratt, 1997). Therefore, finding it in any subcellular fraction does not necessarily prove specific association to a subcellular structure, regardless of the purity of the fraction. To confirm the localization of Hsp90 to the centrosomes we carried out immunofluorescence and immunoelectron microscopy on purified centrosomes and found that Hsp90 is distinctively associated with the centrosomes and is an abundant component of the pericentriolar material (not shown). We also studied the subcellular localization of Hsp90 in whole-mount *Drosophila* embryos by confocal microscopy. We found a significant fraction of Hsp90 distinctively associated to the centrosome before (Figure 2A and B) and after (not shown) cellularization. To determine whether Hsp90 remained in the centrosome beyond embryonic stages we studied mature *Drosophila* testes. We found Hsp90 in the centrosomes throughout spermatogenesis, including the mitotic gonial cells (not shown) and meiotic spermatocytes (Figure 2C–E). In these cells, the centrosomal staining of Hsp90 is clearly distinguishable from the bulk cytoplasmic staining. After meiosis, Hsp90 was also shown to co-localize with the basal bodies of elongating spermatids where a diffuse staining was also visible along the sperm tails (Figure 2F–H). These observations were confirmed by co-localization experiments using γ -tubulin as a centrosomal marker (not shown). The striking and consistent localization of Hsp90 to the centrosome throughout the cell cycle and in different developmental stages identifies Hsp90 as a core centrosomal protein in *Drosophila* throughout development. Moreover, the fact that Hsp90 remains associated to the centrosome throughout the centrosome purification procedure suggests a strong non-microtubule-dependent association with this organelle during embryogenesis.

The published data regarding the subcellular location of Hsp90 in mammalian cell lines are rather contradictory (Pratt and Toft, 1997; Yue *et al.*, 1999). Prompted by our finding of the centrosomal localization of Hsp90 in *Drosophila* and its high evolutionary conservation, we decided to re-examine the subcellular distribution of this protein in vertebrate cells. We performed double-label immunofluorescence microscopy with anti- α -tubulin antibodies and three different anti-Hsp90 antibodies: monoclonal antibodies 16F1 (Lai *et al.*, 1984) and AC88 (Riehl *et al.*, 1985) and polyclonal antibody 771 (Perdew *et al.*, 1993). Five different cell lines were studied, including CES, a primary cell line from chicken (Figure 3A–C), NIH 3T3 (Figure 3D–F), HeLa cells, the mouse embryo fibroblast cell line EFWT and hippocampal neurons

obtained from rat embryos (not shown). In all these cases, a fraction of Hsp90 displayed a clear centrosomal localization throughout the cell cycle. These observations were confirmed by co-localization experiments using γ -tubulin as a centrosomal marker (not shown). The centrosomal localization of Hsp90 was more prominent when the cytoplasmic fraction of this protein had been partially extracted prior to fixation, but could also be observed without extraction. We have not determined the isoform specificity of the centrosome-bound Hsp90 in vertebrate cells. As in purified *Drosophila* centrosomes, the centrosomal localization of Hsp90 in vertebrate cells is maintained after extraction with non-ionic detergents and microtubule depolymerization (Figure 3G–I). Based on these observations, we conclude that Hsp90 is a core centrosomal component in *Drosophila* and vertebrate cells.

Inactivation of Hsp90 disrupts the centrosome cycle and results in abnormal mitotic spindles and aberrant chromosome segregation in *Drosophila*

The centrosomal localization of a protein does not necessarily reflect a centrosomal role (Kalt and Schliwa, 1993). To determine whether Hsp90 performs a centrosomal function we followed a combined genetic and pharmacological approach that included the cytological characterization of the effects brought about by mutation of the Hsp90 *Drosophila* gene (Hsp83) and by treatment of *Drosophila* and mammalian tissue culture cells with geldanamycin. Geldanamycin is a specific competitive inhibitor of Hsp90 that docks to its ATP-binding site (Prodromou *et al.*, 1997; Stebbins *et al.*, 1997). The ATP-binding site of Hsp90 is highly conserved from the bacterial to the human Hsp90 and is important in regulating Hsp90 function (Panaretou *et al.*, 1998).

For the genetic analysis, we focused our attention on hsp83⁵⁸²/hsp83^{9J1} and hsp83⁵⁸²/hsp83^{13F3} individuals, two trans-heterozygous combinations of Hsp90 mutant alleles (van der Straten *et al.*, 1997) that allow for larval and early pupae development. Together with well arranged, euploid mitotic figures (Figure 4A and B), expected in these leaky mutant combinations, the larval brains of these mutants revealed numerous aneuploid (Figure 4D, G and H) and polyploid cells (Figure 4C and E) as well as disorganized anaphases (Figure 4F). Disorganized anaphases were scored as mitotic figures in which sister chromatids were found to be significantly apart from each other, but were not arranged as two well defined groups. Approximately 20% of the anaphases observed in hsp83⁵⁸²/hsp83^{9J1} mutant individuals fall into this category, which includes <3% in control wild-type larvae. Altogether, aneuploid and polyploid cells account for >30% (120 out of 310) of the mitotic figures found in hsp83⁵⁸²/hsp83^{9J1} mutant individuals. This frequency is more than three orders of magnitude higher than the incidence of the same abnormalities in our control flies, hsp83⁵⁸²/+ and hsp83^{9J1}/+, which we have estimated to be <0.1%, in agreement with previous reports (Gatti and Baker, 1989). Thus, chromosome segregation is severely impaired in Hsp90 mutant individuals, even in cases like these two allelic combinations that are not completely deficient for Hsp90 function.

To gain further insight into the mitotic phenotypes caused by loss of Hsp90 function, we studied the

Fig. 2. Subcellular localization of Hsp90 in *Drosophila* cells at different stages of development. (A and B) Nuclear divisions in a *Drosophila* syncytial blastoderm embryo. Hsp90 can be observed at each pole of the mitotic figures in (A) and as two dots on top of the interphase nuclei (B). Hsp90 staining (green channel) and DAPI staining (blue channel). (C–E) *Drosophila* secondary spermatocytes before the onset of the second meiotic division. These cells are going through the short intervening phase between the first and second meiotic divisions and their centrosomes, clearly visible by the Hsp90 staining, are starting to segregate. The individual panels show α -tubulin staining (C), Hsp90 staining (D) and the superimposed images in (E) including DAPI staining in blue. (F–H) *Drosophila* elongating spermatids. The localization of Hsp90 is very distinct in the basal body area of the sperm head (arrows). (F) α -tubulin staining, (G) Hsp90 staining, (H) superimposed images and DAPI staining in blue. Scale bar in all panels, 5 μ m.

behavior of centrosomes and chromosomes in whole-mount preparations of *hsp83⁵⁸²/hsp83^{9J1}* mutant brains as well as in wild-type brains treated with the Hsp90 inhibitor geldanamycin (Figure 5). Whole-mount preparations preserve the three-dimensional structure of the tissue and prevent most of the artefacts caused by squashing. In agreement with what we had observed in squashed preparations, around half of the mitotic cells observed in brains carrying the *hsp83⁵⁸²/hsp83^{9J1}* hypomorph mutant combination were indistinguishable from the wild type.

The remaining half displayed mutant phenotypes that affected chromosome content and arrangement, as well as the centrosomes. Although detailed karyotyping is not possible in these preparations, cells with a DNA content higher than 2N could be observed in *hsp83⁵⁸²/hsp83^{9J1}* mutant brains. We also found disorganized figures that contained condensed chromatin which was randomly dispersed and was not arranged as expected for any of the normal stages of mitosis. More importantly, the centrosome cycle was also found to be severely impaired

Fig. 3. Subcellular localization of Hsp90 in vertebrate cells. Hsp90 localizes in chicken embryo fibroblast cells (A–C) and in mouse NIH 3T3 fibroblasts (D–F) to the MTOC (arrow) and the cytoplasm as revealed by double staining with an anti- α -tubulin antibody (A and D). The localization of Hsp90 is independent of the presence of microtubules as shown by treating NIH 3T3 cells for 1 h with 25 μ M nocodazole. After this treatment, Hsp90 (H) still co-localizes with the centrosomes [(G), arrow head] as can be seen in (I). (A, D and G) α -tubulin staining with the DM1A antibody; (B, E and H) Hsp90 staining with the 16F1 antibody; (C, F and I) superimposed images. Scale bar in all panels, 20 μ m.

in these cells. The abnormalities observed fall into two categories. The first includes mitotic cells containing a single MTOC made of one or two unsegregated centrosomes, leading to the organization of monopolar mitotic figures (Figure 5G–L). The second group includes cells in which the centrosomes fail to be properly assembled, as revealed by the presence of dispersed pericentriolar material (PCM) (Figure 5D–F and M–O) and, in some extreme cases, by the failure of the mitotic PCM marker CP190 to shuttle from the chromatin into the mitotic centrosome (Figure 5D–F).

Inactivation of Hsp90 results in abnormal centrosome maturation and dispersion of the pericentriolar material in human cells

All the mutant phenotypes described before, with the exception of polyploid figures, are phenocopied by treating wild-type brains with geldanamycin, suggesting that Hsp90 is the main target inactivated by this compound and providing additional evidence substantiating its specificity. The absence of polyploid cells in geldanamycin-treated brains is not surprising since the conditions of treatment that were used do not allow more than a single cell cycle to occur. To determine the effect of inhibiting Hsp90 in mammalian cells, we examined non-synchronized cultures of HEK293 and HeLa cells that were treated with geldanamycin for a series of time points from 0 to 30 h (0.25, 0.5, 1, 5, 10, 15, 20, 30 and 36 h). Geldanamycin treatment did not result in any noticeable effect either on the microtubule cytoskeleton or on the morphology and number of centrosomes in interphase cells, as judged by

immunofluorescence microscopy with antibodies against α - and γ -tubulin. However, aberrant mitotic spindles were detected from as early as 10 h of treatment and their frequency increased steadily at least until 36 h after treatment, the longest incubation time that we have applied. Approximately 20% (40/220) of the mitotic cells produced after 15 h treatment, and 70% (247/350) after 36 h, contained aberrant mitotic figures in which the centrosomes, the spindles and the distribution of chromatin were severely disrupted (Figure 6). Typically, the chromosomes were seen condensed and aligned in a metaphase plate and no anaphases were observed, suggesting a block in the metaphase–anaphase transition. Approximately 85% of these figures (205 out of 247) contained misaligned chromosomes, which often were located around the centrosomes, at the opposite side of the metaphase plate (Figure 6F, I, K and L). The frequency of cases that show one or two misaligned chromosomes in control cells is extremely low, ~2% (7 out of 300), and these are always found between the metaphase plate and one of the centrosomes, as expected for chromosomes that have not yet congressed into the metaphase plate. The spindles of geldanamycin-treated cells were rather asymmetric, with unequal amounts of microtubules in each hemi-spindle and spindle poles often detached from the aster microtubules, which were also abnormal in number and distribution. This abnormal distribution and arrangement of chromosomes and microtubules correlates with the presence of severely disrupted centrosomes. In most cases, the two centrosomes of a cell were of very different sizes and had an abnormal shape as revealed by immunostaining with

Fig. 4. Mitotic figures in the brain of Hsp90 mutant third instar larvae carrying the *hsp83⁵⁸²/hsp83⁹¹¹* heteroallelic combination. The DNA was stained with DAPI. (A and B) Well arranged euploid figures indistinguishable from those found in the wild type. (C) Triploid cell, each arrow points at the three copies of each chromosome. (D) Aneuploid cell, the groups of four arrows point at the four copies of the X and fourth chromosomes. (E) Tetraploid cell. The chromosomes are arranged in a circle and the four copies of the fourth chromosome are clearly visible in the center. (F and G) Examples of disorganized anaphases. One of the poles in (G) contains three fourth chromosome chromatids (arrows) while the other pole contains only one (arrow head). (H) Aneuploid anaphase with three fourth chromosome chromatids in each pole (arrows). Scale bar, 5 μ m.

antibodies against γ -tubulin (Figure 6D–I). In other cells the centrosomal material was highly fragmented so that several pieces could be identified near the spindle poles by immunofluorescence with antibodies raised against different PCM components including γ -tubulin and pericentrin (Figure 6H and J–L). Hsp90 can still be detected in the centrosomes of geldanamycin-treated cells (data not shown).

To determine more precisely the nature of the centrosome fragments produced by the geldanamycin treatment, we decided to study whether they were associated with centrioles and to determine whether they were capable of polymerizing microtubules. Immunostaining of control cells with anti-centrin antibodies revealed two pairs of centrioles, each within one of the two centrosomes that

could be observed by immunofluorescence with antibodies against PCM material (Figure 7A–C). A similar situation was found in geldanamycin-treated cells, most of which contained two pairs of centrioles regardless of the extent of centrosome fragmentation, so that the extranumerary pieces of PCM did not contain centrioles (arrow in Figure 7E). To determine whether these centriole-free fragments correspond to functional PCM we carried out a microtubule nucleation assay. To this end, we depolymerized the microtubules of HeLa cells that had previously been treated for 24 h with geldanamycin, and then allowed the microtubules to re-grow for 2–8 min. In mitotic control cells, most of the microtubule re-growth was observed to occur around the two centrosomes (Figure 7G–I). In contrast, microtubule re-growth in mitotic geldanamycin-treated cells took place in multiple foci that were associated to centriole-containing centrosomes and centriole-free PCM fragments of different sizes (Figure 7J–L), suggesting that the PCM fragments produced by inhibition of Hsp90 are competent for microtubule nucleation. From these observations we conclude that one of the functions of this protein is required to maintain centrosome integrity. The presence of two centriole pairs in most geldanamycin-treated cells suggests that centriole replication as such is not affected by the loss of function of Hsp90.

Discussion

Two main conclusions can be drawn from this work: first, that a fraction of Hsp90 is located in the centrosome; and secondly, that Hsp90 is required for centrosome function. The published data regarding the subcellular location of Hsp90 in mammalian cell lines are rather contradictory, and a centrosomal localization of Hsp90 had not been reported previously. Hsp90 has been reported to be cytoplasmic (Yue *et al.*, 1999), cytoplasmic and nuclear (Perdew *et al.*, 1993; Biggiogera *et al.*, 1996) and associated with the cytoskeleton (Czar *et al.*, 1996) or more specifically with microtubules (Redmond *et al.*, 1989). One of the reasons for disagreement may be the reported sensitivity of Hsp90 to different fixation protocols and the extreme conditions that have been used in some of these reports (Czar *et al.*, 1996; Pratt and Toft, 1997). Recently, Hsp90 has been found in centrosome-enriched fractions, similar to the CEF that we use as the starting material for the immunoisolation of centrosomes (Bornens and Moudjou, 1999; Wigley *et al.*, 1999). Nevertheless, as stated by Bornens and Moudjou (1999), given the abundance of Hsp90, its presence in these fractions could not be taken as evidence for its centrosomal localization. We have produced this evidence by immunofluorescence and immunoelectron microscopy observation of the purified centrosome fractions, which show that Hsp90 is a component of the pericentriolar material. This conclusion is further substantiated by our results regarding the subcellular localization of Hsp90 in *Drosophila* and vertebrate cell lines, which show that a fraction of Hsp90 is associated with the centrosome, in a microtubule-independent manner, at different stages of the centrosome cycle during *Drosophila* development and in vertebrate cells. Taken together, these results identify Hsp90 as a conserved, core centrosomal component in higher eukaryotes. We have not quantified the fraction of centrosome-resident Hsp90,

Fig. 5. Centrosomes and chromosomes in whole-mount brain preparations of Hsp90 mutant and geldanamycin-treated wild-type larvae. (A–C) Dividing neuroblasts from control larvae. (D–I) Aberrant mitotic figures induced by geldanamycin in wild-type brains. (J–O) Abnormal mitotic figures observed in *hsp83⁵⁸²/hsp83⁹¹¹* mutant larvae. (A–M) Lane 1, centrosomes revealed by staining with the Rb188 antibody. (B–K) Lane 2, DNA stained with Toto-3. (N) Mitotic chromatin revealed by an antibody against phosphorylated histone. (C–O) Lane 3, merged view of centrosomes and DNA. Chromatin is abnormally arranged and condensed in the neuroblast shown in (D–F). The centrosomal material is also abnormally fragmented and a major fraction of it is still associated with the chromatin (arrowhead). (G–L) Two neuroblasts showing a monopolar mitotic figure containing a single centrosome (arrows). The neuroblast in (J)–(L) is polyploid. (M–O) Polyploid cell containing two centrosomes and an additional centrosome-like structure (arrow). Scale bar in all panels, 5 μ m.

which may be significantly different depending on the species, cell line and developmental stage. Nevertheless, comparing the signal observed in immunofluorescence experiments between extracted and non-extracted cells, we would expect only a minor fraction of the total Hsp90 pool to be centrosomal.

We have also shown that Hsp90 is required for proper

centrosome function. The centrosomal defects observed in loss-of-function conditions for Hsp90 include dispersion of the PCM, failure of some mitotic PCM markers to be recruited to mitotic centrosomes and single MTOCs in mitotic cells. Concomitantly, we have observed that the loss of Hsp90 also results in defects in microtubule organization and chromosome segregation. During the

Fig. 6. The effect of the Hsp90 inhibitor geldanamycin in human cell lines. Projections of a series of confocal laser scanning microscopy images taken through a control HeLa cell (A–C) and HeLa cells that had been incubated in medium containing 1 µg/ml geldanamycin for 30 h (D–L). The centrosomes (blue) were labeled with the anti- γ -tubulin antibody GTU88 (B, E and H) or anti-pericentrin antibody (J–L). Microtubules (green) were visualized with anti- α -tubulin antibody DM1A (A, D and G). DNA (red) was revealed with DAPI (C, F, I–L). (D–I) Two cases of highly asymmetric mitotic spindle containing mislocalized chromosomes (arrow heads) and a single major centrosome decorated with multiple microtubules that grow in opposite direction to the metaphase plate. At the other spindle pole there is a very weak signal of centrosomal material that can barely be detected (arrow). Fragmented centrosome material can be observed near the large centrosome in (H) (double arrow). (J–L) Three examples of geldanamycin-treated cells containing fragmented centrosomal material and mislocalized chromosomes. Scale bar, 8.5 µm. Flow cytometric analysis of HeLa control (M) and HeLa cells that have been treated for 32 h with geldanamycin (N). Note the increase of the G₂ peak in the drug-treated sample.

course of this work, it has been reported that a reduction in Hsp90 function affects microtubules during spermatogenesis in *Drosophila* as well as in yeast cells (Yue *et al.*, 1999). These authors also showed that these effects were

unlikely to be due to a direct interaction between Hsp90 and microtubules. Our observations suggest that these effects are due, at least partially, to abnormal centrosome function. The fact that mitotic centrosomes, which grow

Fig. 7. Centriole localization and microtubule nucleation assay in geldanamycin-treated cells. Control (A–C) and geldanamycin-treated (D–F) HeLa cells double-labeled with the anti- γ -tubulin antibody GTU88 (B and E) and the anti-centrin antibody 20H5 (A and D). A merged view of both cells containing DNA staining with DAPI (red) is shown in (C) and (F). Centrioles are found within each centrosome, but are absent from the centrosomal fragment revealed by the anti- γ -tubulin antibody in (E) (arrow). Microtubule repolymerization assay in control (G–I) and geldanamycin-treated (J–L) cells. Cells were treated for 1 h with 2.5 μ M nocodazole at 37°C and then microtubule re-growth was permitted for 2 min by removing the drug. Cells were double-labeled with the anti- γ -tubulin antibody GTU88 (G and J) and with the anti- α -tubulin antibody N356 (H and K). Control cells displayed a uniform microtubule re-growth from the two centrosomes while the geldanamycin-treated cells (J–L) displayed multiple unequal shaped asters organized around the different γ -tubulin-positive spots present in these cells (J, arrows). Scale bar for all panels, 5 μ m.

significantly at the onset of mitosis, are much more sensitive to the loss of Hsp90 function than interphase centrosomes strongly suggests that the process of centrosome maturation (Lane and Nigg, 1996) may be particularly dependent upon Hsp90. The presence of single MTOCs in mitotic cells with reduced Hsp90 function suggests that it may also be required for centrosome duplication and/or segregation. Interestingly, Hsp82, the yeast homologue of Hsp90, has been shown to play a role in spindle pole body duplication in *Saccharomyces*

cerevisiae (Zarrov *et al.*, 1997). Yeast mutant for the heat-shock transcription factor hsf1-82, which are specifically defective in the expression of the Hsc82 and Hsp82 proteins, contain a single spindle pole body that is unable to duplicate.

Although it seems reasonable that the recruitment of Hsp90 to the centrosome may have some functional relevance, we do not know whether the centrosomal function of Hsp90 is specifically carried out by the centrosome-resident fraction. If the centrosomal role of

Hsp90 is similar to other functions that have been described for this protein, it will probably work by modulating protein structure, thus facilitating interactions and increasing the half-life of one or more centrosomal proteins. Thus, a plausible hypothesis is that the centrosome-resident fraction of Hsp90 interacts with centrosomal proteins that require this interaction for their function. It is also possible that this role is played by the non-centrosomal fraction of Hsp90 through its interaction with centrosomal proteins before they are targeted to the centrosome, or even with non-centrosomal proteins that are required for centrosome function. This centrosome chaperoning activity could be mediated by either Hsp90 on its own, or by an Hsp90-containing chaperoning complex. Four such complexes have been described to date: the Hop and p23 complexes, which are associated with the maturation of steroid hormone receptor, the cdc37 complex that functions in the folding of protein kinases, and the CNS1 complex (reviewed in Caplan, 1999). Indeed, Hsp70, which is a member of two of these complexes, is known to have a centrosomal localization in higher eukaryotes (Perret *et al.*, 1995; Brown *et al.*, 1996). The number of proteins that are known to require Hsp90 for their function is rapidly growing. Recent studies have expanded the original family of Hsp90 substrates—steroid hormone receptors and protein kinases—to include other kinds of proteins like nitric oxide synthase (Garcia-Cardena *et al.*, 1998) and telomerase (Holt *et al.*, 1999). It will now be interesting to identify the centrosomal partners of this essential chaperone.

Materials and methods

Centrosome isolation

Centrosomes were purified from a centrosome-enriched fraction prepared as described previously (Moritz *et al.*, 1995). This fraction was diluted in phosphate-buffered saline (PBS; supplemented with BSA, proteinase inhibitors and DNase) and incubated with the anti- γ -tubulin antibody Rb1011 (Tavosanis *et al.*, 1997) for 1 h. Then the sample was overlaid onto a step gradient of 70, 55 and 35% sucrose (w/v) in buffer 1 (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$ pH 6.8) and centrifuged (1.5 h at 130 000 *g* at 4°C) to remove excess antibody. Centrosome-containing fractions were mixed with PBS (containing BSA, proteinase inhibitors and DNase). Then samples were incubated with magnetic beads (Dyna) carrying anti-rabbit immunoglobins for 1 h. Beads carrying centrosomes were recovered with a magnetic collector and washed with PBS containing 0.1% BSA and 0.5% Triton X-100 and finally with PBS containing 0.5% Triton X-100. The pre-immune serum of the Rb1011 rabbit was used as control for the specificity of this isolation protocol.

MALDI mass spectrometry and nano-electrospray tandem mass spectrometry

The bands of interest were excised from SDS-PAGE gels and in-gel digested with trypsin. A 0.3 μ l aliquot of the supernatant was analyzed by high mass accuracy peptide mass mapping on a Bruker REFLEX MALDI time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) using the fast evaporation technique for matrix preparation (Jensen *et al.*, 1997). A non-redundant database containing >300 000 entries was searched using the Peptide Search algorithm. For P140 the peptide mixture was extracted, desalted on a Poros R2 column and eluted directly into a nano-electrospray needle (Wilm *et al.*, 1996). Tandem mass spectrometry experiments were carried on a triple quadrupole mass spectrometer (API III; PE-Sciex, Ontario, Canada).

Cytology

Immunofluorescence microscopy on isolated centrosomes was performed as described previously (Lange and Gull, 1995). Centrosomes on magnetic beads were fixed in methanol (−20°C) and processed in solution for immunofluorescence microscopy. Beads were collected in a magnetic

collector (Dyna) between all washing and incubation steps. Immunofluorescence on *Drosophila* early embryos and of brain squashes were performed as described previously (Gonzalez and Glover, 1993). Immunofluorescence on *Drosophila* testes was carried out following the protocol described previously (Pisano *et al.*, 1993). Electron microscopy on isolated centrosomes was performed as described by Lange and Gull (1995).

Four different anti-Hsp90 antibodies were used in this work: 771, AC88, 1R2D12p90 and 16F1. In all experiments shown in this paper, the 16F1 antibody was used. The two anti-Hsp90 monoclonal antibodies (AC88 and 16F1) cross-react with *Drosophila* and can reproduce these observations. These anti-Hsp90 antibodies recognize a single band in Western blotting experiments with *Drosophila* extracts. Anti-Hsp90 antibodies were obtained from Biomol (Hamburg, Germany), the anti- γ -tubulin GTU-88 and anti- α -tubulin DM1A antibodies were obtained from Sigma. The anti- α -tubulin antibody N356 was obtained from Amersham. The anti-pericentrin antibody was from Babco (Berkeley, CA). The following antibodies were kindly provided: anti-DNA polymerase II antibody, by P.Becker; anti-Cnn antibody, by T.Kaufman (Li and Kaufman, 1996); anti-CP190 antibody, by W.Whitfield (Whitfield *et al.*, 1988); and anti-centrin antibody, by J.Salisbury (Salisbury *et al.*, 1988).

Microtubule nucleation assay

HeLa cells were treated for 24 h with geldanamycin, followed by an incubation in medium containing 25 μ M nocodazole for 1 h at 37°C and a further incubation in nocodazole-free medium to permit microtubule re-growth. Samples were obtained at different timepoints between 2 and 8 min after the removal of nocodazole.

Flow cytometry

DNA content of mammalian cells was measured after propidium iodide staining using standard methods (Robinson *et al.*, 1999) with a FACScan flow cytometer from Becton-Dickinson. Excitation was at 488 nm and fluorescence emitted was collected using a 585 nm/26 bandpass filter.

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