Molecular Chaperones and the Centrosome

A ROLE FOR TCP-1 IN MICROTIUBULE NUCLEATION*

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Molecular chaperones play an important role in facilitating the proper maturation of many newly synthesized proteins. Here we provide evidence that molecular chaperones also participate in regulating the assembly of the microtubule cytoskeleton. Via indirect immunofluorescence analysis, both hsp 73 and TCP-1 localized within the centrosome in interphase and mitotic cells. These proteins, along with the centrosome-specific protein, pericentrin, were also present within an enriched preparation of centrosomes. Because the centrosome serves as an initiation site for microtubule growth, we examined the ability of cells to regrow their microtubule network in the presence of hsp 73 or TCP-1 specific antibodies. Purified tubulin and GTP were added to cells following the depolymerization and extraction of cellular microtubules. Microtubules were observed to nucleate off the centrosome using this system, even in the presence of anti-hsp 73 antibodies. Incubation with anti-TCP-1 antibodies, however, blocked microtubule regrowth off the centrosome. Similarly, anti-TCP-1 antibodies microinjected into living cells first treated with nocodazole also inhibited the regrowth of the microtubule network following removal of the microtubule poison. Our results complement earlier genetic studies in yeast implicating a role for TCP-1 in microtubule mediated processes, and may help to explain the previously reported mitotic and meiotic abnormalities associated with TCP-1 mutations.

A class of proteins, now being referred to as molecular chaperones, facilitate various aspects of protein maturation. Perhaps the best characterized molecular chaperones are members of the so-called heat shock (hsp) or stress protein family (recently reviewed in Refs. 1 and 2). In animal cells, members of the hsp 70 family (DnaK in bacteria) are distributed throughout various cellular compartments and interact with proteins being synthesized on polyosomes and/or being translocated into organelles. Such an interaction with the hsp 70 proteins likely serves to stabilize and/or maintain the nascent polypeptide in a relatively unfolded state until its synthesis and/or translocation has been completed. Members of the eukaryotic hsp 60 family (GroEL in bacteria) also bind newly synthesized and unfolded polypeptides. This class of molecular chaperones, often called chaperons, are thought to provide a shielded environment in which protein folding and/or higher ordered protein assembly takes place. Although exhibiting only weak homology with other members of the chaperonin family, a eukaryotic cytosolic chaperonin has been identified and, like other members of the chaperonin family, exists as a large (~20 S) particle (3, 4).

Despite structural similarities to other members of the chaperone family, the cytosolic chaperonin exhibits a number of differences as it relates to both its subunit composition, mode of regulation, and apparent substrate specificity. First, at least 8 related subunits, referred to as the TCP-1 family, appear to comprise the cytosolic chaperonin complex in eukaryotic cells. In contrast, chaperonins of both bacteria and plastids contain only 1 or 2 polypeptide subunits. Second, in contrast to the bacterial and plastid chaperones, the family of related TCP-1 proteins do not appear to be up-regulated after heat shock or other metabolic stresses. Finally, while the bacterial GroEL chaperonin appears to function rather promiscuously, facilitating the folding of most any substrate presented to it, so far the cytosolic TCP-1 containing chaperonin has been observed to interact with only a limited number of substrates. Interestingly such substrates for the TCP-1 containing chaperonins are nucleotide binding proteins and include actin, tubulin, and luciferase (4–9).

Previous observations have also provided a connection between the TCP-1 proteins and microtubule related phenomena. In mice, expression of TCP-1 increases markedly during spermatogenesis (10), a process that requires the assembly of complex macromolecular structures including the microtubule core of the flagellum. In addition, mutations within the TCP-1 locus are associated with both microtubule abnormalities and transmission ratio distortions (11). Similarly in yeast, temperature-sensitive mutations of the TCP-1 gene result in a number of alterations in microtubule mediated processes. For example, at the non-permissive temperature the cells exhibit abnormal looking microtubules and give rise to the accumulation of multi-nucleated progeny (12). Taken together, these results suggest that TCP-1 may play an important role in both the folding and the proper assembly of cytoskeleton proteins.

In the course of examining the intracellular locale of hsp 73 and TCP-1, we observed a portion of both proteins to exhibit rather intense staining near the nucleus. Such a locale appeared similar to another protein under study in our laboratory, the so-called pericentrin protein which has recently been shown to be a component of the centrosome (13, 14). Owing to the fact that the centrosome serves as a primary nucleation site for microtubule growth (15), along with the aforementioned observations relating TCP-1 mutations in yeast to microtubule abnormalities, we examined the possible relationship of hsp 73 and TCP-1 with the centrosome. We show here by different means that a portion of both hsp 73 and TCP-1 are, in fact, resident proteins of the centrosome. In addition, evidence is...
presented implicating a role for TCP-1 in mediating the growth of the microtubule network off the centrosome.

**MATERIALS AND METHODS**

Antibodies and Antibody Characterization—A synthetic peptide corresponding to amino acids 42–57 from the mouse TCP-1 protein was synthesized via the high density multiple antigenic peptide system (16), and injected into rabbits. The resultant polyclonal serum, which has been partially characterized previously (17), was analyzed here by different means. Immunoprecipitations were performed from cell lysates harvested under “native” or “denaturing” conditions. For the former, cells were labeled with [35S]methionine (DuPont NEN, specific activity 1100 Ci/mmol) for 16 h and harvested in phosphate-buffered saline (PBS)2 containing 1% Triton X-100 and 2 mM MgCl2 for immunoprecipitations under denaturing conditions, cells were labeled as above, and then solubilized in Laemmli sample buffer containing 1% SDS and heated at 100 °C for 5 min. Following shearing of the released chromatin and subsequent clarification, the lysates were diluted 10-fold in RIPA buffer (1% Triton, 1% deoxycholate in PBS) for subsequent immunoprecipitation analysis. Characterization of the native molecular mass of the 60-kDa antigen was performed by harvesting [35S]methionine-labeled HeLa cells in PBS containing 0.1% Triton X-100. Following clarification (15,000 × g for 5 min), the supernatant was applied to a 20–50% glycerol gradient (in 10 mM Tris, pH 7.4, 10 mM KCl, and 2 mM EGTA), and the sample centrifuged at 28,000 rpm for 40 h in an SW 41 rotor. The gradient was fractionated and the position of the 60-kDa antigen was determined by immunoprecipitation. Other antibodies used included: a rabbit polyclonal antibody to hsp 73 (18); a human autoimmune antibody (19), a rabbit polyclonal antibody, and a rat monoclonal antibody all specific for to pericentrin (14); a mouse monoclonal antibody to α-tubulin (20); and a rat monoclonal antibody specific for TCP-1, designated 23C (Ref. 21, and purchased from the Institute of Cancer Research, Haddow Laboratories, Royal Cancer Hospital, Surrey, UK).

Cell Culture and Immunofluorescence—Cells used in all experiments were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine calf serum at 37 °C. For indirect immunofluorescence experiments, cells were either directly placed into fixative (–20 °C absolute methanol for 3–5 min) or, in some cases, the cells were first extracted with nonionic detergents and then methanol fixed. Extraction was done by incubating the cells in microtubule stabilization buffer (MSB: 80 mM Pipes, pH 6.9, 5 mM EGTA, 1 mM MgCl2) supplemented with 0.05–0.1% Triton X-100 for 2 min at room temperature. Cells were incubated with the antibody of interest for 30 min at 37 °C, the cells washed extensively in phosphate-buffered saline, and the primary antibodies visualized by subsequent incubation with the appropriate antibody conjugated with either fluorescein or rhodamine (all purchased from Cappel Laboratories). All antibodies were diluted into 5 mg/ml bovine serum albumin in PBS.

Isolation and Partial Purification of Centrosomes—Centrosomes were partially purified from [35S]methionine-labeled CHO cells using a similar protocol developed by Mitchison and Kirschner (22) and slightly modified by Bornens et al. (23).

Microinjection Experiments—COS cells growing on glass coverslips were incubated with nocodazole (20 μg/ml) for 90 min. While still in the presence of the drug, the cells were injected with rabbit preimmune antibody, rabbit anti-hsp 73, or rabbit anti-TCP-1. All antibodies used for microinjection were first purified by DEAE-Affi-Gel blue chromatography and used at a concentration of 0.5–1.0 μg/ml in 0.5 × PBS. 15 min after injection of the antibodies, the medium was removed, the cells extensively washed with fresh Dulbecco’s modified Eagle’s medium and then incubated in Dulbecco’s modified Eagle’s medium for an additional 20 min to allow for microtubule regrowth. Coverslips were processed for immunofluorescence as described above.

Microtubule Regrowth in Vitro—COS and CHO cells growing on glass coverslips were treated with nocodazole (20 μg/ml) for 90 min at 37 °C. The medium was then removed and the cells extracted for 2 min at room temperature with MSB supplemented with 0.1% Triton X-100. The extracted cells were washed with fresh MSB and then either preimmune, polyclonal rabbit anti-TCP-1, monoclonal rat anti-TCP-1, or anti-hsp 73 antibodies (2 mg/ml) in MSB were added. Following a 10-min incubation at room temperature the antibody solution was removed, the cells washed with and further incubated in MSB supplemented with 1 mg/ml GTP, 2 mg/ml purified tubulin, and 20% glycerol for 12 min at 37 °C. The cells then were gently washed with MSB, fixed in 2% glutaraldehyde, and processed for immunofluorescence using the antibody–α-tubulin antibody.

**RESULTS**

As an initial step in characterizing the location and function of members of the hsp 73 and TCP-1 families, antibodies specific to each protein were prepared. A rabbit polyclonal antibody was prepared and shown to be specific for hsp 73 (18). For the preparation of a TCP-1 specific antibody, a peptide corresponding to amino acids 45–57 of the mouse TCP-1 protein (Fig. 1A) was synthesized and injected into rabbits. Analysis of
the resultant serum was done via immunoprecipitation using [35S]methionine-labeled HeLa cells solubilized in Laemmli sample buffer containing 1% SDS and heated at 100 °C. Under such conditions, the rabbit anti-TCP-1 antibody precipitated an approximately 60-kDa protein (Fig. 1, panel B, lane 2). Preadsorption of the serum with an excess of the synthetic peptide used to elicit the antibody resulted in an inability of the serum to recognize the 60-kDa protein (Fig. 1B, lane 3). Immunoprecipitation using the rabbit polyclonal antibody to hsp 73 revealed an approximate 70-kDa band (Fig. 1B, lane 4). Further analysis of this 70-kDa protein by two-dimensional gel electrophoresis demonstrated the protein to be identical to the constitutively expressed member of the hsp 70 family, referred to here as hsp 73 (data not shown, see Ref. 18).

To determine whether the antigen recognized by the TCP-1 synthetic peptide antibody existed as an oligomeric species, similar to the situation with other known members of the chaperonin family, [35S]methionine-labeled HeLa cells were solubilized by the addition of a buffer containing 0.1% Triton X-100 and applied to a 20-50% glycerol gradient. Following centrifugation, the gradient was fractionated and aliquots adjusted to a final 1% SDS and heated at 100 °C for 5 min. Immunoprecipitants across the gradient revealed the 60-kDa TCP-1 protein to migrate with an approximate 20 S value (Fig. 1C).

To further confirm the specificity of our rabbit anti-TCP-1 antibody, we examined and compared its substrate specificity with that of a previously described rat monoclonal antibody against TCP-1 (referred to as 23C). CHO cells were labeled for 16 h with [35S]methionine and cell lysates prepared for immunoprecipitation analysis. In our initial experiment, the cells were lysed in a buffer containing non-ionic detergents and then used to examine the substrate specificity of both our rabbit anti-TCP-1 antibody and 23C (Fig. 2). Under these conditions, the rabbit TCP-1 antibody immunoprecipitated a prominent −60-kDa protein (Fig. 2, lane 2) along with a few other proteins. The 23C antibody immunoprecipitated a number of polypeptides with molecular masses between 50 and 70 kDa (Fig. 2, lane 3). Indeed, it is this kind of analysis utilizing the 23C antibody which has resulted in the conclusion that the eukaryotic TCP-1 containing cytosolic chaperonin is comprised of multiple subunits and therefore has been referred to by some (35) as the TCP-1 ring complex (TRiC). However, when the immunoprecipitation analyses were performed using the labeled cells first solubilized in Laemmli sample buffer containing 1% SDS and the lysates then heated at 100 °C, slightly different results were obtained. Now, both our rabbit antibody and the 23C antibody recognized primarily only a single 60-kDa protein (Fig. 2, lanes 5 and 6, respectively).

The above described immunoprecipitation products were analyzed by two-dimensional gel electrophoresis. The immunoprecipitates obtained from the nonionic detergent lysed cells using the 23C antibody (Fig. 2, panel B) and the rabbit peptide antibody (Fig. 2, panel C) revealed the presence of numerous polypeptides. In contrast, the immunoprecipitation products obtained from the cells lysed in buffers containing SDS, using either the 23C antibody (Fig. 2, panel D) or the rabbit peptide antibody (Fig. 2, panel E), revealed only a prominent −60-kDa protein which appeared to be comprised of at least 3 or 4 isoelectric variants (the small amounts of actin and tubulin which also were present likely represent nonspecific background as evidenced by their precipitation using preimmune sera, data not shown).

The intracellular locale of hsp 73 and TCP-1 was determined by indirect immunofluorescence methods. While the majority of both proteins appeared to be present throughout the cytosol, and to a lesser extent inside the nucleus, a relatively localized and rather intense staining pattern also was observed within the perinuclear region of the cells (the latter indicated by arrowheads in Fig. 3). This perinuclear staining pattern appeared remarkably similar to that of another protein under study in our laboratory, specifically, the recently described pericentrin protein which has been shown to be a component of the centrosome. To examine whether a portion of both hsp 73 and TCP-1 might be present within the centrosome, simultaneous staining of the cells with antibodies specific for pericentrin and the two molecular chaperones was performed. To facilitate the analysis, HeLa cells growing on glass coverslips

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**Fig. 2.** The rabbit polyclonal TCP-1 and a monoclonal TCP-1 antibody (23C) precipitate similar proteins under denaturing conditions but not under native conditions. CHO cells were steady state labeled with [35S]methionine for 16 h and then harvested under denaturing conditions (e.g. Laemmli sample buffer containing 1% SDS and heated at 100 °C) or native conditions (cold buffers containing only non-ionic detergents). Immunoprecipitation analyses were performed using either the rabbit polyclonal anti-TCP-1 or the rat monoclonal 23C TCP-1 specific antibody. Duplicate immunoprecipitate samples were analyzed by both one- and two-dimensional gel electrophoresis. Panel A, analysis of the immunoprecipitates by one-dimensional SDS-PAGE. Immunoprecipitations from cell lysates solubilized under native conditions (lanes 1-3) or from cell lysates solubilized under denaturing conditions (lanes 4-6). Lanes 1 and 4, no added antibody; lanes 2 and 5, the rabbit anti-TCP-1 antibody; lanes 3 and 6, the rat monoclonal 23C anti-TCP-1 antibody. Molecular mass markers are indicated on the left and the arrowhead indicates the position of the approximate 60-kDa TCP-1 protein. Panels B-E, analysis of the immunoprecipititates by two-dimensional gel electrophoresis using 70% pH 5–7 and 30% pH 3–10 ampholines (the left side of each panel represents the acid side of the gel). In panel B is shown the rat monoclonal 23C immunoprecipitation product obtained from cells lysed under native conditions; in panel C the rabbit anti-TCP-1 immunoprecipitation product obtained from cells lysed under native conditions; and in panel E the rabbit anti-TCP-1 immunoprecipitation product obtained from cells lysed under denaturing conditions. The position of the TCP-1 protein(s) is indicated by a bracket while the positions of both actin and tubulin are indicated by an asterisk (*).
were first lightly extracted with buffers containing nonionic detergents to remove the majority of the cytosolic and nuclear forms of hsp 73 and TCP-1. Previous studies have shown that the centrosome, and many of its associated components, are not grossly affected by such extraction with non-ionic detergents (14). Double label staining using a human autoimmune sera (5051) specific for pericentrin, along with either antibodies specific for hsp 73 or TCP-1 revealed that a portion of hsp 73 and TCP-1 were in fact present very near or within the centrosome (Fig. 4). Staining of the centrosome by antibodies to the two molecular chaperones was observed in interphase cells as well as in cells which had entered mitosis. Although not shown, we have observed a similar distribution for hsp 73 and TCP-1 within the centrosome of other animal cells including the two monkey cell lines, CV-1 and COS, rat embryo fibroblasts, baby hamster kidney, mouse 3T3, and Chinese hamster ovary cells.

To further explore the apparent centrosomal locale of both hsp 73 and TCP-1, an enriched preparation of centrosomes was prepared and examined for the presence of the two molecular chaperones (23). Briefly, [35S]methionine-labeled CHO cells were treated with the microfilament poison cytochalasin and the microtubule depolymerizing agent nocodazole. Following lysis of the cells and subsequent clarification of the lysate, the resultant supernatant was applied to a discontinuous sucrose gradient, the material centrifuged at 100,000 × g for 2 min and the gradient then fractionated. As is shown in Fig. 5, panel A, the vast majority of radioactive material was present within the first half of the gradient. In addition, a distinct peak of radioactive material was observed near the bottom of the gradient (fractions 4–7), this representing material within the 50–70% sucrose interface where centrosomes have previously been shown to migrate using this procedure (23). To confirm that the material present within fractions 4–7 contained a relatively dense particle corresponding to the centrosome, the fractions were pooled and diluted 4-fold with buffer. The diluted material then was centrifuged at 14,000 × g for 20–30 min and the resultant supernatant and pellet examined by SDS-PAGE. As is shown in Fig. 5B, the vast majority of the radiolabeled proteins were found within the pellet, consistent with the proteins being present within a dense particle. Both hsp 73 and TCP-1, along with a few other proteins including actin and tubulin (as well as other unidentified proteins) were found to be present within the enriched preparation of centrosomes (Fig. 5D).

These partially purified centrosomes were centrifuged onto glass coverslips and examined by indirect immunofluorescence, again using antibodies to hsp 73, TCP-1, and the antibody specific for the centrosome protein, pericentrin. As was found with the detergent-extracted cells, coincident staining of pericentrin and hsp 73 (Fig. 6, A and B, respectively), as well as coincident staining of pericentrin and TCP-1 (Fig. 6, C and D, respectively) was observed.

Previous work has established that the centrosome serves both as an anchor for microtubules and as a site for nucleation of microtubule growth (15, 34). For example, when cells are treated with nocodazole, the microtubules are observed to depolymerize. Upon removal of the drug, the microtubules are
observed to regrow with such regrowth emanating from the centrosome (14, 22). To address the possible role played by either hsp 73 or TCP-1 in facilitating the regrowth of the microtubule network, we initiated “blocking” experiments utilizing antibodies to both molecular chaperones. Cells were treated with nocodazole to depolymerize the microtubule network and the cells then injected with either preimmune, anti-hsp 73 or anti-TCP-1 antibodies. Following their injection, the cells were extensively washed to remove the nocodazole, and then further incubated for various times to allow for the regrowth of the microtubule network. In those cells injected with either preimmune or anti-hsp 73 antibodies, regrowth of the microtubules from the centrosome was unaffected (data not shown).

In contrast to the lack of effects following injection of either the preimmune or hsp 73 antibodies, cells injected with the rabbit anti-TCP-1 antibodies appeared unable to support new microtubule growth. COS cells growing on glass coverslips were treated with nocodazole and injected with the anti-TCP-1 antibody as described above. Ten min after the injections, the cells were washed with and further incubated in fresh culture medium for either 10 or 30 min. This time, however, the cells were first lightly extracted with nonionic detergent to facilitate the removal of any nonpolymerized tubulin. Following their fixation, the identification of the injected cells, along with the distribution of the microtubules, was determined by double label staining exactly as described earlier. Note that little or no microtubule regrowth was observed in those cells injected with anti-TCP-1 and then allowed either a 10-min (Fig. 7, D-F) or 30 min (Fig. 7, G-I) regrowth period. In contrast, by 10 min, the un.injected cells had initiated microtubule aster formation (Fig. 7F) and by 30 min had regrown their complete microtubule network (Fig. 7I).

We next performed similar in vitro blocking experiments using permeabilized cells. In our first experiment, COS cells were treated with nocodazole to depolymerize the microtubule network and the cells then detergent extracted to remove the free 6 S tubulin. Antibodies (preimmune as a control, anti-hsp 73 or anti-TCP-1) were added to the extracted cells, and the ability of the cells to support microtubule regrowth following addition of purified tubulin was examined. In those cells incubated with preimmune antibodies, new microtubule growth (as assayed by staining with the anti-tubulin antibody) was seen emanating from a single site, likely the centrosome (Fig. 8A). Incubation of the cells with the anti-hsp 73 antibody again had no obvious effect on microtubule regrowth (Fig. 8B). In contrast, in two separate experiments, incubation of the extracted cells with the anti-TCP-1 antibody blocked the regrowth of the microtubules (Fig. 8, C1 and C2). Note, however, that prior incubation of the extracted cells with the anti-TCP-1 antibody did not prevent subsequent staining of the centrosome with the anti-tubulin antibody (note the localized perinuclear staining in Fig. 8C2). Such staining likely represents that portion of tubulin present within the centriolar region of the centrosome.

To further confirm these results we examined the effects of incubating the cells with the rat monoclonal antibody (23C) specific for TCP-1 (21). In vitro regrowth experiments, identical to those shown in Fig. 8, were performed using Chinese hamster ovary cells (CHO). Similar to our results using the rabbit polyclonal anti-TCP-1 sera, addition of the 23C antibody prior to the addition of purified tubulin resulted in an inhibition of microtubule growth (Fig. 9B).

**DISCUSSION**

Our studies, in summary, demonstrate that a subpopulation of two molecular chaperones, hsp 73 and TCP-1, co-localize with and likely are integral components of the centrosome in eukaryotic cells. This conclusion was reached, in part, on the basis of double label immunofluorescence experiments showing that antibodies to these two molecular chaperones stained perinuclear structures in a fashion identical to that using antibodies which recognize a defined centrosomal antigen, pericentrin. Using this approach, we previously observed the presence of hsp 73 in centrosomes of both mitotic and interphase cells (24). Likewise, using an antibody that recognized both the
constitutive (hsp 73) and stress inducible (hsp 72) forms of hsp 70, Rattner (25) showed what appeared to be centrosomal staining in mitotic cells. Here, we have performed additional experiments to verify that hsp 73 and TCP-1 are indeed centrosomal components. For example, both hsp 73 and TCP-1 were found to be present within an enriched preparation of centrosomes isolated by conventional means. Moreover, we have found that treatment of the isolated centrosomes with high concentrations of salt did not result in the release of the centrosomal antigen pericentrin, hsp 73, or TCP-1, results indicative that all three of these proteins likely are integral components of the organelle (data not shown). Finally, for at

**Fig. 6. Hsp 73 and TCP-1 co-localize with pericentrin in isolated centrosomes.** Centrosome fractions prepared from CHO cells as described in the legend to Fig. 4 (fractions 4–7) were pooled, diluted in centrosome isolation buffer, and then centrifuged onto glass coverslips. The coverslips were incubated with antibodies to both hsp 73 (rabbit) and pericentrin (rat), or with antibodies to TCP-1 (rabbit) and pericentrin (rat). Primary antibodies were visualized by subsequent incubation with fluorescein-conjugated goat anti-rabbit and rhodamine-conjugated goat anti-rat antibodies. Panels A and B, centrosomes simultaneously stained for pericentrin (panel A) and hsp 73 (panel B). Panels C and D, centrosomes simultaneously stained for both pericentrin (panel C) and TCP-1 (panel D). Arrowheads indicate representative centrosomes being recognized by both antibodies.

A

B

C

D

Panels A and B, centrosomes simultaneously stained for pericentrin (panel A) and hsp 73 (panel B). Panels C and D, centrosomes simultaneously stained for both pericentrin (panel C) and TCP-1 (panel D). Arrowheads indicate representative centrosomes being recognized by both antibodies.
least TCP-1 we have shown that both our own antibody, as well as a previously described TCP-1 antibody (23C) appeared effective in inhibiting the growth of microtubules off the centrosome, be it in detergent permeabilized cells or in living cells microinjected with the anti-TCP-1 antibody.

Some discussion regarding the properties of our polyclonal TCP-1 antibody merits inclusion here. As was shown in Figs. 1 and 2, our antibody, when used in immunoprecipitation studies of cell lysates first subjected to SDS and heating, precipitated the same TCP-1 antigen as that recognized by the previously described 23C anti-TCP-1 antibody. However, when the analysis was performed using cells lysed under “nondenaturing” conditions (e.g. nonionic detergents and no heating) the 23C antibody precipitated the TCP-1 antigen along with a number of other proteins. This collection of co-precipitating proteins has been suggested to represent a heteromeric TCP-1 containing chaperonin complex (35) and as such has been given the name TRiC. In contrast, our rabbit antibody did not precipitate a TRiC-like complex from the cells lysed under nondenaturing conditions. One possible explanation for this difference is that the binding of our antibody to the TRiC somehow results in the dissociation of the heteromeric TCP-1 containing particle. Alternatively, there may exist two forms of the TCP-1 protein, one present within TRiC, and the other perhaps present in monomeric form. As was shown in Fig. 1, we do routinely observe what appears to be two forms of the TCP-1 protein, one which migrates in the velocity sedimentation gradients with an apparent mass of 20 S, and the other which remains at the top of the gradient and therefore which might represent the “free” or monomeric form of the protein. If so, one wonders what form of TCP-1 is present within the centrosome. For example, does the centrosome contain the entire TCP-1 containing chaperonin complex TRiC, or simply the TCP-1 protein itself?

Could our results showing compromises in the ability of the cells to initiate growth of the microtubules off the centrosome following addition/injection of anti-TCP-1 antibodies be explained by simple steric hindrance? For example, upon binding of the TCP-1 antibody to the centrosome might the subsequent recruitment of 6 S tubulin (or other essential components such as microtubule-associated proteins) be hindered from reaching the centrosome? Although difficult to rule out, we did show that in cells first incubated with either anti-hsp 73 or anti-TCP-1 (either of which bound to the centrosome) we could subsequently stain the centrosome with the antibody to tubulin (Fig. 8). Hence, the tubulin antibody presumably is still able to bind to that portion of tubulin within the centriolar component of the centrosome, even when the organelle had first been engaged with the antibodies to the two different molecular chaperones. Similarly, we have performed all of the other possible permutations involving sequential addition of the three different antibodies that were shown here to stain components present within the centrosome. For example, incubation first with antibodies to pericentrin did not prevent subsequent staining of the same centrosome with either hsp 73 or TCP-1 antibodies, and vice versa. Thus we think it unlikely that the TCP-1 antibody, when engaged with the centrosome, is simply pre-
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Fig. 8. Incubation of detergent permeabilized and nocodazole-treated cells with anti-TCP-1 antibody blocks the re-growth of the microtubules. HeLa cells growing on glass coverslips were treated with nocodazole (20 μg/ml) for 90 min at 37°C. The medium was removed and the cells extracted with microtubule stabilization buffer supplemented with 0.1% Triton X-100 for 2 min at room temperature. Following their extraction and subsequent washing with MSB, the cells were incubated with rabbit preimmune sera, rabbit anti-hsp 73, or the rabbit anti-TCP-1 antibody. Following a 10-min incubation at room temperature, the antibody solution was removed, the cells washed with MSB buffer and then incubated with MSB containing 1 mM GTP, 2 mg/ml tubulin, and 20% glycerol. After 12 min at 37°C the cells were washed with MSB, fixed with 2% glutaraldehyde in MSB, and processed for immunofluorescence using the anti-α-tubulin antibody followed by rhodamine conjugated goat anti-mouse antibody. Shown are the immunofluorescent photographs of the tubulin staining. Panel A, cells incubated with rabbit pre-immune serum; panel B, cells incubated with rabbit anti-hsp 73; panel C, cells incubated with rabbit anti-TCP-1 (C1 and C2 represent two different experiments using the anti-TCP-1 antibody). Note the formation of microtubule asters only in those cells incubated with either the preimmune or hsp 73 antibodies.

Fig. 9. The previously characterized rat monoclonal antibody specific for TCP-1 also inhibits in vitro microtubule regrowth in CHO cells. In vitro microtubule regrowth experiments, identical to those described in the legend to Fig. 8, were performed using either a control antibody (preimmune) or a purified preparation of the rat monoclonal antibody (23C) specific for TCP-1. Shown is the pattern of tubulin staining. Panel A, cells incubated with the control antibody; panel B, cells incubated with the rat monoclonal 23C anti-TCP-1 antibody.

venting the access of other components, like tubulin, to the centrosome.

Both our experimental approach and some of our results examining the locale and function of TCP-1 within the centrosome appear similar to previous studies examining a newly identified member of the tubulin family, γ-tubulin (26). Via indirect immunofluorescence methods γ-tubulin was shown to be present within the centrosome of both interphase and mitotic cells. In addition, injection of γ-tubulin antibodies into living cells was shown to block the re-growth of the microtubules off the centrosome. Interestingly, like TCP-1, γ-tubulin exists in vivo as a large complex, on the order of 25 S (27). Moreover, both γ-tubulin as well as another centrosomal component, the vertebrate actin-related protein are dependent on the TCP-1 chaperonin for their folding and/or higher ordered assembly (8). While we do not yet know how all of these observations fit together, we suspect that they are more than coincidental.

Molecular chaperones are important components in the pathways by which newly synthesized proteins acquire their properly folded state. Therefore, we did consider the possibility that microtubule regrowth might require new protein synthesis and that we were simply inhibiting the proper maturation of such proteins as a result of the injected TCP-1 antibodies. However, a number of observations are inconsistent with this notion. First, we found that prior addition of the protein synthesis inhibitor cycloheximide had no effect on the ability of nocodazole-treated cells to regrow their microtubule network following subsequent removal of the microtubule poison (data not shown). Second, cells permeabilized via the addition of nonionic detergents were still competent to support microtubule growth off the centrosome upon addition of purified tubulin and GTP. Finally, at least in vitro, an enriched preparation of 6 S tubulin is competent to assemble into microtubules provided that an energy source is supplied. Indeed, it is this latter result which might lead one to question why molecular chaperones would be needed at all to facilitate the growth of microtubules in vivo. While we still do not have a satisfactory answer to this fundamental question, it is important to point out that a similar question was raised when molecular chaperones were first suggested to be important components in the pathways of protein folding inside the cell. Here a significant amount of work had established that a purified protein, when first rendered unfolded via treatment with protein chaotropes, could spontaneously refold upon removal of the chaotrope (36). In vivo, however, the possibility that an unfolded protein (e.g., newly synthesized protein) will interact with other macromolecules during its folding process, thereby leading to nonproductive folding or aggregation, seems quite high. Consequently, molecular chaperones have been suggested to participate in the pathway of protein folding in vivo, not by providing any direct information for the folding process, but rather by reducing the probability of inappropriate intra- or intermolecular interactions which might lead to nonproductive folding and/or aggregation. Exactly how this scenario might apply to the apparent role of TCP-1 in mediating the growth of the microtubules off the centrosome is still unclear to us at this time. Using various in vitro approaches, we hope to begin addressing these types of questions in the near future.

We should mention that there are a number of other observations which link members of the molecular chaperone family with the cytoskeleton, and in particular the microtubules. For example, we and others have shown previously that hsp 73 co-purifies with microtubules during repeated rounds of tubulin assembly/disassembly in vitro (29–31). Whether hsp 73 is in fact a true microtubule-associated protein (i.e., a MAP), required for microtubule growth, is not known, but is now being addressed in our laboratory. We now have preliminary evidence that TCP-1 is present at modest amounts in three-time cycled preparations of microtubules. Even more intriguing are recent studies implicating an involvement of the TCP-1 containing chaperonin in facilitating the folding of both newly synthesized actin and tubulin (6–8). Finally and most germane
to the results presented here are previous genetic studies showing a connection between TCP-1 and microtubule-related phenomena. In the mouse, where the TCP-1 locus was first identified, mutations in the locus were associated with transmission distortions (11), perhaps indicative of microtubule abnormalities. In addition, the TCP-1 protein was shown to be expressed at high levels during spermatogenesis, a process that requires the assembly of complex macromolecular structures, including the microtubule core of the flagellum. Perhaps more in line with the observations presented here are those studies in yeast showing that mutants harboring a cold-sensitive mutation in TCP-1 exhibited a number of microtubule-related abnormalities (12, 32). Such cells, at their restrictive temperature, failed to grow exponentially, had an impaired generation time, and exhibited rather striking abnormalities in normal cell division events. Moreover, these TCP-1 mutants exhibited a large number of abnormal microtubule staining patterns, and gave rise to both anucleate and multinucleate progeny. Thus, these earlier genetic observations, coupled with the biochemical studies shown here, are indicative that a functional TCP-1 protein may be important for the normal function of the centrosome, especially as it relates to the establishment of a proper mitotic/meiotic spindle. Perhaps one or more molecular chaperones, like TCP-1 and hsp73, participate directly in facilitating various macromolecular rearrangements which accompany the establishment of mitotic and meiotic spindles and the organization of cytoplasmic microtubule arrays in general (see the following paper (28) for further discussion).

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