Requirement of Hsp90 for centrosomal function reflects its regulation of Polo kinase stability

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We have previously shown that the molecular chaperone heat shock protein 90 (Hsp90) is required to ensure proper centrosome function in *Drosophila* and vertebrate cells. This observation led to the hypothesis that this chaperone could be required for the stability of one or more centrosomal proteins. We have found that one of these is Polo, a protein kinase known to regulate several aspects of cell division including centrosome maturation and function. Inhibition of Hsp90 results in the inactivation of Polo kinase activity. It also leads to a loss in the ability of cytoplasmic extracts to complement the failure of salt-stripped preparations of centrosomes to nucleate microtubules. This effect can be rescued upon addition of active recombinant Polo. We also show that Polo and Hsp90 are part of a complex and conclude that stabilization of Polo is one of the mechanisms by which Hsp90 contributes to the maintenance of functional centrosomes.
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Introduction

We have previously identified heat shock protein 83 (Hsp83; Cutforth and Rubin, 1994; van der Straten *et al.*, 1997) as a centrosomal component in *Drosophila* and vertebrate cell lines (Lange *et al.*, 2000). Hsp83 is 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). Hsp90 is a chaperone known to maintain the activity of a large number of proteins, including members of signal transduction pathways, and cell cycle regulatory proteins such as Raf, steroid hormone receptors and Wee1 (Aligue *et al.*, 1994; Cutforth and Rubin, 1994; Nathan and Lindquist, 1995; Nathan *et al.*, 1997; Pratt and Toft, 1997). A fraction of the total pool of Hsp90 is localized in the centrosome throughout the cell cycle at different stages of development in *Drosophila*. The centrosomal localization of Hsp90 does not depend on microtubules. Moreover, disruption of Hsp90 function, either by mutations or by

treatment with the Hsp90 inhibitor geldanamycin, results in abnormal centrosome segregation and maturation, aberrant cell division spindles and impaired chromosome segregation. Therefore, Hsp90 behaves as a core centrosomal component and is required to ensure proper centrosome function (Lange *et al.*, 2000).

Hsp90 is known to modulate protein structure, thus increasing the half-life of proteins and facilitating their interactions (Nathan and Lindquist, 1995; Pratt, 1997; Buchner, 1999). Inhibition of Hsp90 by geldanamycin has been shown to decrease the half-life of Hsp90-interacting proteins like Src, Raf and steroid receptor (Coke and Litwack, 1978; Moudgil and John, 1980; Barnett *et al.*, 1983; Holbrook *et al.*, 1983). Geldanamycin is a specific competitive inhibitor of Hsp90 that docks to its highly conserved ATP binding site, which is important in regulating Hsp90 function (Prodromou *et al.*, 1997; Stebbins *et al.*, 1997; Panaretou *et al.*, 1998). Geldanamycin-bound Hsp90 cannot form heterocomplexes and this results in destabilization of the proteins that require Hsp90. Consequently, it has been proposed that this property of geldanamycin can be used to identify proteins that interact with Hsp90 and that might be inactivated or downregulated following treatment of live cells with this drug (Pratt and Toft, 1997). Such a method has been used successfully to demonstrate Hsp90 association with mutants of p53 (Puca *et al.*, 1972) and the reverse transcriptase of hepatitis B virus (Baulieu and Jung, 1972).

We have followed this approach to test the hypothesis that the centrosomal defects produced by inhibition of Hsp90 may be a consequence of the inactivation of a centrosomal protein whose stability requires Hsp90. Among 15 centrosomal proteins studied, we have found that the protein kinase Polo is very sensitive to Hsp90 inhibition. Interestingly, the reported centrosomal phenotypes produced by inhibition of Polo in different systems bear striking similarity to the effects of geldanamycin treatment. We have also found that the ability of cytoplasmic extracts to complement salt-stripped centrosomes is lost following Hsp90 inactivation, but can be rescued by the addition of recombinant Polo. These observations strongly suggest that stabilization of Polo is one of the mechanisms by which Hsp90 contributes to maintaining functional centrosomes.

Results

Hsp90 is required to maintain the levels of Polo

To identify the centrosomal proteins that may require Hsp90 for their function we followed the stability of a panel of proteins, which have been described as structural or regulatory components of centrosomes, in *Drosophila* SL2 cells treated with geldanamycin. Geldanamycin

Fig. 1. Stability of centrosomal proteins following inhibition of Hsp90 and heat shock. Cell extracts obtained from *Drosophila* SL2 cells treated with the Hsp90 inhibitor geldanamycin (A) or heat shock (B) were prepared at different time points, run in SDS-PAGE gels and blotted with antibodies against a panel of centrosomal proteins. Of 15 centrosomal proteins examined, Polo was destabilized following geldanamycin treatment, Hsp90, Hsp70 and Cnn increased slightly and the rest of the proteins did not change. Heat shock, which also leads to an increase in Hsp90, does not have any noticeable effect on the levels of Polo (B). Immunoprecipitated Polo from heat shocked cells maintains kinase activity (C). Heat shock-induced centrosome dispersion can be observed as early as 1 h (D). The centrosomal marker CP190, microtubules and DNA are shown in red, green and blue. The spindle poles are labelled with arrowheads.

treatment of SL2 cells results in aberrant centrosome and mitotic spindle morphology (data not shown) comparable to the effects that it shows upon mammalian cells (Lange *et al.*, 2000). Of 15 centrosomal proteins studied (see Materials and methods), only the Polo protein kinase followed a rapid degradation kinetics. This enzyme exhibited a reduction of its normal protein levels by 50% in 24 h and >90% after 48 h of geldanamycin treatment (Figure 1A). Geldanamycin treatment also results in an increase in the levels of Hsp70, Hsp90 and, to a lesser extent, centrosomin (Cnn). The moderate, but significant upregulation of Cnn is difficult to interpret since we are not aware of other examples of proteins upregulated by treatment with geldanamycin. It could be either a direct consequence of the destabilization of Polo or other proteins that require Hsp90, or an indirect consequence of the dispersion of centrosomal material and its failure to accumulate into organized centrosomes.

The upregulation of Hsp70 and Hsp90 following geldanamycin treatment has been reported previously in mammalian cells (Loo *et al.*, 1998) and has been interpreted as a stress response to the presence of this drug (Zou *et al.*, 1998). Indeed, heat shock also results in an increase in the levels of Hsp70 and Hsp90 and is known to induce the reversible dispersion of the pericentriolar material (Debec *et al.*, 1990). Thus, to rule out a general stress response as the cause of the degradation of Polo in

geldanamycin-treated cells, we followed the effect of heat shock on the levels of the enzyme in total cell extracts (Figure 1B). We found that heat shock does not have any noticeable effect on Polo levels, which remain fairly constant, while Hsp90 levels increase >5-fold. We then immunoprecipitated Polo from these extracts and assayed its kinase activity. We found that the Polo kinase activity is fairly stable even after 9 h of heat shock (Figure 1C), much longer than it takes for centrosomes to get disrupted under these conditions (Figure 1D). Thus, the degradation of Polo in geldanamycin-treated cells does not appear to be a consequence of a general stress response. Moreover, the effect upon centrosome function of heat shock seems not to be mediated by the inactivation of Polo. Interestingly, stress induced by cadmium chloride or hydrogen peroxide treatment, which upregulates most heat shock proteins, neither leads to dispersion of the centrosome nor affects its microtubule nucleation capacity (Debec *et al.*, 1990). Therefore, dispersion of the centrosome is not an automatic consequence of stress.

Hsp90 and Polo are part of a complex

Having shown that maintenance of the levels of Polo requires functional Hsp90, we wished to determine whether these two proteins interact. We first observed that Hsp90 and Polo co-localize in purified *Drosophila* embryo centrosomes that are competent to polymerize

Fig. 2. Polo co-localizes with Hsp90 in purified centrosomes and co-immunoprecipitates with Hsp90. (A) Immunofluorescence showing co-localization of Hsp90 (green) and Polo (blue) in purified *Drosophila* centrosomes that are competent to organize microtubule asters (red). (B) Total homogenate and a fraction immunoprecipitated with an anti-myc antibody (IP α -myc) prepared from control cells (lanes 1 and 3) and cells carrying a myc-tagged version of Hsp90 (lanes 2 and 4). These samples were blotted with antibodies against myc, Hsp90 and Polo. Polo co-immunoprecipitates with overexpressed myc-Hsp90 (lane 4). (C) A fraction immunoprecipitated with an anti-Polo (IP α -Polo) prepared from SL2 cells, and blotted with anti-Hsp90 antibodies. The asterisk corresponds to the heavy chain of immunoglobulin. The IP α -Polo fraction was also assayed for kinase activity. Hsp90 co-immunoprecipitates with the endogenous Polo (lane 6).

microtubules (Figure 2A). We then studied whether these two proteins could be found in the same complex. To this end we overexpressed myc-tagged *Drosophila* Hsp90 in SL2 cells and determined whether Polo could co-immunoprecipitate with Hsp90 (Figure 2B). In total extracts, myc can be detected in cells transfected with the myc-Hsp90 (lane 2), but not in the control (lane 1). Hsp90 and Polo can also be observed in these extracts. In the anti-myc-derived immunoprecipitates, myc and Hsp90 can be observed in the transfected cells (lane 4), but not in the control (lane 3). The two bands revealed with the anti-Hsp90 antibody are due to dimerization of tagged (low mobility band) and endogenous non-tagged Hsp90 molecules. The presence of Polo in lane 4 suggests that Polo co-immunoprecipitated with Hsp90. We also studied the presence of Hsp90 in immunoprecipitates obtained with anti-Polo antibodies from SL2 cells that do not over-express either of these two proteins (Figure 2C) and found that Hsp90 co-immunoprecipitates with Polo (lane 6). These observations suggest that Polo and Hsp90 form part of a complex. The co-immunoprecipitation of Polo and Hsp90 is not disrupted by heat shock (not shown) as expected from the stability of Polo under heat shock conditions.

Inhibition of Hsp90 results in inactivation and proteasome-mediated degradation of Polo

To determine whether the degradation of Polo that follows geldanamycin treatment in SL2 cells is dependent upon the

proteasome, we studied the course of Polo degradation in geldanamycin-treated cells in the presence and absence of the proteasome inhibitor lactacystin. To minimize apoptosis, lactacystin was used at a concentration of 1 μ M, that is one-tenth of its IC₅₀. Even at these relatively low levels, lactacystin is able to significantly retard the rate of Polo degradation. This observation suggests that the degradation of Polo induced by geldanamycin is mediated by the proteasome (Figure 3A). We then decided to determine the kinase activity of the Polo protein that had been prevented from degradation following geldanamycin treatment by inhibiting the proteasome. With this aim we determined the casein kinase activity of the Polo protein immunoprecipitated from cells treated with geldanamycin and lactacystin (Figure 3B). Polo kinase activity is lost as early as 24 h from the start of geldanamycin treatment, even though the levels of Polo protein remain hardly affected. These observations strongly suggest that the eventual degradation of Polo in geldanamycin-treated cells is preceded by the inactivation of the Polo kinase activity.

The ability of cytoplasmic extracts to complement salt-stripped centrosomes is lost following Hsp90 inactivation, but can be rescued by the addition of recombinant Polo

In vitro studies have demonstrated that preparations of centrosomes become unable to nucleate asters of microtubules following treatment with high salt concentrations. This can be restored by the provision of cytoplasmic

Fig. 3. Polo degradation is mediated by the proteasome. **(A)** Inhibition of the proteasome with lactacystin significantly slows down the rate of Polo degradation in geldanamycin-treated cells. **(B)** The kinase activity of Polo drops dramatically as early as 24 h after geldanamycin treatment, even when Polo degradation is retarded by lactacystin.

fractions containing specifically the γ -tubulin ring complex and Asp protein (Moritz *et al.*, 1998; Avides and Glover, 1999). Recent studies indicate that the Asp protein is a substrate of Polo kinase and suggest that Asp has to become phosphorylated to be able to nucleate microtubule organizing centre (MTOC) activity at the centrosome (do Carmo Avides *et al.*, 2001). This would predict that cytoplasm from cells treated to inactivate Hsp90 would be unable to provide such activity. To test this hypothesis we examined the ability of cytoplasmic extracts of cells treated with geldanamycin to complement the loss of MTOC activity of salt-stripped centrosomes (Figure 4A and B). We found that microtubule nucleation is markedly less efficient in extracts derived from cells in which Hsp90 had been inhibited with geldanamycin for 24 h than in control untreated cells, and is essentially absent in extracts derived from cells treated for 48 h. This correlates with the activity and stability of Polo kinase in these extracts and so is consistent with a requirement for Polo to phosphorylate components that are removed from centrosomes by the salt-stripping process.

To establish whether the inactivation of Polo alone could account for the loss of complementing activity in geldanamycin-treated cytoplasmic extracts, we quantified the ability of pure recombinant Polo to rescue this effect. We found that the addition of Polo in the range 1–10 nM, to extracts that had been treated with geldanamycin for 48 h, rescues up to 70% of the control activity (Figure 4B). Therefore, Polo degradation seems to be one of the major causes of the abnormal centrosomes induced by geldana-

mycin treatment. The failure of recombinant Polo to rescue 100% of the control aster formation activity suggests, but does not prove, that other activities may also be involved.

Discussion

We have shown previously that Hsp90 is required for proper centrosome function (Lange *et al.*, 2000). The centrosomal defects observed in loss-of-function conditions for Hsp90 include dispersion of the pericentriolar material (PCM), failure of some mitotic PCM markers to be recruited to mitotic centrosomes, and the presence of only a single MTOC in mitotic cells. Concomitantly, in agreement with previous observations (Yue *et al.*, 1999), we have observed that the loss of Hsp90 also results in defects in microtubule organization and chromosome segregation (Lange *et al.*, 2000). The fact that mitotic centrosomes, which grow 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 this chaperone may also be required for centrosome duplication and/or segregation.

Hsp90 is a molecular chaperone known to interact with proteins to modulate their structure, increase their half-life and facilitate protein–protein interactions. Thus, a plausible hypothesis to interpret the centrosomal defects brought about by Hsp90 inhibition is that Hsp90 may interact with one or more centrosomal proteins that require this interaction for their function. Our current observations indicate that one such protein is Polo kinase. This conclusion is fully consistent with the striking similarity between the phenotypes induced by inhibition of Hsp90 and those that have been reported in cells with reduced Polo function. *Drosophila* Polo (Llamazares *et al.*, 1991) is the founder member of the polo-like kinase (Plk) family, which has homologues in a wide range of organisms (for reviews see Glover *et al.*, 1998; Nigg, 1998). The Plks are found at a number of cellular locations during mitosis, including the spindle pole bodies of yeasts and the centrosomes of metazoans. Females homozygous for *polo*¹, the first mutant allele of this gene isolated in *Drosophila*, give rise to embryos that show abnormal mitotic spindles and in which centrosomal components fail to organize into discrete structures (Sunkel and Glover, 1988; Riparbelli *et al.*, 2000). In both *Drosophila* and human cells, reduction of Plk kinase function results in a failure of centrosome segregation that leads to the formation of monopolar spindles (Llamazares *et al.*, 1991; Lane and Nigg, 1996). A similar phenotype is observed by mutation in *plol*, the fission yeast homologue of *polo* (Ohkura *et al.*, 1995). Moreover, inhibition of human Plk-1 by microinjection of blocking antibodies arrests cells in mitosis and results in small centrosomes unable to undergo the increase in size that precedes the onset of mitosis (Lane and Nigg, 1996). Identical results can be observed in human Hs68 cells treated with geldanamycin (G.de Cárcer, unpublished).

The similarity between the phenotypes produced by the loss of Polo or Hsp90, together with the remarkable

Fig. 4. The complementing activity of cytoplasmic extracts required to restore microtubule polymerization in salt-stripped centrosomes is inhibited by geldanamycin and rescued by Polo. (A) Immunofluorescence showing the ability of SL2 cell extracts to complement KI-extracted centrosomes (ctrl). Treatment with geldanamycin progressively inhibits the complementation activity (24 h, 48 h), but the effect can be reversed by the addition of 10 nM recombinant Polo (48 h + 10 nM Polo). Centrosomes and microtubules were stained with anti- γ -tubulin (green) and anti- α -tubulin (red), respectively. (B) Quantification of the inhibition produced by geldanamycin and the effect of recombinant Polo added to the extracts. Geldanamycin treatment reduces the complementing activity of cell extracts by 30% after 24 h and >90% after 48 h. Increasing concentrations of Polo added to these extracts can rescue up to 70% of the control complementing activity.

destabilization of Polo following the loss of Hsp90 function, strongly suggests that the centrosome alterations produced by inhibition of Hsp90 are due, at least partially, to the inactivation of Polo. This conclusion is strengthened by the ability of Polo to rescue the inhibitory effect of geldanamycin upon the complementation activity of cytoplasmic extracts to restore microtubule nucleation from salt-stripped centrosome cores. Therefore, notwithstanding the possible involvement of other Hsp90 partners that we have not yet identified, we conclude that stabilization of Polo is one of the mechanisms by which Hsp90 contributes to the maintenance of functional centrosomes.

The mitotic centrosomes of *polo¹* have been shown to fail to accumulate the mitosis-specific phospho-epitopes identified in wild-type centrosomes by the MPM2 antibody (Logarinho and Sunkel, 1998). Inhibition of Hsp90 also prevents the accumulation of these mitosis-specific phospho-epitopes (G.de Cárcer, unpublished). One of the MPM2 phospho-epitopes that requires functional Polo for its accumulation is the phosphorylated form of the Asp protein (do Carmo Avides *et al.*, 2001). Asp is a 220 kDa microtubule-associated protein found at the spindle poles from prophase to early telophase (Saunders *et al.*, 1997; Avides and Glover, 1999). Asp is required to restore the microtubule nucleating activity of isolated preparations of

centrosomes. This activity is not provided by the Asp protein supplied by extracts derived from *polo* mutant embryos. Our present finding that the complementing activity of cell extracts is also lost following the destabilization of Polo kinase induced by geldanamycin treatment strongly suggests that phosphorylation of Asp by Polo is required for centrosome function. This hypothesis is consistent with the similarities between the phenotypes of *asp* and *polo* mutants and their synergic interaction (Gonzalez *et al.*, 1998).

The number of proteins that are known to require Hsp90 for their function is growing rapidly. Recent studies have expanded the original family of Hsp90 substrates—steroid hormone receptors and protein kinases—to include other classes of proteins such as nitric oxide synthase (Garcia-Cardena *et al.*, 1998) and telomerase (Holt *et al.*, 1999). Hsp90 has also been shown to form complexes with and be required for the function of weel (Aligue *et al.*, 1994) and cdc2 (Muñoz and Jimenez, 1999), two kinases that play a major role in the control of the cell cycle. In this work we have shown that an additional mechanism by which Hsp90 can facilitate cell cycle regulation is by maintaining the level of Plks. Our work is consistent with that of Simizu and Osada (2000) who showed that mammalian Plk1 interacts with Hsp90, and that the instability of Plk1 in some human tumours and its insensitivity to geldanamycin

may be due to mutations in the C-terminal non-catalytic domain.

Although our work serves to emphasize the role of Plks in regulating the centrosome cycle, we should not overlook the functions of the enzyme at other mitotic stages. We have also found that geldanamycin-treated HeLa cells tend to arrest at metaphase (Lange *et al.*, 2000). In such cells Cdc27, a component of the anaphase-promoting complex suggested by Kotani *et al.* (1998) to be phosphorylated and activated by Plk1, is found in its unphosphorylated form and levels of Plk1 are reduced (G.de Cárcer, unpublished data). This points to a requirement for Hsp90 to stabilize polo kinase to regulate the metaphase–anaphase transition. Hence, Hsp90 seems to serve as a chaperone of this essential mitotic kinase, and perhaps other centrosomal and cell cycle regulatory proteins yet to be identified, throughout mitotic progression.

Materials and methods

Antibodies

The following antibodies were used: mouse monoclonal MA294 anti-Polo (Llamazares *et al.*, 1991); rabbit Rb1011 anti- γ -tubulin (Tavosanis *et al.*, 1997); mouse monoclonal GTU-88 anti- γ -tubulin (Sigma); rabbit anti-Cp60 (Kellogg *et al.*, 1995); rabbit Rb188 anti-Cp190 (Whitfield *et al.*, 1988); rabbit anti-Cnn (Li and Kaufman, 1996); mouse monoclonal N356 anti- α -tubulin (Amersham); rat monoclonal 16F1 anti-Hsp90 α (Stressgen); mouse monoclonal anti-Hsp70 (Stressgen); rabbit anti-LK6 (Kidd and Raff, 1997), anti-centrin (Salisbury *et al.*, 1988); rabbit anti-TCP-1 α (Stressgen); rabbit anti-14-3-3 β (Santa Cruz Technologies); rabbit MA8 anti-D-TACC (Gergely *et al.*, 2000); sheep anti-PP4 (Helps *et al.*, 1998) and rabbit ANGT anti-*Drosophila* Nek2 (C.Gonzalez and C.E.Sunkel, unpublished).

Cell culture and drug treatments

Drosophila Schneider cells (SL2) were maintained at 25°C in Schneider medium (Gibco-BRL) supplemented with 10% fetal bovine serum. Where indicated, 1.78 μ M geldanamycin (Sigma) and 1 or 10 μ M lactacystin (Calbiochem) were added to the culture medium. For heat shock treatment, SL2 cells were shifted to 37°C for the times indicated.

Cell extracts, western blots and immunoprecipitation assays

Cells were harvested from Petri dishes, washed in phosphate-buffered saline (PBS) and resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 0.5 M NaCl, 6 mM EGTA, 6 mM EDTA, 0.1% NP-40) supplemented with 1 μ g/ml of aprotinin, 1 μ g/ml of leupeptin, 1 μ g/ml of pepstatin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM sodium β -glycerophosphate, 1 mM sodium fluoride and 1 mM sodium orthovanadate. Cell lysates were spun down and the proteins in the supernatant were resolved in 10% gradient SDS–polyacrylamide gels and transferred on to nitrocellulose membranes. Blots were incubated in blocking buffer [10% milk in PBS with 0.05% Tween-20 (PBST)] for 1 h and then incubated in PBST containing the primary antibody dilution for 1 h. They were then washed in PBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 45 min. After several washes, blots were developed with ECL chemiluminescence reagent (Amersham).

Drosophila SL2 cells were transfected using the Effectene kit (Qiagen) with the pACT5 expression vector carrying Hsp83–myc under the control of the actin promoter (van der Straten *et al.*, 1997). Cells were lysed 48 h after transfection in lysis buffer. Two hundred micrograms of each extract were incubated with 0.2 μ g of rabbit polyclonal anti-myc (Signal Transduction) for 2 h at 4°C. Protein A–Sepharose beads (Amersham) were then added to the extracts, after which the beads were washed three times with 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% TX-100 and boiled in sample buffer. Finally, samples were run in SDS–PAGE gels.

Purification of centrosomes and complementation assays

Purification of centrosomes from *Drosophila* embryos, immunofluorescence and complementation assays on KI-treated centrosomes were

carried out as described by Moritz *et al.* (1995) and Moritz and Alberts (1999). Complementation extracts were prepared from *Drosophila* SL2 cells treated with geldanamycin for 24 and 48 h. Cells were centrifuged at low speed on a bench-top centrifuge and washed twice in buffer A (50 mM K-HEPES pH 7.6, 100 mM KCl, 1 mM MgCl₂, 1 mM Na₃EGTA, 10% glycerol, 100 μ M nocodazole, 1 mM PMSF; 1:100 protease inhibitor stock). Protease inhibitor stock is 10 mM benzamidine–HCl, 0.1 mg/ml phenanthroline, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin. The pellet was resuspended in cold buffer A (typically 200 μ l of buffer for each ml of culture) and homogenized using a Dounce homogenizer at 4°C. The extract was cleared by centrifugation (twice) at top speed for 30 min in a refrigerated Eppendorf centrifuge. Polo rescue experiments were made, supplementing cell extracts that had been treated with geldanamycin for 48 h with increasing concentrations of His-tagged recombinant Polo obtained from a baculovirus expression system (Qian *et al.*, 1998).

Polo kinase assays

Drosophila SL2 extracts, either Hsp90 inhibited or heat shocked, were prepared as described for centrosome complementation assay. Extracts were immunoprecipitated with Dynabeads (Dyna) coated with the anti-Polo monoclonal antibody MA294 (typically using 200 μ l of extract and 20 μ l of antibody). The beads were extensively washed in buffer A and then resuspended in 50 μ l of kinase buffer (10 mM K-HEPES pH 7.5, 75 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM EGTA). Dephosphorylated casein (1 μ l of a 20 mg/ml stock) was mixed with 10 μ l of the immunoprecipitate, 1 μ l 2 mM ATP and 0.5 μ l [γ -³²P]ATP. Reactions were incubated for 20 min at room temperature and analysed by SDS–PAGE and autoradiography.

Immunofluorescence microscopy

Drosophila SL2 were spun down on to coverslips, fixed in 4% formaldehyde in PBS for 10 min, and permeabilized in cold (–20°C) methanol. They were then incubated with glycine 20 mM/PBS for 10 min, followed by a further incubation with bovine serum albumin 3% in PBS for 20 min. Microtubules and centrosomes were stained with antibodies against α -tubulin (Amersham N356) and Cp190, respectively, and the corresponding fluorescein and Texas-Red-tagged secondary antibodies. Chromosomes were counter-stained with 4'-6'-diamidino-2-phenylindole (2 μ g/ml). The cells were mounted with Mowiol and analysed with a Leica epifluorescence microscope.

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