A constitutive 70 kDa heat-shock protein is localized on the fibres of spindles and asters at metaphase in an ATP-dependent manner: a new chaperone role is proposed

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In the present study, double immunofluorescence and immunoblot analysis have been used to show that centrosomes, isolated from *Paracentrotus lividus* sea urchin embryos at the first mitotic metaphase, contain the constitutive chaperone, heat-shock protein (HSP) 70. More specifically, we demonstrate that centrosomes contain only the HSP70-d isoform, which is one of the four isoforms identified in *P. lividus* . We also provide evidence that p34(cell division control kinase-2) and t complex polypeptide-1 (TCP-1) α, a subunit of the TCP-1 complex, are localized on the centrosomes. Furthermore, inhibition of TCP-1 *in vivo*, via microinjecting an anti-(TCP-1α) antibody into *P. lividus* eggs before fertilization, either impaired mitosis or induced severe malformations in more than 50 % of embryos. In addition, we have isolated the whole mitotic apparatus and shown that HSP70 localizes on the fibres of spindles and asters, and binds them in an ATP-dependent manner. These observations suggest that HSP70 has a chaperone role in assisting the TCP-1 complex in tubulin folding, when localized on centrosomes, and during the assembling and disassembling of the mitotic apparatus, when localized on the fibres of spindles and asters.

Key words: *Paracentrotus lividus* sea urchin, chaperone activity, constitutive HSP70, mitosis.

INTRODUCTION

The large family of 70 kDa heat-shock or stress proteins (HSP) is the most evolutionary conserved and most prominent among all of the stress proteins [1,2]. They are rapidly synthesized when cells are stressed by heat or by a wide range of adverse stress conditions in order to provide cytoprotection against macromolecular damage [3–5].

Constitutive members of this family in non-stressed cells have a role as molecular chaperones [6–9] and have been localized in several cellular compartments, including the centrosomes. In HeLa cells, the constitutive form, HSP73, localizes on the centrosome during mitotic cell division, whereas a portion of the inducible form, HSP72, collects there following heat shock [10]. Other data [11] have confirmed the presence of constitutive HSP73 in centrosomes after heat shock, and it has been suggested that HSP73 facilitates the recovery and reorganization of the centrosomal structure after heat treatment. Constitutive HSP70 has also been described in dinoflagellate centrosomes [12] and in HeLa centrosomes with which the proteasome machinery is dynamically associated [13]. As yet, a specific role of HSP70 in mitosis has not yet been assigned.

Evidence for an essential role of constitutive HSP70 in mitosis can be found in experiments performed by blocking its function, such as experiments that we have described previously [14] using the sea urchin as a model of embryonic development. In *Paracentrotus lividus* sea urchin embryos, inhibiting constitutive HSP70 by microinjecting an anti-(HSP70) antibody into eggs before fertilization impairs mitosis and embryo development. In whole-mount, non-injected embryos, the majority of constitutive HSP70 is clearly detected on the asters of the mitotic apparatus during early cleavage. In some injected embryos, constitutive HSP70 is partially concentrated in an eccentric zone of the zygote; in other cases it is concentrated on and around irregularly positioned asters. In both cases, one of the two asters has a regular shape, whereas the other is incomplete. It is likely that, in *P. lividus* zygotes, the protein localizes on the centrosomal structure, which, in animal cells, is known to be the microtubule-organizing centre [15]. In order to explain more fully the localization of constitutive HSP70 on the mitotic apparatus and to investigate its potential role as a chaperone, we isolated intact spindles with asters and centrosomes from first-metaphase *P. lividus* embryos. Both experiments confirmed the presence of constitutive HSP70 in the isolated structures. We also found that constitutive HSP70 was located on the mitotic apparatus in an ATP-dependent manner, and that only one of the four constitutive HSP70 isoforms in *P. lividus* [16] was contained in isolated centrosomes. Finally, we have shown that p34(cell division control kinase-2) [(cdc2); also known as cyclin-dependent kinase (CDK) 1] and the t-complex protein, t complex polypeptide-1 (TCP-1), co-localized there. We therefore suggest that the HSP70-d isoform has a specific role during the assembling and disassembling of the mitotic spindle, which, during early development in *P. lividus* embryos, occurs every 30 min.

MATERIALS AND METHODS

Embryo culture

P. lividus eggs were fertilized in filtered seawater containing p-aminobenzoic acid (PABA) and then suspended in Ca^{2+}-free artificial seawater at 18 °C with overhead stirring at 25 rev./min The fertilization envelope was mechanically stripped from the embryo 8–10 min after fertilization by passing it through a 75

Abbreviations used: AP, alkaline phosphatase; cdc2, cell division control kinase-2; CDK, cyclin-dependent kinase; HSP, heat-shock protein; PABA, p-aminobenzoic acid; TCP-1, t complex polypeptide-1; TRiC, TCP-1 ring complex.

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mesh Nitex filter cloth. Embryos were collected and suspended at a concentration of approx. 10^4/ml in filtered seawater at 18 °C with overhead stirring at 25 rev./min and cultured until the required developmental stage as previously described [14].

Embryos for protein analysis were collected at the appropriate stage and lysed in 50 mM Tris/HCl (pH 7.4) containing 1 mM EDTA, 150 mM NaCl, 10 % (v/v) glycerol, 1 % (v/v) Nonidet P40, 0.1 mM PMSF and 1 mM dithiothreitol.

Microinjection of eggs

Unfertilized eggs were dejelled, attached on to protamine sulphate-treated Petri dishes and microinjected with 50 pl of PBS containing either a monoclonal anti-(TCP-1α) antibody [raised against TCP-1α, a subunit of the chaperonine TCP-1 ring complex (TRiC)] (StressGen), anti-γ-tubulin antibody (Sigma), anti-mouse IgG antibody (Promega) or 15 mM sodium azide as described previously [14]. The antibody concentrations used in the assay were 2 μg/μl. At least ten batches of 50 eggs were microinjected for each antibody or sodium azide treatment. The eggs were then fertilized and incubated as above.

Centrosomal preparation

After the removal of the fertilization envelope, centrosomes were prepared as described previously [17]. The pellet was resuspended in a solution containing 50 % (v/v) glycerol, 10 mM Pipes, 1 mM $MgSO_4$, 1 mM EGTA and 1 mM PMSF (pH 6.8) and kept at −80 °C.

Double immunofluorescence staining

Centrosomal preparations were spread on to polylysine-treated slides and fixed with methanol. After washing three times with PBS, the slides were incubated for 1 h at 20 °C in a humidified chamber with 50 μl of PBS containing rabbit polyclonal anti-γ-tubulin or mouse monoclonal anti-(HSP70) antibodies at a dilution of 1:5 and 1:50 respectively (Sigma). The slides were then rinsed with PBS and incubated for 30 min with fluorescein-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG (Sigma) diluted in PBS containing 0.1 % BSA. Unbound antibody was removed by washing with PBS followed by distilled water. Slides were mounted in 2 % 1,4 diazabicyclo (2,2,2) octane ('Dabco') in 90 % (v/v) glycerol. Samples were examined using an Olympus BX-50 microscope equipped with a vario Cam B/W camera and elaborated by image-pro/plus 3.0 Media Cybernetics.

Gel electrophoresis

Samples (50 μg of protein) of total lysates from first-metaphase embryos or isolated centrosomes were resolved by one-dimensional SDS/PAGE using 10 % (w/v) or 12 % (w/v) acrylamide gels for 75 min at 100 V as described previously [18]. High-range rainbow molecular-mass markers (Amersham Pharmacia Biotech) were used. Following electrophoresis, proteins were subjected to either Western blotting or Coomassie Blue staining.

For two-dimensional gel electrophoresis, samples of centrosomal preparations or total protein lysates (250 μg) were resolved on a one-dimension gel in 1.6 % pH 5–7 and 0.4 % pH 3–6 ampholytes (Amersham Pharmacia Biotech) and run overnight at 300 V as previously described [19]. For the second dimension, proteins were separated by SDS/PAGE on 10 % (w/v) acrylamide gels for 90 min at 100 V.

Western blotting

Following one- or two-dimensional electrophoresis, proteins were transferred on to an ECL®-Hybond membrane (Amersham Pharmacia-Biotech) as described previously [20]. Membranes were incubated with monoclonal antibodies against HSP70 (1:3000 dilution), γ-tubulin (1:1000 dilution), β-tubulin (1:1000 dilution), the Pro-Ser-Thr-Ala-Ile-Arg (PSTAIR) sequence of p34(cdc2) (1:1000 dilution) (Sigma) and TCP-1α (1:1000 dilution; StressGen). Alkaline phosphatase (AP)-conjugated secondary antibodies were used to detect antigen–antibody complexes.

Isolation of intact mitotic apparatus, ATP or GTP treatment and immunoassay

Eggs were fertilized, the fertilization envelope removed and the embryo allowed to develop until the first metaphase as described above. The mitotic apparatus was then isolated from these embryos as described previously [21]. Aliquots of the isolated mitotic apparatus were subjected to treatment with either ATP or GTP at a final concentration of 0.5 or 20 mM for 15 min at 10 °C. Aliquots of treated and control samples were spread on to polylysine-treated slides and the samples were fixed in PBS containing 2.5 % glutaraldehyde for 30 min. The slides were washed three times with PBS, treated with methanol for 5 s and incubated for 1 h at 20 °C in a humidified chamber with 50 μl of PBS alone or PBS containing monoclonal anti-β-tubulin or anti-(HSP70) antibodies. After incubation, the slides were washed with PBS and an anti-mouse AP-conjugated secondary antibody was added for 1 h. Staining was performed using nitroblue tetrazolium ('NBT'; 75 mg/ml) and 5-bromo-4-chloroindol-3-yl phosphate ('BCIP'; 50 mg/ml).

RESULTS

Isolation and characterization of centrosomes

In order to determine the presence of constitutive HSP70 in the centrosomal structure, we isolated centrosomes from first-metaphase *P. lividus* embryos [17]. In these embryos, the first metaphase occurs 50 min after fertilization and all the embryos are synchronized. Once isolated, the centrosomal structure was identified by the presence of γ-tubulin, which, at metaphase, is typically found there [22,23]. An anti-γ-tubulin antibody, which identifies a 48 kDa protein, the molecular mass of γ-tubulins of other species, was used in immunoblot analysis of isolated centrosomes after separation by SDS/PAGE. As shown in Figure 1(A), a protein band of 48 kDa (lane 1) was found in our preparation of *P. lividus* embryo centrosomes. Coomassie Blue staining of gels after SDS/PAGE showed that a different pattern of proteins was obtained from isolated centrosomes and total zygote embryo lysates. As expected, the total protein lysate contained a complex series of proteins (Figure 1B, lane 1), whereas fewer protein bands were present in the centrosomes; at least some of the protein bands from total lysates were absent in the centrosomal preparation (Figure 1B, lane 2). It has previously been described [13,24] that the protein composition of centrosomes is complex and may change according to its function. Our data have identified centrosomes in the embryo preparation and demonstrated that the well-conserved γ-tubulin was also present in *P. lividus* embryos.

Isolated centrosomes contain a portion of constitutive HSP70

The same preparation of centrosomes was also analysed by Western blotting using an anti-(HSP70) antibody to determine

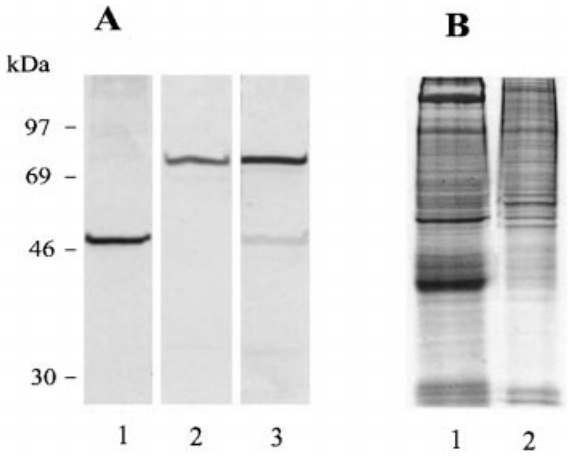

Figure 1 Identification of centrosomes by the presence of γ-tubulin and localization of HSP70

Centrosomes and total lysate were prepared from 50-min embryos and subjected to SDS/PAGE using 10% gels. (**A**) Western-blot analysis was carried out using antisera against either γ-tubulin (lane 1) or HSP70 (lane 2) alone or in combination (lane 3). Lanes 1 and 2, centrosomes (50 μg); lane 3, total lysate (50 μg). (**B**) Coomassie Blue staining. Lane 1, total lysate (50 μg); lane 2, isolated centrosomes (50 μg). Molecular mass-markers (in kDa) are indicated on the left.

the presence of constitutive HSP70. As shown in Figure 1(A) (lane 2), immunoreactivity corresponding to constitutive HSP70 was identified, which, in *P. lividus* embryos, has been found to be a 75 kDa protein [16]. Moreover, double immunoreaction with anti-γ-tubulin and anti-(HSP70) antibodies was carried out on the total lysate of first-metaphase zygote embryos using the same amount of proteins as in the centrosomal preparation (Figure 1A, lane 3). HSP70 expression in the isolated centrosome was weaker than that of the whole embryo lysates; a possible explanation for this is that only a portion of HSP70 was localized on the centrosomes. Indeed, imunohistochemical analysis revealed that, in whole-mount first-metaphase embryos, constitutive HSP70 appeared dispersed in the cytoplasm and only a portion was concentrated on the asters of the mitotic apparatus [14].

Co-localization of γ-tubulin and constitutive HSP70 by double immunofluorescence of centrosomes

To confirm further the presence of both HSP70 and γ-tubulin in isolated centrosomes, we carried out double immunofluorescence experiments and identified both proteins on the isolated centrosomes (Figures 2A and 2B). To determine if the two proteins had the same localization, the images from Figures 2(A) and 2(B) were superimposed. The resulting image (Figure 2C) indicated that HSP70 and γ-tubulin were indeed co-localized, as shown by the yellow staining.

Only one isoform among the constitutive HSP70 isoforms of *P. lividus* is contained on isolated centrosomes

Four constitutive HSP70 isoforms have been identified in *P. lividus* embryos to date, although the function of these isoforms remain to be assigned [16]. In order to investigate if all the isoforms are present in the centrosomes, isolated centrosomes and total lysates from first-metaphase embryos were analysed by two-dimensional PAGE and Western blotting using the anti-(HSP70) antibody. Total embryo lysate (Figure 3A) contained all four HSP70 isoforms, which were previously named a, b, c and d [16], whereas only the d isoform was detected in the isolated centrosomes (Figure 3B). This result suggested that the constitutive HSP70-d isoform may play a specific chaperone role for the proteins located on this structure. It may also help to explain why the constitutive HSP70 band in the centrosomal preparation, shown in Figure 1(A), is weaker than that of total lysate. In addition, the presence of only one constitutive HSP70 isoform also further indicated that the centrosomes had not been contaminated by cytosol.

TCP-1 and p34(cdc2) co-localize on isolated centrosomes from first-metaphase embryos

After identifying one constitutive HSP70 isoform on isolated first-metaphase centrosomes, we proceeded to assess its role in

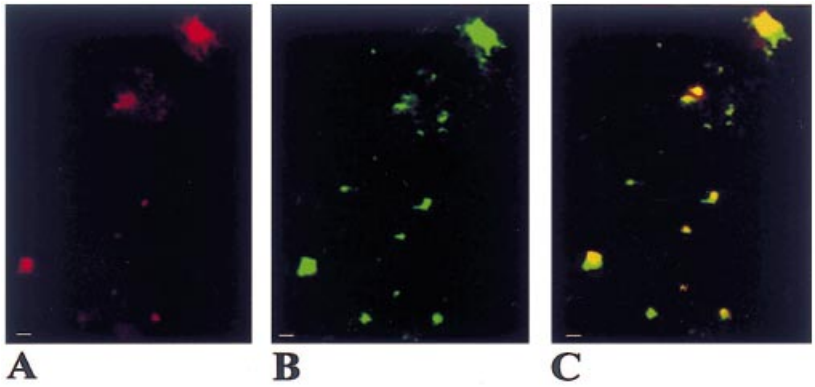

Figure 2 Constitutive HSP70 and γ-tubulin co-localization

Isolated centrosomes were immunostained with antisera raised against γ-tubulin (**A**) and HSP70 (**B**), and fluoresence detected following the addition of rhodamine-conjugated anti-rabbit IgG and fluorescein-conjugated anti-mouse IgG respectively. (**C**) The images from (**A**) and (**B**) were superimposed. Scale bar, 4 μm.

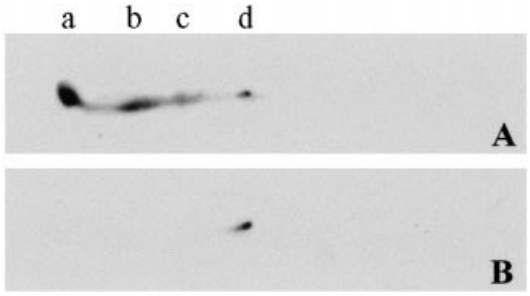

Figure 3 Localization of a specific HSP70 isoform in centrosomes

Total lysate from 50-min embryos (**A**) and isolated centrosomes (**B**) were subjected to two-dimensional gel electrophoresis, and subjected to Western blot-analysis using the anti-(HSP70) antibody. a–d represent the four different HSP70 isoforms found in *P. lividus* embryos.

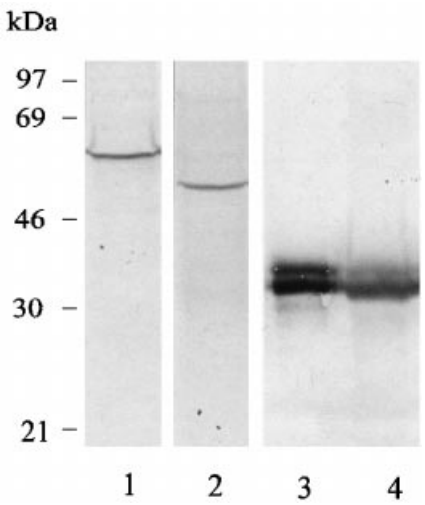

Figure 4 Localization of TCP-1 and p34(cdc2) in centrosomes

Aliquots of isolated centrosomes (lanes 1–3) and total lysate from 50-min embryos (lane 4) were resolved by SDS/PAGE on 10% (lanes 1, 2) or 12% (lanes 3, 4) gels. Proteins were subjected to Western-blot analysis with antibodies against TCP-1α (lane 1), β-tubulin (lane 2) and the Pro-Ser-Thr-Ala-Ile-Arg sequence of p34(cdc2) (lanes 3 and 4). Molecular mass-markers (in kDa) are indicated on the left.

mitosis. To do so, we attempted to determine the presence of the TCP-1 complex and p34(cdc2), which are involved in tubulin folding and in cell cycle progression respectively. Isolated centrosomes were assayed by immunoblot analysis for the presence of β-tubulin and TCP-1α. Two immunoreactive bands were identified; one corresponding to the 60 kDa TCP-1α protein and the other to the 48 kDa β-tubulin protein (Figure 4, lanes 1 and 2 respectively). The localization of TCP-1α on centrosomes suggested the presence of the hetero-oligomeric TCP-1 complex and its possible involvement in α/β- and γ-tubulin folding during the microtubule nucleation, as has been described for other cells [25–28]. To clarify the role of the TCP-1 complex, we performed experiments to block its function by microinjecting the anti-(TCP-1α) antibody into *P. lividus* eggs. After fertilization, we observed that 7–10% of the embryos stopped developing immediately and died and 30–42% of the embryos developed irregularly during early cleavage stages (Figure 5A), whereas the rest of the embryos were normal or only slightly irregular (Figure 5B). Later in development, some of the irregular embryos continued to develop, but their shape remained irregular, whereas others regained an almost normal shape. In contrast, the microinjection of the anti-γ-tubulin antibody totally blocked (100%) development (Figure 5C). Microinjection of an anti-IgG antibody or sodium azide had no effect (Figure 5D). Therefore the results indicate that, in *P. lividus* embryos, the anti-(TCP-1α) antibody is only able to block partially the function of the whole TCP-1 complex *in vivo*, which is known to be a large hetero-oligomeric structure. Therefore our results suggest that HSP70-d in centrosomes, together with the TCP-1 complex, may have a chaperone role in the process of tubulin folding.

To ascertain if HSP70-d was also involved in the cell cycle, we assayed isolated centrosomes for the presence of p34(cdc2) using an antibody which recognizes its conserved Pro-Ser-Thr-Ala-Ile-Arg epitope-domain, common also to CDK2 and CDK3. Three immunoreactive bands were identified with molecular masses of 34, 32 and 31 kDa (Figure 4, lane 3), corresponding to the molecular masses of p34(cdc2), CDK2 and CDK3 respectively. These proteins had different levels of expression in both total embryo lysate and isolated centrosomes (Figure 4, lanes 3 and 4 respectively). Therefore this demonstrated that p34(cdc2), together with CDK2 and CDK3, co-localized with HSP70-d in isolated first-metaphase centrosomes, and that they were present in differing concentrations. Even though p34(cdc2) is highly concentrated on centrosomes at this stage of the cell cycle, we have not found a direct link between HSP70-d and p34(cdc2) in isolated centrosomes. Although it is likely that the reason we failed to co-immunoprecipitate them is because HSP70 does not play a chaperone role in p34(cdc2) activity, we cannot rule out the possibility that the p34(cdc2) epitopes are inaccessible.

HSP70-d isoform is located on isolated mitotic spindles in an ATP-dependent manner

In order to verify the hypothesis that, besides being located on centrosomes, HSP70-d is also located on the spindle fibres, we isolated the whole mitotic apparatus [21]. Immunostaining with an anti-β-tubulin antibody demonstrated isolated spindles with asters, all of which were intact in our preparation (Figure 6A). The same preparation assayed with the anti-(HSP70) antibody showed categorically that HSP70-d was located on the fibres of isolated asters and spindles (Figures 6B and 6C). Moreover, this finding confirmed our previous observation [14] obtained by immunostaining whole embryos, in which constitutive HSP70 was concentrated on the asters of spindles. To determine antibody specificity and to exclude antibody trapping in the isolated mitotic structure, we assayed the preparation using the secondary antibody alone. In the absence of the primary antibody, no staining was observed and the mitotic structures alone were detected using phase-contrast microscopy (Figure 6D).

In order to define whether HSP70-d functions in an ATP-dependent manner on the mitotic apparatus, we treated the mitotic preparation with different concentrations of ATP or GTP. It has previously been shown [29,30] that, in the presence of ATP, HSP70 is able to detach itself from its substrate. Treatment with 20 mM ATP for 15 min removed most of the HSP70 from the fibres of the mitotic apparatus, as shown by the weak immunostaining with the anti-(HSP70) antibody (Figure 6E). We also observed that, after ATP-treatment, the mitotic structures became relaxed, irregular in shape and torn. Treatment with 0.5 mM ATP did not produce any effect; HSP70

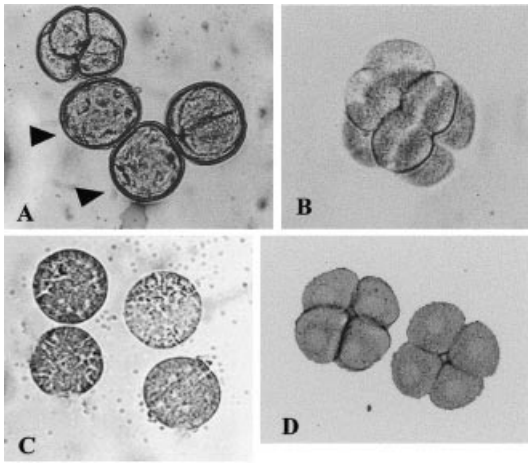

Figure 5 Effect of microinjecting anti-(TCP-1α) and anti-γ-tubulin antibodies in sea urchin embryos

The eggs were microinjected before fertilization with antibodies against TCP-1α (**A** and **B**), γ-tubulin (**C**), monoclonal anti-IgG or 15 mM sodium azide (**D**). (**A**) Embryos that stopped developing (7–10%, filled triangles) or have severe malformations (30–42%) are shown. (**B**) Embryos that develop irregularly. (**C**) Embryos whose development is immediately blocked are shown. (**D**) Normally developing embryos.

was not removed and the spindles and asters were stained as in Figure 6(A) (results not shown). To establish whether the HSP70 removal was due to the specific addition of ATP in mitotic preparation, we performed the same assay using 20 mM GTP instead of ATP. GTP treatment did not affect the HSP70 localization on the mitotic apparatus (Figure 6F). We suggest that HSP70 localized on the mitotic apparatus has a chaperone role, particularly for proteins that constitute the spindles.

DISCUSSION

The results in the present study not only clarify, but are also consistent with data from our previous study [14]. In that study, we found that constitutive HSP70 was ubiquitous in the unfertilized egg and moved within the cell, concentrating around the zygote nucleus soon after fertilization. Subsequently, it localized at the two opposite poles and finally appeared during mitosis on the asters. However, it was not clear if constitutive HSP70 was also present on the spindles, but we hypothesized that a portion of HSP70 was on the centrosomes where the spindles and asters form. In the present study, we confirmed this hypothesis by isolating centrosomes. This conclusion was reached, in part, by characterizing isolated centrosomes as containing γ-tubulin and having a protein pattern different from that of the whole embryo lysate. The protein pattern of centrosomes may appear relatively complex, but it has been described as a 'landing pad' for many proteins, such as those devoted to microtubule organization, kinases, components of proteasomal machinery and proteins related to the cell-cycle and involved in centrosome replication [31]. Using double immunofluorescence, we demonstrated the co-localization of γ-tubulin and constitutive HSP70 on the centrosomes. In addition, using two-dimensional PAGE and Western blotting, we found that, of the four isoforms known to be present in *P. lividus* embryos [16], only the HSP70-d isoform was present in centrosomes. Therefore what role does HSP70-d play in centrosomes? One possible explanation is that HSP70-d assists proteins that are either stable or transient components of this structure. To verify this, we attempted to identify the presence of the TRiC/TCP-1 complex, which is involved in tubulin folding during microtubule nucleation [28]. If the complex was present in *P. lividus* centrosomes, HSP70-d may have a role in assisting the tubulins engaged in folding. Using Western-blot analysis, we observed for the first time in *P. lividus* the presence of TCP-1α, which is a subunit of the hetero-oligomeric complex. In order to verify the involvement of TCP-

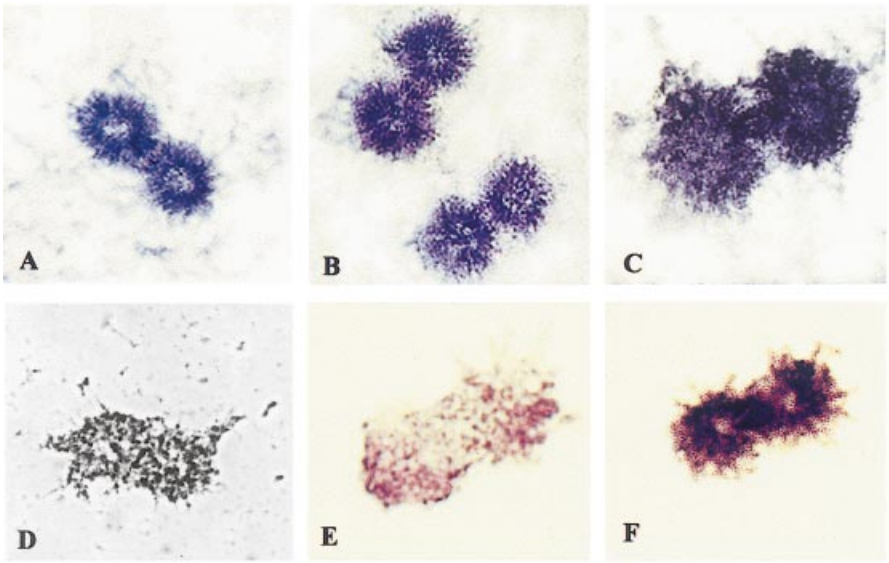

Figure 6 Localization of HSP70-d on the isolated mitotic apparatus in an ATP-dependent manner

Immunostaining was performed on spindles with asters isolated from 50-min embryos. Anti-β-tubulin (**A**) and anti-(HSP70) antibodies (**B**, **C**, **E** and **F**) were used for immunostaining. (**D**) The primary antibody, anti-(HSP70), was omitted and the isolated spindle was visualized using a phase-contrast microscope. ATP- (**E**) or GTP-treated (**F**) mitotic apparatus. Photographs were taken at 20 $\times$ magnification (**A**, **B**, **D** and **F**) and at 40 $\times$ magnification (**C** and **E**).

1 in tubulin folding, we performed experiments to block its function *in vivo* by microinjecting unfertilized eggs with the anti-(TCP-1α) antibody and observed embryo development after fertilization. We expected a total inhibition of TCP-1 function with its consequent effect on development. Surprisingly, only a few embryos were blocked at the zygote stage, whereas many developed with varying degrees of damage, or were not affected at all. Microinjection of antibodies *per se* did not affect embryonic development as antibodies not involved in mitosis, such as anti-IgG, did not affect development. In contrast, microinjection of an antibody involved in mitosis, such as anti-γ-tubulin, completely blocked embryonic development. Thus we were unable to completely block TCP-1 using an antibody that is directed towards one subunit of the large hetero-oligomeric complex. An explanation for this may be that the interaction between the antibody and TCP-1α depends upon the conformational state of the TCP-1 complex, which is known to be altered by substrate binding or by nucleotide interchange [27]. One consequence may be a total or partial inhibition of tubulin folding in centrosomes, and this causes either the interruption of embryo development or malformation of the embryo. We suggest that HSP70 assists the centrosomal chaperonine TCP-1 complex in tubulin folding.

The next step in our study involved exploring isolated centrosomes for the presence of p34(cdc2), a protein involved in the cell cycle. This protein was examined as it has previously been described in centrosomes [22] and has been linked with constitutive HSP70-2 in mouse spermatocyte meiosis [32]. HSP70-2 is expressed at high levels in mouse spermatocytes and was found in wild-type male mice with p34(cdc2) during the meiotic cell cycle, but not in homozygous mutant male mice. This suggests that HSP70-2 is required for the assembly of the functional (p34)cdc2–cyclinB1 complex. In the present study we detected p34(cdc2) by Western blot in *P. lividus* centrosomal proteins, but immunoprecipitation failed to demonstrate a direct link between the p34(cdc2) and HSP70-d. Therefore, we suggest that HSP70-d and p34(cdc2) are not associated in centrosomes isolated from first-metaphase *P. lividus* embryos. Nevertheless, we cannot exclude a link between these two proteins in a different phase of the cell cycle.

Another question raised by HSP70 immunolocalization in whole sea urchin embryos is whether HSP70 is also located on the fibres of mitotic spindles. It is important to underline that, in *P. lividus* embryos, the mitotic apparatus is about 40 μm long at the first division and it is assembled and disassembled every 30 min during the early cleavage stages. To determine the location of HSP70, we isolated intact *P. lividus* mitotic apparatus, as observed by phase-contrast microscopy and anti-β tubulin immunostaining. We have shown that immunostaining with the anti-(HSP70) antibody unequivocally localized constitutive HSP70 on the fibres of spindles and asters, which were clearly stained. In the presence of ATP, constitutive HSP70 became detached from the fibres of the mitotic apparatus, thus demonstrating that its localization was ATP-dependent. Indeed, it is known that ATP-bound HSP70 has a high dissociation constant for its substrates. In contrast, the presence of GTP did not affect the mitotic scaffolding. Our observation of hundreds of mitotic spindles indicated that the entire structure became less compact, and sometimes broken, in the presence of ATP. These observations enable us to hypothesize that constitutive HSP70 has a role in the building and polymerization of the spindle fibres, and assisting tubulins and/or other proteins involved in the organization of spindle-pole motors. Another possibility is that constitutive HSP70 has a chaperone role in the depolymerization of the spindle fibres when the mitotic structure disassembles. Our findings in the present study suggest that HSP70 is necessary for the mitotic apparatus, and is ATP-dependent. Furthermore, the results from the present study explain more fully our previous results [14] obtained by anti-(HSP70) antibody microinjection in eggs prior to fertilization. In that study, we found that HSP70 inhibition produced both the arrest of embryo mitosis and the formation of irregular or broken asters that are localized in eccentric zones; an effect dependent upon the amount of anti-(HSP70) antibody microinjected. Therefore we have found that in the absence of HSP70, due to either antibody inhibition or ATP treatment, the mitotic apparatus does not form or becomes irregular and broken. A similar result, but related to meiosis, was obtained in HSP70-2 $-/-$ mutant mice, where the absence of constitutive HSP70-2 caused the arrest of spermatogenesis at the pachytene stage, as synaptonemal complexes, where HSP70-2 is normally localized, failed to desynapse [33]. Indeed, it was suggested that HSP70 plays a critical role in meiosis. Our findings, together with these considerations, strongly suggest that HSP70 has a similar chaperone role for proteins of the mitotic apparatus in *P. lividus* embryos.

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