Selected Subunits of the Cytosolic Chaperonin Associate with Microtubules Assembled in Vitro*

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The molecular chaperone activities of the only known chaperonin in the eukaryotic cytosol (cytosolic chaperonin containing T-complex polypeptide 1 (CCT)) appear to be relatively specialized; the main folding substrates in vivo and in vitro are identified as tubulins and actins. CCT is unique among chaperonins in the complexity of its hetero-oligomeric structure, containing eight different, although related, gene products. In addition to their known ability to bind to and promote correct folding of newly synthesized and denatured tubulins, we show here that CCT subunits α, γ, ζ, and θ also associated with in vitro assembled microtubules, i.e. behaved as microtubule-associated proteins. This nucleotide-dependent association between microtubules and CCT polypeptides (Kd ~ 0.1 µM CCT subunit) did not appear to involve whole oligomeric chaperonin particles, but rather free CCT subunits. Removal of the tubulin COOH termini by subtilisin digestion caused all eight CCT subunits to associate with the microtubule polymer, thus highlighting the non-chaperonin nature of the selective CCT subunit association with normal microtubules.

Molecular chaperones are a diverse group of proteins that assist the correct folding and intracellular targeting of newly synthesized polypeptides (1) and can modulate the oligomerization and polymerization of folded native proteins (e.g. Ref. 2). The chaperonins are a family of molecular chaperones that are characterized by their oligomeric structure, namely a double torus of ~60 kDa subunits (3) enclosing a central cavity within which the folding substrate may be sequestered (4–6). Chaperonin-assisted protein folding proceeds by ATP-driven, alternating cycles of substrate binding and release, ultimately resulting in a native, or near-native, protein that is no longer recognized by the chaperonin (7, 8). The cytosolic chaperonin containing T-complex polypeptide 1 (CCT)1 is the only known chaperonin in the cytosol of eukaryotes (9, 10). The eight-membered rings of the CCT double torus consist usually of eight distinct but related (~30% identity) gene products, CCTα, -β, -γ, -δ, -ε, -ζ, -η, and -θ (11). In yeast, these eight subunits are encoded by essential genes, and mutations in individual subunits lead to defects in the functioning of the cytoskeleton, most commonly manifested as arrest in mitosis (reviewed in Ref. 12). There is both in vivo (10) and in vitro (13–15) evidence that major substrates of the cytosolic chaperonin are tubulins and actins. In addition to assisting folding of newly synthesized tubulins and actins, the CCTα subunit appears to be a component of the centrosome and essential for nucleated microtubule assembly from this organelle (16). That this process can take place in a permeabilized cell system, in the absence of protein synthesis, suggests that CCTα at least may also be involved in facilitating the polymerization of fully folded tubulins; which, if any, other CCT subunits are required remains to be determined. Indeed, whether CCT subunits always and only exist in cells as components of a single type of 20 S oligomeric particle or whether free subunits and assemblies of variable subunit composition have some functional roles is not yet resolved. We have presented data that support the latter possibility (17).

In view of the close functional relationship between CCT subunits and the synthesis and assembly of tubulins, it seemed appropriate to determine whether CCT subunits could be detected as microtubule-associated proteins (MAPs). MAPs are defined operationally as proteins that copurify with tubulins to a constant stoichiometry during microtubule assembly (18, 19). Several MAPs appear to fulfill structural roles in microtubule function, e.g. by modulating the stability of microtubules and by forming side-arm structures important for maintaining the integrity of the cytoskeleton (e.g. Refs. 20–22). Such structural MAPs commonly promote the in vitro assembly of tubulin into microtubules. Other MAPs are more transiently associated with the microtubule, such as the ATP-driven motor molecules kinesin and cytoplasmic dynein, which are required for the microtubule-based motility so critical for the functioning and proliferation of eukaryotic cells (23, 24). The association of these latter MAPs with microtubules is often detected only in vitro when microtubules are polymerized in the presence of a non-hydrolyzable ATP analog such as AMPPNP (25). Indeed, a number of stringent experimental criteria have been developed over the years by which a protein may be defined as a MAP. Two of these criteria, copolymerization through temperature-dependent cycles of microtubule assembly and disassembly (18, 19) and copurification with Taxol-assembled microtubule protein prepared and extracted under carefully defined centrifugation conditions (26), have been used in this investigation of the association of CCT subunits with microtubules.

Initially, we detected CCT subunits in standard mammalian brain MAP preparations and have since examined in more detail the CCT content of MAPs in the P19 cell line. This pluripotent mouse embryonal carcinoma cell line has the ability to differentiate into a variety of phenotypes (27). Of particular interest to us, in view of the presence of CCT subunits in standard brain MAP preparations, is the neuronal differentiation pathway that is induced by aggregation in the presence of submicromolar levels of retinoic acid. The use of this cell line, in conjunction with the plant alkaloid Taxol (28) to induce microtubule assembly, permitted a much greater flexibility in experimental conditions, including microtubule polymerization.

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‡ The abbreviations used are: CCT, cytosolic chaperonin containing T-complex polypeptide 1; MAP, microtubule-associated protein; AMP-PNP, adenosyl-5’-yl imidodiphosphate; P19N, P19 neuron; P19EC, P19 embryonal carcinoma; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); PAGE, polyacrylamide gel electrophoresis.
in the absence of added nucleotide. We report here that CCT subunits do indeed copurify with microtubules. The subunit proportions of the microtubule-associated CCT subunits differ from those in the parental tissue/cell lysate and from those in the bulk 20 S chaperonin particles purified thereof. These microtubule-associated CCT subunits are not assembled in a chaperonin-sized particle. We discuss the possible role free CCT subunits may play in the molecular chaperone activities of the eukaryotic cytosolic chaperonin and identify a conserved sequence in CCT subunits that may contribute to their binding to microtubules.

MATERIALS AND METHODS

Cells—The P19 mouse embryonal carcinoma cell line was obtained from Prof. Peter Andrews (University of Sheffield, Sheffield, United Kingdom) and maintained at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Paisley, UK) supplemented with 2 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 10% (v/v) fetal bovine serum (heat-inactivated; Sigma, Poole, UK). Cells were radiolabeled overnight (17 h) by replacing normal maintenance medium with 5 ml of medium containing only a 5% normal methionine and cysteine content and supplemented with 100 μCi of [35S]methionine (Trans-[35S]-label, ICN). Retinoic acid-induced differentiation in this MAP preparation (Fig. 1, lanes 3, 5) was at 35 °C in 90% PEM buffer for 15 min. Polymerization mixtures were then underlaid with 500 μl of 10% sucrose in the appropriate buffer and centrifuged at 30,000 × g for 30 min at 25 °C. After removal of the supernatant and careful washing, the pellet was resuspended in 200 μl of warm assembly buffer. MAPs were further purified away from tubulins (Fig. 1, lanes 3, 5) in 20 μl of MAPs (lane 4), 5 μg of phosphocellulose-purified tubulin (lane 3), and 5 μg of MAPs (lane 4), 5 μg of phosphocellulose-purified tubulin (lane 2), 5 μg of phosphocellulose-purified tubulin (lane 3), and 5 μg of MAPs (lane 4), and 5 μg of MAPs (lane 3). Molecular mass markers are 205, 116, 94, 65, 45, and 29 kDa.

Antibodies—Rabbit polyclonal antibodies were raised against key-homomemoycin conjugates (33) of peptide sequences taken from COOH-terminal sequences of murine CCT subunits (11) and from a sequence near the COOH terminus of the constitutive form of hsp70 (hsc70) (34). Sera were affinity-purified over the appropriate immobilized peptide (17).

RESULTS

CCT Subunits Are Present in Rat Brain MAP Preparations—Microtubules purified by three cycles of temperature-dependent assembly/disassembly were largely composed of α- and β-tubulins and the high molecular mass MAPs characteristic of brain microtubules (Fig. 1a, lane 2). The MAPs persisting through three microtubule assembly/disassembly cycles were further purified away from tubulins (Fig. 1a, lane 3) by phosphocellulose chromatography. This MAP preparation (Fig. 1a, lane 4) was predominately the high molecular mass MAP groups MAP1 and MAP2, but also contained small amounts of many other proteins. The Coomassie Blue-stained SDS-polyacrylamide gel resolution of these minor components was very different from the profile of polypeptides present in the original brain extract (Fig. 1a, lanes 1 and 4). Thus, their presence in this highly purified MAP preparation signified their selective purification along with tubulins during microtubule assembly/disassembly cycles. Several subunits of the cytoplasmic chaperonin CCT were detectable by immunoblotting in this MAP preparation (Fig. 1, lanes 1–4, lanes 1, 2, 3, 4). However, when compared with the CCT subunit profiles of the original brain extract (Fig. 1, lanes 1, 2, 3, 4), the CCTβ (Fig. 1c) and CCTε (Fig. 1d) subunits were depleted, whereas CCTγ (Fig. 1f, lower

**Fig. 1.** CCT subunits are present in MAPs prepared from threecycled rat brain microtubule protein. Shown are SDS-PAGE resolutions on 9% acrylamide gels detected by the following. a, Coomassie Blue staining of 15 μg of rat brain extract protein (lane 1), 5 μg of threecycled microtubule protein (lane 2), 5 μg of phosphocellulose purified tubulin (lane 3), and 5 μg of MAPs (lane 4). b–f, immunoblot detection of CCTα (b), CCTβ (c), CCTγ (upper band) and CCTδ (d), CCTε (e), and hsc70 (upper band) and CCTγ (f) 20 μg of rat brain extract protein (lanes 1), 5 μg of phosphocellulose-purified tubulin (lanes 2), and 5 μg of MAPs (lanes 3). Molecular mass markers are 205, 116, 94, 65, 45, and 29 kDa.

**Tubulin and MAP Purification from Thrice-cycled Rat Brain Microtubules—** Rat brains were homogenized in an equal volume of ice-cold 0.1 M Pipes-NaOH, pH 6.9, containing 2 mM EGTA, 1 mM MgCl2, and 1 mM NaN3 (90% PEM buffer) containing the protease inhibitors leupeptin (5 μg/ml), pepstatin (1 μg/ml), and phenylmethylsulfonyl fluoride (0.2 mM). The washed cell pellets were resuspended in 2–3 volumes of extraction buffer and homogenized by 20 passes in a glass-glass homogenizer. Homogenates were then centrifuged at 100,000 × g for 90 min at 4 °C in a Beckman TL100 benchtop ultracentrifuge. The resulting supernatants are referred to as the cell extracts.

20 S CCT Particle Purification—Chaperonin particles were purified as described (21) by fractionation on a sucrose gradient (10–40%) resolution of P19 cell and rat testis extracts. Sucrose gradient fractions containing 20 S particles were then concentrated and separated from proteasomes by anion-exchange chromatography over Resource Q (Amersham Pharmacia Biotech), eluting bound CCT with 300 mM NaCl in column buffer (17).
That stabilizes microtubules by binding to self-assembly are, however, very restrictive and can seldom be applied without modifications to other tissues or to cultured cells. Because we wished to determine the association of CCT subunits with microtubules under a wider variety of conditions, we examined MAPs purified with the aid of Taxol from the P19 cell lysates of the Taxol procedure are that it can be performed with small amounts of tissue or cells and under a wide variety of buffer conditions, including the absence of nucleotides. To obtain tubulin in such a way as to span adjacent protomofilaments in the tubule wall (39) and has been used to promote microtubule assembly in non-neuronal tissue and cell extracts. The two major advantages of the Taxol procedure are that it can be performed with small amounts of tissue or cells and under a wide variety of conditions.

Although P19N cell extracts were able to support self-assembly of microtubules in the presence of GTP, presumably due to the induction in these cells of microtubule assembly-promoting MAPs such as MAP2 and tau, Taxol was required for microtubule formation in embryonal carcinoma cell extracts (Fig. 2, a and b, lanes 2 and 7). Since it is generally held that a single round of Taxol-induced polymerization using defined centrifugation conditions yields microtubules at least as pure as those obtained after two or three cycles of the temperature-dependent assembly/disassembly procedure (26), we examined microtubules isolated from P19 cells by a single Taxol-induced assembly. This premise was confirmed by comparing MAPs isolated from mammalian tissues at low concentrations of tubulin.

MAPs from P19 Cells Contain CCT Subunits—Brain tissue is peculiarly amenable to purification of microtubule proteins by self-assembly because of its high concentrations of tubulin and microtubule-stabilizing MAPs such as MAP2 and tau. The conditions required for purification of microtubule proteins by self-assembly are, however, very restrictive and can seldom be applied without modifications to other tissues or to cultured cells. Because we wished to determine the association of CCT subunits with microtubules under a wider variety of conditions, we examined MAPs purified with the aid of Taxol from the P19 mouse embryonal carcinoma cell line. Taxol is a plant alkaloid that stabilizes microtubules by binding to \( \beta \)-tubulin in such a way as to span adjacent protofilaments in the tubule wall (39) and has been widely used to promote microtubule assembly in non-neuronal tissue and cell extracts. The two major advantages of the Taxol procedure are that it can be performed with small amounts of tissue or cells and under a wide variety of buffer conditions, including the absence of nucleotides.

In this study, we examined MAPs purified both from the rapidly proliferating, undifferentiated P19EC cells and from post-mitotic neuronal cultures (P19N) that were in fact >90% neurons with the residue <10% fibroblast-like cells. At the stage of differentiation examined, these neurons expressed many neuron-specific proteins, including the characteristic neuronal MAPs, MAP2, and tau, none of which were detectable in the undifferentiated embryonal carcinoma cells (data not shown).

Although P19EC cells were able to support self-assembly of microtubules in the presence of GTP, presumably due to the induction in these cells of microtubule assembly-promoting MAPs such as MAP2 and tau, Taxol was required for microtubule formation in embryonal carcinoma cell extracts (Fig. 2, a and b, lanes 2 and 7). Since it is generally held that a single round of Taxol-induced polymerization using defined centrifugation conditions yields microtubules at least as pure as those obtained after two or three cycles of the temperature-dependent assembly/disassembly procedure (26), we examined microtubules isolated from P19 cells by a single Taxol-induced assembly.

This premise was confirmed by comparing MAPs prepared by the Taxol procedure from rat brain extract (Fig. 2a, Br lane) with MAPs isolated from threecycled brain microtubules (Fig. 1a, lane 4), which contained a similar array of polypeptides. MAPs were readily liberated from these Taxol-stabilized microtubules by exposure to mild (0.36 M) salt treatment. Proteins displaced from Taxol-stabilized microtubules were shown in Fig. 2a (lanes 3 and 8, respectively). There were marked differences in the MAP profiles from P19EC and P19N microtubules are shown in Fig. 2a (lanes 3 and 8, respectively). There were marked differences in the MAP profiles from P19EC and P19N microtubules, and the compositions of both these MAP preparations were very different from the parental cell lysate profiles (Fig. 2a, lanes 1 and 6, respectively). Both these observations point to the selective nature of this MAP isolation procedure. Immunoblot analyses of these MAP preparations demonstrated the presence of CCT subunits (Fig. 2, c–i, lanes 3 and 8), although, as with brain MAP preparations, the relative contents of the CCT\( \beta \) and CCT\( \tau \) subunits were reduced compared with the corresponding cell lysates (Fig. 2, c–i, compare lanes 3 with lanes 1 and 8 with lanes 6). Once again, the levels of most CCT subunits in P19 cell MAPs were similar, relative to cell lysate control, to those of hsc70 (Fig. 2j). As a percentage of their total cell extract content (P19EC cells), the amounts of CCT subunits copurifying with MAPs varied from 0.4% for CCT\( \beta \) to 3% for CCT\( \tau \), with other CCT subunits at ~2%. In the case of P19 neurons, these percentages were approximately doubled because the cell lysate content of most...
CCT subunits in P19 neurons was less than half that in embryonal carcinoma cells.

The above P19 cell MAP preparations were from microtubules prepared in the absence of added nucleotides. Since all CCT subunits are ATP-binding proteins, it was considered of interest to determine whether nucleotides modulated CCT subunit-microtubule associations. Polymerization in the presence of the non-hydrolyzable ATP analog AMP-PNP increased the amounts of CCT subunits associated with P19EC and P19N microtubules (Fig. 2, c–i, lanes 4 and 9), even though the amounts of tubulin polymerized were somewhat lower under these conditions (Fig. 2b). It should also be noted that polymerization in the presence of AMP-PNP led to marked changes in the MAP profiles (Fig. 2a, lanes 4 and 9), with the prominent appearance of kinesin heavy chain (asterisks, identified only on the basis of size and properties) and possibly cytoplasmic dynein (arrows). Additionally along with kinesin, CCT subunits were displaced from the Taxol-stabilized P19 microtubules by exposure to MgATP alone (Fig. 2, c–i, lanes 4 and 9); subsequent exposure to mild salt MAP-eluting treatment displaced additional MAP polypeptides (Fig. 2a, lanes 5 and 10), although CCT subunits were not among these (c–i, lanes 5 and 10). The amounts of CCT subunits associated with microtubules could also be increased by introducing exogenous phosphocellulose-purified brain tubulin into the P19 extract polymerization mixtures (data not shown). Hsc70 was also detected in P19 cell MAP preparations (Fig. 2j). However, hsc70 association with tubulin was not increased by the presence of AMP-PNP. Furthermore, hsc70 was not so readily displaced from microtubules by ATP as were CCT subunits (Fig. 2, c–j, compare lanes 4 and 5 and lanes 9 and 10). This latter observation is similar to the partial release of hsp70 from flagellar axonomes by ATP reported by Bloch and Johnson (40).

**CCT Subunits in MAP Preparations Are Not Assembled in Chaperonin-sized Oligomers**—The levels of CCT subunits associated with polymerizing microtubules were non-stoichiometric compared with those in either cell extracts or purified P19 20 S CCT chaperonin particles (see below). This raised the question of whether the CCT subunits in MAP preparations were in a chaperonin particle of unusual subunit composition or free subunits or smaller oligomers. Electron microscopic examination of negatively stained P19EC cell MAP preparations (Fig. 3f) failed to identify any characteristic ring structures so easily discerned in partially purified (20 S) microtubules if present in abundance, sucrose gradient-purified P19EC chaperonin was added to salt-extracted, Taxol-purified P19EC microtubules (i.e. tubulin-only microtubules) (Fig. 3e). Chaperonin rings were readily observed in such a mixture (Fig. 3f).

The absence of normal CCT chaperonin particles in MAP preparations was confirmed by sucrose gradient analysis. The 20 S chaperonin particle normally fractionates at 20–22% sucrose on our 10–40% (w/v) sucrose gradient resolutions (17, 41). Exposure of P19EC cell extract to the salt concentrations used to displace CCTs from Taxol-stabilized microtubules (0.36 M) did not cause any change in this fractionation position (Fig. 4a, arrow). Immunoblot analyses of equivalent sucrose gradient fractionation of MAP preparations clearly showed the great majority of CCT subunits in MAPs migrating at the top of the gradient (Fig. 4, b–d), as did the majority of MAP polypeptides (Fig. 4e). Small amounts of the CCTγ subunit and, on prolonged exposures for ECL detection, very small amounts of the CCTγ and CCTθ subunits could be detected in the 20 S particle position of the gradient. We have noticed a tendency for free CCT subunits to reassemble into 20 S particles (data not shown) over a time span similar to the centrifugation time involved in the sucrose gradient resolution, and so these small amounts of 20 S particles may have re-formed from free CCT subunits in MAPs during the course of the experiment. However, it would appear that the bulk, and possibly all, of the CCT subunits isolated as MAPs were as free subunits, or “microcomplexes” (42), rather than as chaperonin particles.

**Subunits of Purified CCT Cosediment with Microtubules Polymerized from Pure Tubulin**—The presence of CCT subunits in MAP preparations could be due to direct interaction with tubulin or, more indirectly, to interaction with other MAPs, including folding cofactors (43, 44). To distinguish between these two possibilities, sedimentation analysis of polymerization mixtures, using rat brain phosphocellulose-purified tubulin with purified P19EC and rat testis CCTs, was carried out. The components of the polymerized material from such incubations are shown in Fig. 5a. Under the centrifugation conditions employed, P19 20 S CCT particles did not sediment (Fig.
The selective association of CCT subunits with tubulin is dependent on polymerization. In gel filtration analysis of CCT/dimeric tubulin mixtures with tubulin levels below the critical concentration for microtubule assembly, only CCTs could be detected in the tubulin dimer-containing fractions (data not shown).

In addition to some CCT subunits, several other polypeptides present in small amounts in purified P19EC CCT chaperonin were concentrated into the assembled microtubule pellets (Fig. 5a, lane 4, arrows). The identities of these polypeptides at 183, 167, 154, 102, 92, and 40 kDa and the doublet centered at 72 kDa are not known. Neither band in the 72-kDa doublet was recognized by our rabbit anti-hsc70 polyclonal antibody. A similar range of polypeptides, also present in small amounts in the CCT preparation but strikingly concentrated into assembled microtubules, was detected in CCT purified from rat testis (data not shown).

The binding of MAPs such as MAP2, tau, and cytoplasmic dynein to tubulin involves the highly acidic COOH termini of tubulins. Removal of this domain by limited subtilisin digestion abolishes binding of MAP2 and cytoplasmic dynein without diminishing the ability of the residual tubulin to polymerize (e.g. Ref. 32). When subtilisin-digested tubulin was polymerized in the presence of CCT, the subunits associated with the microtubules formed (Fig. 6). However, the selectivity in the associating CCT subunits, which we had found with whole tubulin, was no longer operative. The CCTβ and CCTε subunits, which did not associate with polymerizing whole tubulin, were associated with subtilisin-digested tubulin polymers to the same extent as the other CCT subunits. It may be the case that removal of the tubulin COOH-terminal domains generates a tubulin or microtubule species recognizable as a folding substrate by the oligomeric assembly of CCT subunits, i.e. by the CCT chaperonin particle, required for the correct folding of newly synthesized tubulins. We further note that, in contrast to the lack of effect of CCT on the yield of whole tubulin polymer, subtilisin-digested tubulin polymerization was significantly enhanced by the presence of CCT (Fig. 6a). This and the loss of selectivity in microtubule-associated CCT subunits after subtilisin digestion of tubulin highlight the non-chaperonin-like nature of the selective association of CCT subunits with polymerizing whole tubulin and also argue against the observed association merely arising from CCT whole complex binding to denatured tubulin in the preparation.

**DISCUSSION**

In pilot studies to confirm and extend the findings of Brown et al. (16), we observed, by immunofluorescence microscopy, both CCTα and CCTγ as components enriched at the centrosome (data not shown) and, by immunoblotting, CCT components in brain MAP preparations. This report addresses the basic question of whether or not CCT subunits are MAPs. Some CCT subunits certainly do fulfill the generally accepted biochemical criteria for defining proteins as MAPs. The association of CCT subunits with microtubules is, in several ways, similar to that between microtubules and the hsc/hsp70 family of molecular chaperones (37, 38, 45). Neither molecular chaperone (CCT nor hsc70) is quantitatively removed from cell extracts by assembling microtubules (Ref. 38 and this report). Immunofluorescence detection of both chaperones produces diffuse staining in the cytoplasm (9, 16, 17, 40) rather than a microtubular fibrous staining exhibited by antibodies to some of the structural MAPs (e.g. MAP4 in Ref. 46) or the punctate, vesicular cytoplasmic staining reported for the motor protein kinesin (e.g. Ref. 47). Neither CCT subunits nor hsc70 stimulates tubulin assembly in vitro (Ref. 38 and this report); and finally, both chaperones are dissociated from microtubules by ATP (Refs. 38 and 40 and this report), although we found CCT...
subunits to be more readily displaced than hsc70. We therefore conclude that certain CCT subunits behave as MAPs in a similar way to members of the hsc/hsp70 family that are already classified as MAPs (37, 38, 45).

The presence of partially denatured tubulin molecules in standard microtubule preparations might be expected to attract the attentions of molecular chaperones, particularly the CCT chaperonin, and this could explain their behavior as MAPs. However, the tubulin/actin folding activity of CCT is understood to require the 20 S chaperonin particle containing all eight CCT subunits (6, 12, 15, 42). The data in this present report suggest that removal of the tubulin COOH termini (residues 439–451 and 434–445 for α- and β-tubulins, respectively) (48) by subtilisin generates a form of tubulin that is indeed recognized as a folding substrate for the CCT chaperonin containing all eight subunits. Dobrynski et al. (49) have similarly reported that in vitro translation of β-tubulin mRNA lacking 27 residues from the COOH terminus causes arrest of the resulting polypeptide on the CCT complex. Thus, the presence of selected CCT subunits in MAP preparations possibly points to functions of some CCT subunits in association with microtubules other than, or in addition to, those undertaken by these subunits when they are incorporated into the core 20 S chaperonin particle. Indeed, we have shown here that the CCT subunits associated with microtubules appear to be free subunits or microassemblies rather than 20 S oligomeric CCT, although from their fractionation position in sucrose gradients, it seems more likely that these microtubule-associated CCT subunits are actually free subunits rather than the microassemblies described by Liou and Willison (42).

The dissociation constants for the binding of selected CCT subunits to polymerized tubulin are ≅0.1 μM (e.g. Fig. 5f). Since the concentration of CCT in the cytosol is between 1 and 2 μM (9, 50, 51), there should be significant CCT subunit binding to microtubules in the cell, even if only 5% of CCT is in the form of free subunits (42); we have more recent evidence, however, that in cells, the proportion of CCT in the form of free subunits is likely to be substantially >5%.2 The low stoichiometry of CCT subunit binding to microtubules is not without precedent.

FIG. 5. Selected CCT subunits cosediment with polymerized pure tubulin. a–d, shown are SDS-PAGE resolutions on 9% acrylamide gels of sucrose gradient-purified P19EC CCT (lanes 1) and of material sedimented at 30,000 × g for 30 min at 25 °C following incubation of 200 μg/ml P19EC CCT alone (lanes 2), 920 μg/ml phosphocellulose-purified tubulin alone (lanes 3), and 200 μg/ml P19EC CCT plus 920 μg/ml tubulin at 35 °C for 15 min with 20 μM Taxol (lanes 4). Gel loadings were 0.1 μg of CCT (lanes 1) and 1% of the total pelleted material (lanes 2–4). Proteins were detected by silver staining (a) and by immunoblotting and probing for CCTα (b), CCTγ (upper band) and CCTδ (c), and CCTζ (d). Molecular mass markers are 205, 116, 94, 68, 45, and 29 kDa. e and f, Scatchard analyses of CCT-microtubule binding. e, radiolabeled P19 CCT (11,300 dpm/μg of protein), purified by sucrose gradient and anion-exchange resolutions, at 8–45 μg/ml was incubated with 240 μg/ml tubulin and 20 μM Taxol at 35 °C for 15 min. Radioactivity in the washed and resuspended microtubule pellets (125 μg/ml protein) was determined. f, purified P19 CCT at 13.5–67.5 μg/ml was incubated with 125 μg/ml tubulin and 20 μM Taxol for 15 min at 35 °C. CCTζ content in the washed and resuspended microtubule pellets (24 μg/ml protein) was measured by immunoblot analysis together with a standard range of CCT concentrations.

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among MAPs (for example, the STOP protein (52)). Restriction of binding to a specific subset of tubulin molecules, e.g. GTP-tubulin or some post-translationally modified tubulin, could explain the low stoichiometry observed. At present, we can only speculate on the functions of these free CCT subunits. The observation that most are associated only with polymerizing tubulin may indicate a role for these subunits in facilitating tubulin polymerization in the intracellular environment, a role already suggested by the requirement for CCTα in centrosome-nucleated microtubule growth (16). Alternatively, the association of CCT subunits with microtubules may relate to microtubule-based transport or motility, a function already suggested for microtubule-associated hsc70 (38).

If some CCT subunits can associate with microtubules in a way other than via the proposed subunit apical domains that recognize unfolded protein substrates including tubulins (50), what region of the subunits might bind to microtubules? Using synthetic oligonucleotides devised for the purpose of probing the VPGGGG motif present in MAP2, MAP4, and tau sequences (54) is compared with CCT subunit sequences from Kubota et al. (11).

formation changes in the CCT subunits consequent to ATP hydrolysis could well affect accessibility of this motif for binding to microtubules. However, the synthetic peptide VPGGGA did not compete with CCT subunits for binding to microtubules (data not shown), and so if this motif is involved in CCT subunit association with microtubules, other as yet unidentified regions of the CCT subunits must also be required for binding to occur. Alternatively, as we noted with polymerization of pure tubulin in the presence of purified CCT, certain polypeptides in these CCT preparations other than CCT subunits were quite strikingly concentrated into assembling microtubules (Fig. 5). It is quite plausible that some of these proteins, which may be naturally associated with the CCT chaperonin, are the actual mediators of interactions between CCT subunits and microtubules.

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