Chaperonin-mediated Folding of Vertebrate Actin-Related Protein and γ-Tubulin

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Abstract. The folding of actin and tubulin is mediated via interaction with a heteromeric toroidal complex (cytoplasmic chaperonin) that hydrolyzes ATP as part of the reaction whereby native proteins are ultimately released. Vertebrate actin–related protein (actin-RPV) (also termed centractin) and γ-tubulin are two proteins that are distantly related to actin and tubulin, respectively: γ-tubulin is exclusively located at the centrosome, while actin-RPV is conspicuously abundant at the same site. Here we show that actin-RPV and γ-tubulin are both folded via interaction with the same chaperonin that mediates the folding of β-actin and α- and β-tubulin. In each case, the unfolded polypeptide forms a binary complex with cytoplasmic chaperonin and is released as a soluble, monomeric protein in the presence of Mg-ATP and the presence or absence of Mg-GTP. In contrast to α- and β-tubulin, the folding of γ-tubulin does not require the presence of cofactors in addition to chaperonin itself. Monomeric actin-RPV produced in in vitro folding reactions cocycles efficiently with native brain actin, while in vitro folded γ-tubulin binds to polymerized microtubules in a manner consistent with interaction with microtubule ends. Both monomeric actin-RPV and γ-tubulin bind to columns of immobilized nucleotide: monomeric actin-RPV has no marked preference for ATP or GTP, while γ-tubulin shows some preference for GTP binding. We show that actin-RPV and γ-tubulin compete with one another, and with β-actin or α-tubulin, for binary complex formation with cytoplasmic chaperonin.

I T is generally accepted that the information contained in the linear sequence of amino acids of a given protein is sufficient to dictate its functional three dimensional structure (Anfinsen, 1973; Jaenicke, 1991). Though in some cases protein folding is a spontaneous process, it is now clear that many proteins assume their correct three dimensional structure via interaction with a class of proteins or protein complexes known as molecular chaperones (for review see Rothman, 1989; Ellis et al., 1991; Gething and Sambrook, 1992). Chaperones use the energy of ATP hydrolysis as part of the mechanism whereby correctly folded proteins are generated, and are thought to function by stabilizing the conformation of intermediates that ultimately lead to the formation of native protein. Two major classes of chaperones have been defined. The first, exemplified by the heat-shock protein hsp70, function as monomers or dimers, and interact with polypeptides so as to maintain them in an unfolded or partially unfolded state (for review see Welch, 1991). The second, exemplified by the heat-shock protein hsp60, function as multisubunit toroidal complexes that provide a sequestered environment in which facilitated protein folding can take place (Bochkareva et al., 1988; Cheng et al., 1989; Martin et al., 1991; Goloubinoff et al., 1989; Ostermann et

al., 1989; Viitanen et al., 1992; Phipps et al., 1993). These latter complexes are termed chaperonins.

In the cytoplasm of eukaryotic cells, the folding of actin and tubulin are facilitated via interaction with a multisubunit heteromeric chaperonin (Gao et al., 1992, 1993; Frydman et al., 1992; Yaffe et al., 1992). The folding reaction takes place in two steps: the rapid, ATP-independent formation of a binary complex between the target protein and chaperonin, followed by a slower, ATP-dependent release of the native protein (Gao et al., 1992, 1993; Frydman et al., 1992). To investigate the potential involvement of the same chaperonin in the folding of actin- and tubulin-related polypeptides, we decided to see whether two recently described proteins, actin-RPV (Lees-Miller et al., 1992) (also termed centractin [Clark and Meyer, 1992]) and γ-tubulin (Oakley and Oakley, 1989; Oakley et al., 1990; Zheng et al., 1991; Stearns et al., 1991; Joshi et al., 1992), can be folded via interaction with cytoplasmic chaperonin. These proteins are of particular interest because of their distinctive subcellular localization: γ-tubulin is present exclusively at the centrosome (Zheng et al., 1991; Stearns et al., 1991), while actin-RPV is abundantly present at the same location (Clark and Meyer, 1992) and also in association with the dynactin complex, a multisubunit complex involved in microtubule based vesicle motility (Lees-Miller et al., 1992). Microinjection of anti-γ-tubulin antibodies strongly suggests that γ-tubulin is es-

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sential for microtubule nucleation at the centrosome (Joshi et al., 1992); however, the solubility and other properties of this low-abundance protein are completely unknown. Here we show that actin-RPV and γ-tubulin both undergo facilitated folding via interaction with cytoplasmic chaperonin, followed by their release as soluble monomeric proteins. We describe the properties of these proteins in terms of their ability to bind nucleotide, to coassemble with their homologs, and to compete with other cytoskeletal proteins for association with cytoplasmic chaperonin.

Materials and Methods

Expression of Labeled Actin-RPV and γ-Tubulin in E. coli

A full length cloned cDNA encoding human γ-tubulin (Zheng et al., 1991) was engineered into the *E. coli* expression vector pET11a (Studier et al., 1990) in a three-way ligation containing the 4.3-kb *NdeI-EcoRI* fragment derived from the pET11a vector, a 1.5-kb *BglI-EcoRI* fragment encoding sequences downstream from nucleotide 38 of the γ-tubulin cDNA, and the synthetic oligonucleotides 5'TATGCCGAGGGAAATCATCACCCTACAG-TTGGGCCAGTG3' and 5'TGGCCCAACTGCAGGGTGATGATTTCCC-TCGGCA3', included so as to provide sequences encoding amino acids 1-13. The resulting clone and a cloned cDNA encoding the complete coding region of human actin-RPV in pET8c (Lees-Miller et al., 1992) were expressed in *E. coli* BL21DE3 as [^{35}S]-methionine-labeled proteins under conditions where host mRNA synthesis was inhibited by incubation in the presence of rifampicin (Studier et al., 1990; Gao et al., 1992,1993). The insoluble recombinant proteins were purified from host *E. coli* cells and denatured by solubilization in guanidine-HCl as described previously (Gao et al., 1992); specific radioactivities were in the range 2-5 × 10^6 cpm/μg.

Preparation of Cytoplasmic Chaperonin

Biochemically homogeneous cytoplasmic chaperonin was prepared either from rabbit reticulocyte lysate or from bovine testis by the methods described (Gao et al., 1992; Frydman et al., 1992). The biological activity of each preparation was checked by assaying its ability to facilitate the folding of β-actin in vitro as described previously (Gao et al., 1992).

Purification of Actin and Tubulin

Actin. Actin was extracted from homogenized mouse brains as described (Levilliers et al., 1984). After two cycles of polymerization/depolymerization, the product was flash-frozen at 1.25 mg/ml in liquid N$_2$ and stored at −70°C.

Tubulin. Tubulin was prepared from bovine brain by three cycles of assembly and disassembly (Shelanski et al., 1973) followed by chromatography on phosphocellulose (Weingarten et al., 1975). The product was concentrated by ultrafiltration and stored at −70°C in 0.1 M Pipes, pH 6.9, 0.5 mM EGTA, 0.25 mM MgCl$_2$, 3.4 M glycerol, and 0.2 mM GTP at a concentration of 6 mg/ml.

In Vitro Translation and Folding Reactions

RNA was transcribed from recombinant pET plasmids containing cDNAs encoding complete actin-RPV or γ-tubulin polypeptides (described above) using T7 polymerase (Melton et al., 1984). Aliquots (1 μg) of these RNAs were added to micrococcal nuclease-treated rabbit reticulocyte lysate (25 μl) (Promega Corp., Madison, WI) supplemented with 10 μCi [^{35}S]-methionine and incubated for 1 h at 30°C. In vitro folding reactions were performed by rapidly diluting labeled, denatured actin-RPV or γ-tubulin (prepared as described above) at a ratio of 1:100 into either reticulocyte lysate that had been cleared of particulate material by centrifugation for 15 min at 200,000 g, or into buffer A (20 mM MES, pH 6.9, 0.1 M KCl, 1 mM MgCl$_2$, 0.5 mM EGTA, 1 mM DTT) supplemented with 1 mM ATP and containing purified chaperonin (6 pmol). In some experiments (see text), the in vitro folding reactions also contained 1 mM GTP. Control folding reactions done in buffer A without chaperonin were supplemented with BSA (0.8 mg/ml). Folding reactions were incubated for 2 h at 30°C and applied to a Superose 6 HR 10-30 gel filtration column (Pharmacia LKB Biotech-

nology, Piscataway, NJ) equilibrated and run at 4°C in buffer A without nucleotide and containing 10% glycerol.

Nucleotide-binding Properties of In Vitro Generated Actin-RPV and γ-Tubulin

Material from in vitro translation reactions emerging from the Superose 6 column (see above) with an apparent molecular weight of 40-45 kD was divided into two equal portions; each was applied to a column of either agarose-bound ATP or GTP (Sigma Chem. Co., St. Louis, MO) (0.5 × 4.0 cm) equilibrated in buffer A. The columns were extensively washed with buffer A until no further radioactivity was detectable in the column eluate. Material bound to the columns was eluted with 5 mM Mg-ATP in buffer A (in the case of ATP-agarose) or 5 mM Mg-GTP in buffer A (in the case of GTP-agarose), and quantitated by scintillation counting.

Coassembly Reactions

Actin-RPV. Labeled monomeric actin-RPV synthesized by translation in vitro or the products of an in vitro folding reaction done with purified chaperonin were fractionated on a Superose 6 gel-filtration column. Material emerging from this column with an apparent molecular weight of 40-45 kD was incubated with unlabeled mouse brain globular actin (0.85 μg/ml) in a buffer (buffer B) containing 5 mM Tris-HCl, pH 7.2, 0.2 mM CaCl$_2$, 0.5 mM 2-mercaptoethanol, 0.005% NaN$_3$ and 0.2 mM ATP. Polymerization was induced by the addition of KCl to 0.1 M and MgCl$_2$ to 1 mM, followed by incubation at 25°C for 1 h. Polymerized material was pelleted by centrifugation at 200,000 g for 30 min at 20°C. The pellet was rinsed and resuspended in the initial volume of ice-cold buffer B, and incubated on ice overnight. Aggregated material was removed by centrifugation at 200,000 g for 10 min at 4°C, polymerization reinduced in the supernatant material, and the polymerized material recovered as described above. Aliquots of supernatants and resuspended pellets were analyzed by electrophoresis on SDS-polyacrylamide gels followed by fluorography.

γ-tubulin. Bovine brain phosphocellulose-purified tubulin (Weingarten et al., 1975) (4 mg/ml) was assembled in 0.1 M Pipes, pH 6.9, 6 mM MgCl$_2$, 0.5 mM EGTA, 0.5 mM GTP (buffer C) containing 30% glycerol at 37°C for 15 min. The products of a γ-tubulin in vitro translation reaction or a γ-tubulin in vitro folding reaction done with homogeneous cytoplasmic chaperonin were resolved on Superose 6 as described above. Material emerging from the column with an apparent molecular weight of 40-45 kD was added either to assembled microtubules or to unpolymerized tubulin. The mixtures were incubated for 15 min at 37°C, loaded onto cushions of 30% sucrose in buffer C, and centrifuged at 200,000 g for 30 min at 37°C. Pellets were rinsed with warm buffer C and depolymerized by resuspension in ice-cold buffer C for 30 min. After clarification of depolymerized material, a second polymerization reaction was initiated by addition of an equimolar amount of taxol and incubation at 37°C for 15 min. Microtubules were then sedimented as described above. Aliquots of resuspended pellets and supernatants were analyzed by autoradiography after SDS-PAGE. In experiments to determine the binding of γ-tubulin to microtubule ends, phosphocellulose-purified tubulin (60 μM) was polymerized by the addition of 1.5 molar equivalents of taxol. In some experiments, the assembled microtubules were sheared by repeated passage through a 25-gauge needle. Microtubules were diluted with buffer C containing 40 μM taxol before the addition of in vitro generated γ-tubulin and incubated for 10 min at 37°C. Reaction products were analyzed by sedimentation through sucrose cushions as described above.

Competition Experiments

Unlabeled target proteins for use in competition experiments were expressed in 25-ml cultures of *E. coli* BL21DE3 as described (Gao et al., 1993). Labeled, denatured actin-RPV or γ-tubulin was added to increasing amounts of unlabeled, denatured competitor (see text), and the mixtures used as probes in dilution assays containing purified cytoplasmic chaperonin such that each reaction contained 0.4 pmol of labeled target protein. Dilution assays were done essentially as described above, except that the dilution buffer contained 20 mM MES, pH 6.9, 20 mM KCl, 2 mM MgCl$_2$, 1 mM EGTA, 1 mM DTT. After incubation for 10 min at 20°C to allow binary complex formation, the reaction products were analyzed on non-denaturing composite agarose/polyacrylamide gels containing 0.5% agarose and 3.0% polyacrylamide, 80 mM MES, pH 6.9, 1 mM EDTA. The gels were run in the same buffer in a Miniprotean gel apparatus (Biorad Labs., Richmond, CA) at 90 V for 1 h, stained with Coomassie blue, destained, and fluorographed.
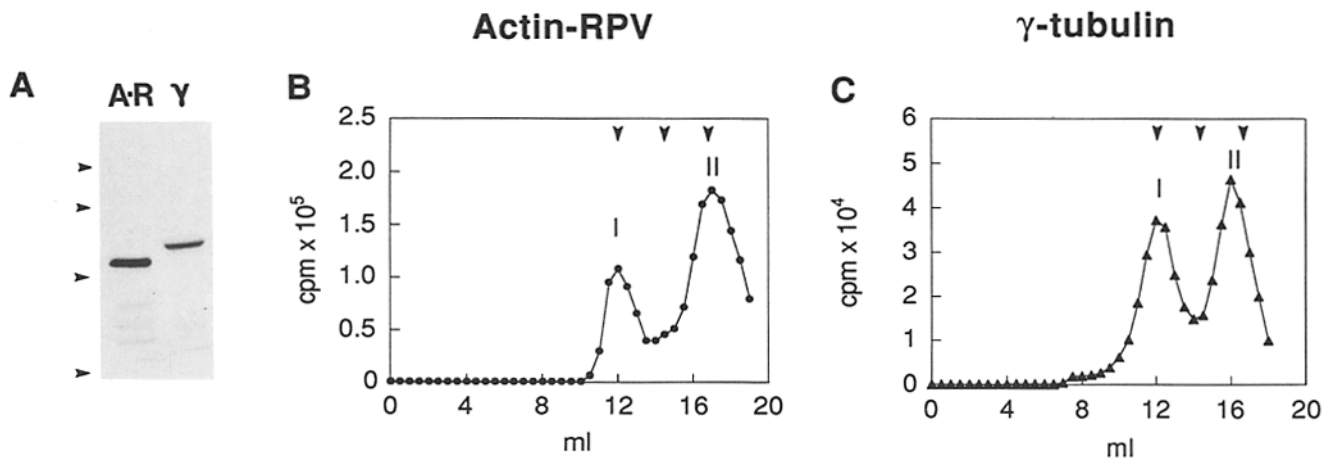
Actin-RPV

γ-tubulin

Figure 1. Gel-filtration analysis of in vitro translated actin-RPV and γ-tubulin. (*A*) Analysis on a 8% SDS-polyacrylamide gel of in vitro translation reactions containing mRNA encoding human actin-RPV (lane marked *A-R*) and mRNA encoding human γ-tubulin (lane marked γ). Location of molecular weight markers (97, 66, 44, and 21 kD) are shown at the left. (*B* and *C*) Elution profiles of the same in vitro translation reactions shown in A on a Superose 6 gel-filtration column. Arrows show the location of molecular size markers (thyroglobulin, 670 kD; immunoglobulin G, 158 kD; and ovalbumin, 44 kD) run under identical conditions on the same column.

Results

Expression of Actin-RPV and γ-Tubulin in Reticulocyte Lysate

We first characterized the products of in vitro translation reactions containing mRNA encoding actin-RPV or γ-tubulin (Fig. 1*A*). The products of the in vitro translation reactions were analyzed under native conditions by gel filtration on Superose 6. In the case of both actin-RPV and γ-tubulin, ~40% of the labeled protein appeared in a peak with an apparent molecular weight of ~700 kD (Fig. 1, *B* and *C* peak I). The behavior of this material on gel filtration was identical to that of binary complexes formed between β-actin or α-, or β-tubulin and cytoplasmic chaperonin (Gao et al., 1992, 1993; Yaffe et al., 1992). In addition, a second peak containing ~60% of the labeled protein emerged with an apparent molecular weight of 40–45 kD (Fig. 1, *B* and *C* peak II), consistent with the generation of monomeric actin-RPV or γ-tubulin.

Chaperonin-mediated Folding of Actin-RPV and γ-Tubulin in vitro

The data presented in Fig. 1 suggested that, as in the case of β-actin and α- and β-tubulin, newly synthesized actin-RPV and γ-tubulin polypeptides become associated with cytoplasmic chaperonin as binary complexes which are then discharged as native monomeric proteins. To see whether this was indeed the case, we did folding reactions in which labeled, denatured actin-RPV or γ-tubulin were used as target proteins in folding reactions performed in vitro. Labeled, denatured target proteins for use in these in vitro folding reactions were generated by expressing cDNAs encoding the complete actin-RPV or γ-tubulin polypeptides in *E. coli* in the presence of [³⁵S]methionine under conditions where host protein synthesis was suppressed (Studier et al., 1990). This resulted in the accumulation of large quantities of recombinant protein (Fig. 2 *A*). The insoluble nature of the bacterially expressed products enabled us to purify them from the bulk of host proteins, so as to yield material that
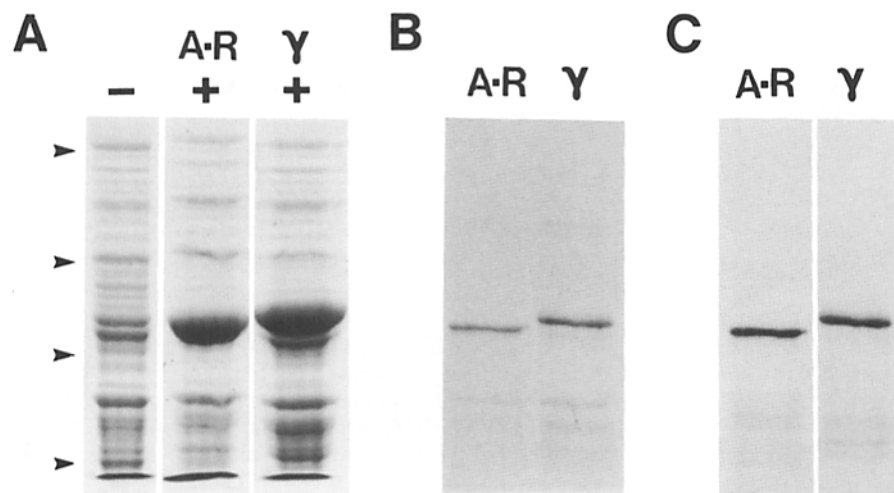

Figure 2. Expression of labeled actin-RPV and γ-tubulin in *E. coli*. Analysis on a 8% SDS-polyacrylamide gel of total extracts of host *E. coli* cells or purified insoluble inclusion bodies. (*A*) Lanes marked (−), total extract of uninduced cells; lanes marked *A-R* and γ (+), total extract of cells expressing actin-RPV and γ-tubulin, respectively. (*B*) Purified inclusion bodies from cells expressing actin-RPV (*A-R*) and γ-tubulin (γ), respectively. (*C*) Autoradiograph after fluorography of the material shown in *B*. Arrows indicate the location of the same molecular size markers shown in Fig. 1 *A*.
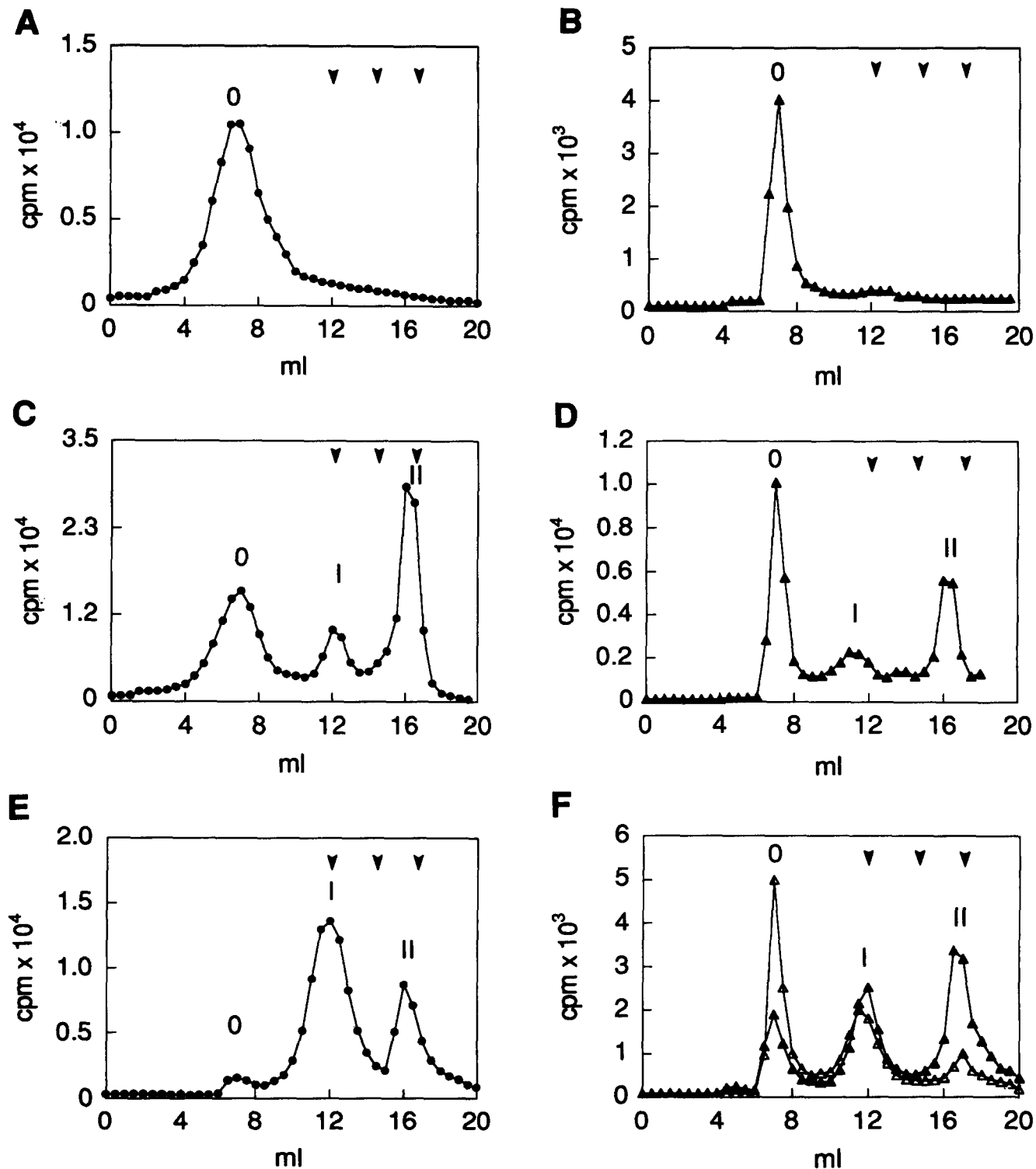
Actin-RPV   **γ-tubulin**

Figure 3. Gel-filtration analyses of the products of in vitro folding reactions. Elution profiles of in vitro folding reactions done using labeled, denatured actin-RPV (*A*, *C*, and *E*) or γ-tubulin (*B*, *D*, and *F*) as target protein and applied to a Superose 6 gel-filtration column. Folding reactions were done in which target proteins were diluted into buffer containing BSA (*A* and *B*), unfractionated rabbit reticulocyte lysate (*C* and *D*), or homogeneous cytoplasmic chaperonin supplemented with Mg-ATP and/or Mg-GTP (*E* and *F*). In *F*, analysis of γ-tubulin in vitro folding reactions done with Mg-ATP and Mg-GTP (▲) or with Mg-ATP alone (△) are shown. Arrows indicate the location of the same molecular size markers shown in Fig. 1, *B* and *C*.

was at least 80% biochemically pure as judged by Coomassie blue staining of an SDS-polyacrylamide gel (Fig. 2 B), and at least 90% radiochemically pure as judged by autoradiography of the same material (Fig. 2 C). The labeled bacterially expressed insoluble polypeptides were denatured with guanidine-HCl and urea and used as target proteins in folding assays in which they were rapidly diluted from denaturant. After incubation at 30°C, the reaction products were analyzed by gel filtration chromatography.

As a control, we first investigated the effect of diluting the labeled, denatured target proteins into buffer containing BSA as a potential stabilizing protein. These experiments resulted in the emergence of all the radioactivity in the column void volume, presumably reflecting the generation of aggregates of high molecular mass (Fig. 3, A and B, peak 0). In contrast, when the same target proteins were diluted into unfractionated reticulocyte lysate, three peaks emerged. The first (peak 0) appeared in the column void volume, a second (peak I) with an apparent molecular weight of 700 kD, and a third (peak II) with an apparent molecular weight of 40–45 kD; the relative abundance of radioactivity in these peaks was 41, 17, and 42% in the case of actin-RPV (Fig. 3 C) and 43, 23, and 34% in the case of γ-tubulin (Fig. 3 D). Peaks I and II correspond in apparent molecular mass with the products obtained in the in vitro translation experiments described in Fig. 1. Thus, both in vitro translation and protein folding reactions in unfractionated reticulocyte lysate resulted in the production of complexes of high molecular mass as well as species that behaved as soluble monomers.

To test whether the high molecular weight species were binary complexes formed between radiolabeled target proteins and cytoplasmic chaperonin, we did in vitro folding reactions in which the labeled, denatured target proteins were diluted into biochemically homogeneous cytoplasmic chaperonin and Mg-ATP. The results of these experiments are shown in Fig. 3, E and F. Upon gel-filtration analysis of the actin-RPV reaction products, 66% of the radioactivity emerged with an apparent molecular weight of ∼700 kD, and 29% with an apparent molecular weight of 40–45 kD; the corresponding figures for a γ-tubulin folding reaction (done in the presence of Mg-ATP and Mg-GTP, see below) were 34 and 47%, respectively. We conclude that cytoplasmic chaperonin alone is capable of folding denatured actin-RPV or γ-tubulin, and that the resulting soluble proteins behave as monomers upon gel filtration.

Since α- and β-tubulin, the homologs of γ-tubulin, are both GTP-binding proteins, we did γ-tubulin in vitro folding reactions using purified cytoplasmic chaperonin and Mg-ATP both with and without the addition of Mg-GTP (Fig. 3 F). The inclusion of Mg-GTP in the folding reactions greatly enhanced the yield of soluble 40–45 kD product compared with a reaction that contained Mg-ATP alone (Fig. 3 F, peak II). These data suggested that γ-tubulin, like α- and β-tubulin, is a GTP-binding protein; this notion was further investigated in experiments described below.

Coassembly Properties of In Vitro Generated Polypeptides

To determine whether the 40–45 kD products produced in our in vitro translation and chaperonin-mediated folding reactions were correctly folded, we tested their ability to copolymerize with either actin (in the case of actin-RPV) or

tubulin (in the case of γ-tubulin). The 40–45 kD protein produced by in vitro translation of actin-RPV mRNA (Fig. 1 B) was cocycled with mouse brain actin, and the polymerized and nonpolymerized material separated by centrifugation. Material in the supernatants and pellets obtained after the first and second polymerization/depolymerization cycles was examined by SDS-PAGE. Approximately 80% of the labeled actin-RPV copolymerized with actin filaments after the first cycle; after the second cycle, ∼95% of the labeled actin-RPV was contained in the pellet (Fig. 4 A). In a parallel experiment done with actin-RPV produced in an in vitro chaperonin-mediated folding reaction, ∼95% of the labeled actin-RPV was found in the pellet after either the first or second polymerization cycle (Fig. 4 B). The polymerized material obtained in these experiments might represent copolymers with unlabeled brain actin; alternatively, it could represent polymers of actin-RPV on its own. To distinguish between these two possibilities, we did a control experiment in which actin-RPV generated via a chaperonin-mediated in vitro folding reaction was incubated in the absence of carrier actin under conditions in which actin monomers polymerize (Fig. 4 C). No detectable radioactivity was found to sediment in a manner consistent with the behavior of polymerized material. From these data, we conclude that actin-RPV produced either by translation in reticulocyte lysate or by folding in vitro by homogeneous cytoplasmic chaperonin can efficiently copolymerize with native actin.

We investigated the biological activity of γ-tubulin produced in our translation and in vitro folding reactions in parallel experiments by assaying its ability to cocycle with phosphocellulose-purified brain tubulin. After depolymerization of the first cycle pellet, tubulin was assembled into microtubules by the addition of taxol. At the end of each polymerization reaction, polymerized material was sedimented through a sucrose cushion, a procedure that allows only the cosedimentation of proteins that are tightly bound to microtubules (Melki et al., 1991). After the first cycle, ∼40% of the labeled γ-tubulin produced by in vitro translation was found in the pellet; upon depolymerization and addition of taxol, virtually all the radioactivity was found to cosediment with microtubules (Fig. 4 D). In the corresponding experiment done with material produced in an in vitro folding reaction containing purified chaperonin, ∼80% of the labeled γ-tubulin was found in the pellet after the first cycle, whereas after the second cycle, the pellet contained ∼95% of the labeled protein (Fig. 4 E).

Because of its centrosomal location, it has been proposed that the function of γ-tubulin is to nucleate microtubules (Oakley et al., 1990; Stearns et al., 1991; Joshi et al., 1992). This concept implies that γ-tubulin can interact with microtubule minus ends. To test this notion, we did an experiment in which a constant amount of labeled, in vitro generated monomeric γ-tubulin was assayed for its ability to bind to taxol-stabilized microtubules over a range of concentrations. A constant amount of γ-tubulin was incubated with different concentrations of microtubules averaging either 11 ± 3 μm or 1.2 ± 0.4 μm in length (as measured by electron microscopy; the latter polymers were generated by shearing through a narrow gauge needle) and the reaction products analyzed by sedimentation through a sucrose cushion. This experiment (Fig. 5) showed that the extent to which γ-tubulin bound to microtubules was highly dependent on the num-

Actin-RPV

A 1st 2nd   **B** 1st 2nd   **C**

γ-tubulin
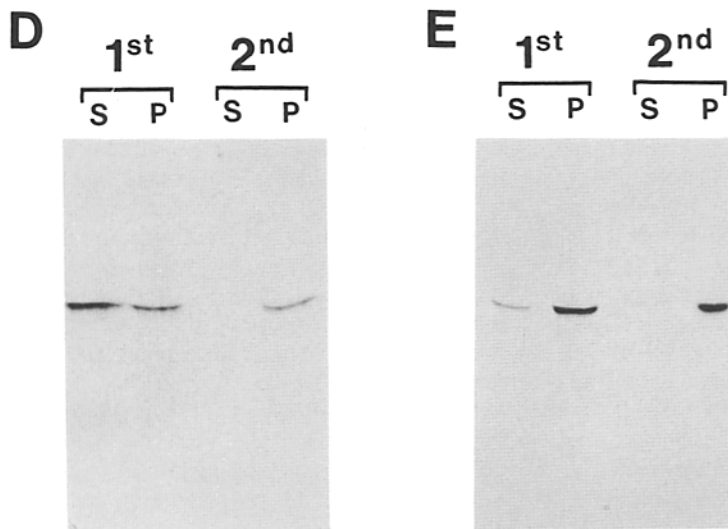
D 1st 2nd   **E** 1st 2nd
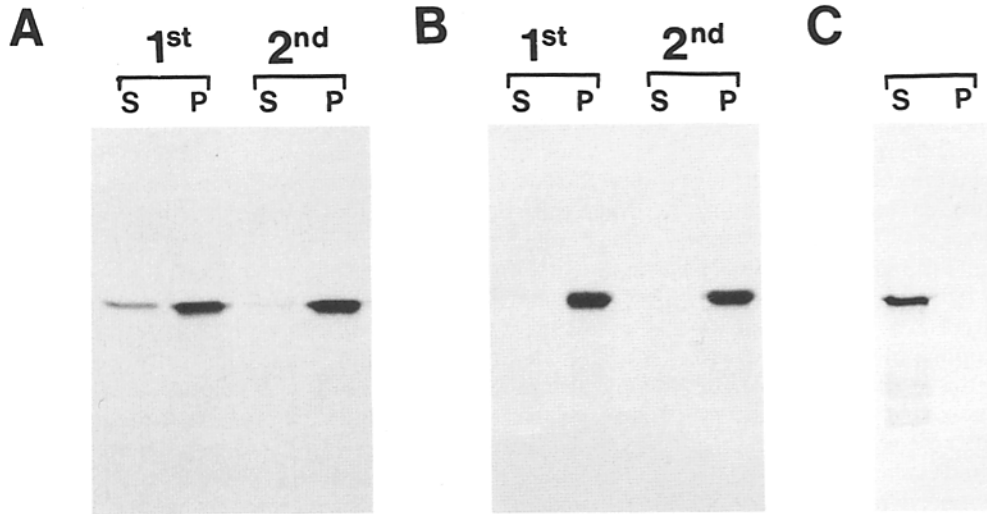
Figure 4. Cocycling with brain actin or tubulin of labeled actin-RPV or γ-tubulin translated or folded in vitro. Analysis on 8% SDS-polyacrylamide gels of the supernatants (*S*) and pellets (*P*) derived from cocycling experiments in which labeled actin-RPV (*A-C*) or γ-tubulin (*D* and *E*) produced either by translation in vitro (*A* and *D*) or by folding in vitro (*B, C,* and *E*) were tested for their ability to cocycle through one (*1st*) and two (*2nd*) cycles of polymerization and depolymerization with unlabeled mouse brain actin (in the case of actin-RPV) or unlabeled bovine brain tubulin (in the case of γ-tubulin).

ber of available ends. These data, and the copolymerization experiments described above (Fig. 4, *D* and *E*), strongly suggest that the products of our γ-tubulin translation and in vitro folding reactions behave as native polypeptides, and are consistent with the interaction of γ-tubulin with microtubules ends.

Nucleotide-binding Properties of Actin-RPV and γ-Tubulin

Actin-RPV and γ-tubulin share ~50% and 35% amino acid identity with β-actin and β-tubulin, respectively (Lees-Miller et al., 1992; Clark and Meyer, 1992; Oakley and Oakley, 1989; Zheng et al., 1991). Both might therefore be expected to be nucleotide-binding proteins; in the case of actin-RPV, 14 out of 15 residues that bind Ca-ATP in actin

are conserved (Lees-Miller et al., 1992). However, the nucleotide-binding properties of actin-RPV and γ-tubulin are unknown. We therefore measured the binding of in vitro folded actin-RPV and γ-tubulin to agarose-bound ATP and GTP. For comparison, we measured the ability of β-actin to bind to these columns: 52% of in vitro translated β-actin bound to ATP-agarose, while 42% of the same material bound to GTP-agarose (Table I). As a negative control, we examined the binding of in vitro synthesized β-globin to the same immobilized nucleotide columns: no measurable binding was observed. In the case of actin-RPV, 33 and 27%, respectively, of the labeled protein bound to ATP- or GTP-agarose; the corresponding figures for γ-tubulin were 20 and 32%. The relatively low binding efficiencies in all these experiments were not a result of kinetically limited exchange reactions, since repassage of the non-binding fractions over

Table I. Binding of Monomeric β-Globin, β-Actin, Centractin, and γ-Tubulin to ATP- and GTP-Agarose

Agarose-bound nucleotide	Relative amount of bound* protein (%)			
	β-globin	β-actin	Centractin	γ-tubulin
ATP	0	52	33	20
GTP	0	40	27	32

* Measured as the yield of material eluted from the column with Mg-ATP (in the case of ATP-agarose) or Mg-GTP (in the case of GTP-agarose).

fresh nucleotide-agarose columns failed to result in the retention of any further material. It is possible that only a portion of the monomeric products emerging from the gel-filtration columns was native; in addition, it is conceivable that significant denaturation occurred during experimental manipulations after the in vitro folding reactions. Nonetheless, our data suggest that actin-RPV and γ-tubulin are both nucleoside triphosphate–binding proteins, though neither shows an absolute specificity for ATP or GTP.

Interaction between Chaperonin and Actin-RPV or γ-Tubulin

The data described above demonstrate that actin-RPV and γ-tubulin are both folded via interaction with the same chaperonin that folds β-actin and α- and β-tubulin. There is evidence that chaperonin-mediated folding reactions take place within the central cavity that runs along the axis of chaperonin complexes (Creighton, 1991; Langer et al., 1992a,b; Braig et al., 1993), but the nature and specificity of target protein/chaperonin interactions is unknown. We therefore decided to test the ability of actin-RPV and γ-tubulin to compete with one another or with β-actin or α-tubulin for interaction with cytoplasmic chaperonin. In these experiments, increasing quantities of unlabeled denatured competitor protein were added to the labeled, denatured actin-RPV or γ-tubulin probes before dilution into a reaction containing purified chaperonin. The yield of binary complex was then visualized after resolution of the reaction products on a nondenaturing gel (Gao et al., 1993) (Fig. 6). In negative control reactions in which actin-RPV or γ-tubulin was competed with unlabeled α-globin, a polypeptide that does not, on its own, form a binary complex with cytoplasmic chaperonin (Gao et al., 1993), no decrease in the yield of binary complex was detected in the presence of high concentrations of added unlabeled competitor (Fig. 6, A and F). In contrast, a marked decline in the yield of binary complex was detected in competition reactions done with increasing concentrations of unlabeled homolog (Fig. 6, B and G). Competition of the actin-RPV and γ-tubulin probes with one another or with β-actin or α-tubulin all resulted in a similar decline in the yield of labeled binary complex (Fig. 6, C–E; H–J). From these data, we conclude that all these cytoskeletal proteins interact with the same or overlapping sites on the cytoplasmic chaperonin.
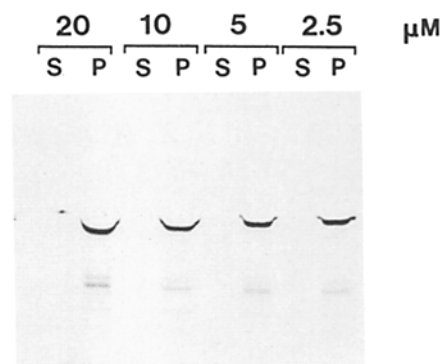
Discussion

The chaperonin-mediated folding of β-actin and α- and β-tubulin proceeds via the formation of a binary complex consisting of the target protein bound to chaperonin. These binary complexes have an apparent molecular weight of 700
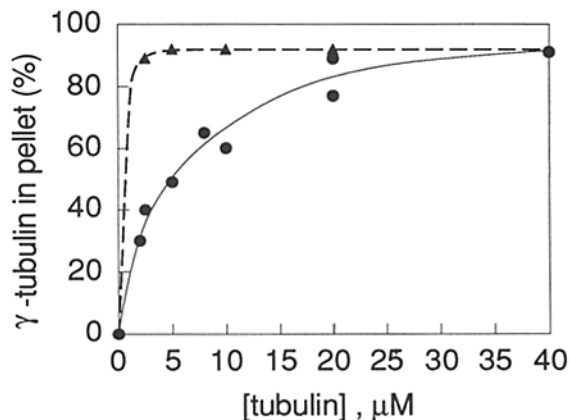
A
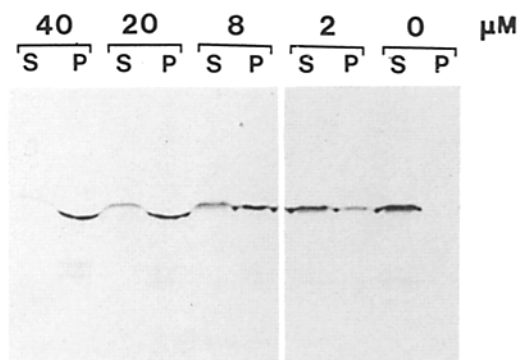
B

C

Figure 5. Binding of γ-tubulin to microtubules as a function of polymerized tubulin concentration and number of polymer ends. Analysis on an 8% SDS-polyacrylamide gel of the supernatants (S) and pellets (P) derived from experiments in which a constant amount of monomeric γ-tubulin generated in vitro was incubated with varying concentrations of taxol-stabilized microtubules (shown in μM in the figure) and the resulting polymers sedimented through sucrose cushions. (A) Unsheared microtubules (average length 11 ± 3 μm). (B) Sheared microtubules (average length 1.2 ± 0.4 μm). (C) Quantitation of the data shown in A (*closed circles, solid line*) and B (*closed triangles, dashed line*).
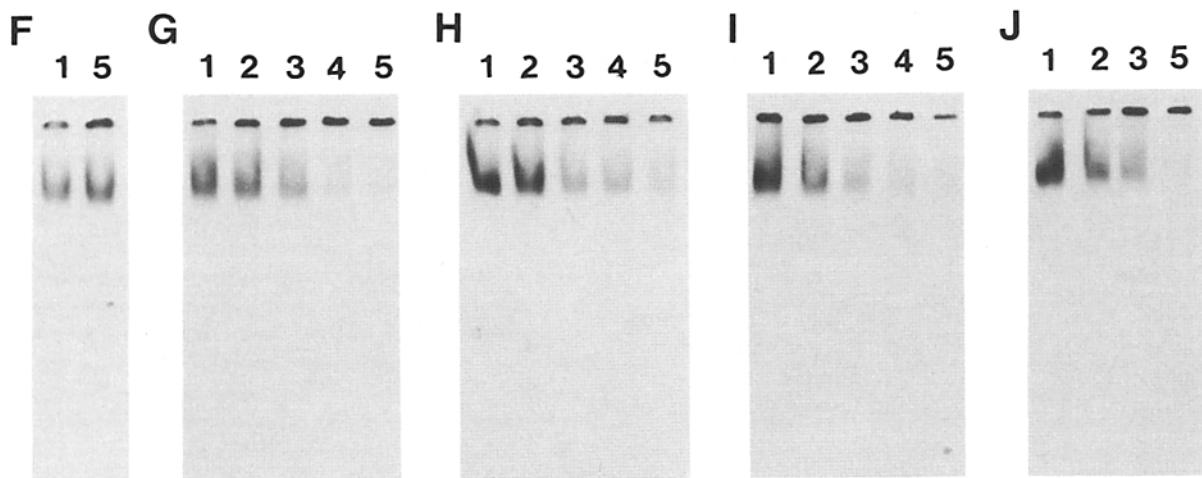
Actin-RPV
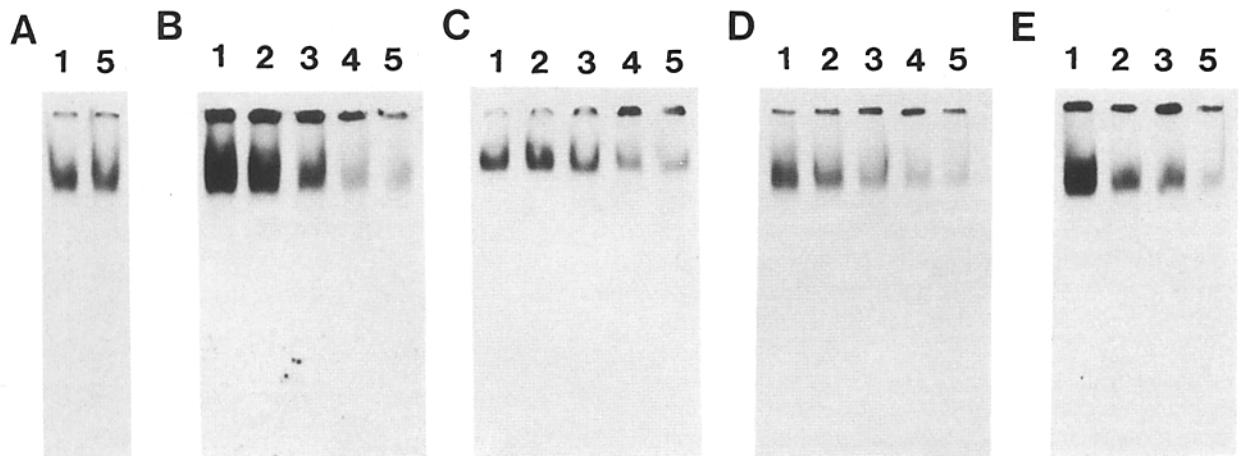
γ-tubulin

Figure 6. Competition among cytoskeletal proteins for association with cytoplasmic chaperonin. Analysis by native agarose/PAGE of binary complexes formed between cytoplasmic chaperonin and either labeled denatured actin-RPV (*A–E*) or γ-tubulin (*F–J*) in the presence of unlabeled competitor protein. Competition reactions shown are with unlabeled denatured α-globin (*A* and *F*), actin-RPV (*B* and *I*), γ-tubulin (*D* and *G*), β-actin (*C* and *H*), α-tubulin (*E* and *J*). Lanes marked 1–5 represent reactions in which equal amounts of labeled denatured target proteins were competed with a fivefold (lanes *1*), tenfold (lanes *2*), 20-fold (lanes *3*), 30-fold (lanes *4*), or 40-fold (lanes *5*) molar excess of unlabeled denatured competitor protein.

kD; in the presence of Mg-ATP, Mg-GTP, and (in the case of tubulin) additional protein cofactors, the native proteins are released (Gao et al., 1992, 1993). The appearance of a 700-kD species together with monomeric actin-RPV or γ-tubulin in our in vitro translation reactions was therefore suggestive of the existence of synthetic intermediates consisting of de novo synthesized unfolded polypeptides bound to chaperonin, a conclusion that was confirmed by the generation of identical products in in vitro folding reactions containing homogeneous chaperonin and Mg-ATP (Fig. 3). The soluble nature of monomeric actin-RPV was to be expected, since it is found throughout the cytoplasm, though concentrated at the centrosome (Clark and Meyer, 1992). The generation of soluble monomeric γ-tubulin was more surprising, given the restricted localization of this protein at the

centrosome (Oakley et al., 1990; Zheng et al., 1991; Horio et al., 1991; Stearns et al., 1991). Immunofluorescence experiments have shown that the cytoplasmic chaperonin is diffusely distributed throughout the cytosol (Gao et al., 1992; Lewis et al., 1992). Though our data clearly establish that the facilitated folding of actin-RPV and γ-tubulin can be mediated by interaction with cytoplasmic chaperonin, the mechanism whereby the soluble monomeric proteins become incorporated into the centrosome remains unknown.

Previous analyses of actin and α- and β-tubulin folding reactions have shown that while actin is efficiently folded via interaction with chaperonin alone, the proper folding of α- and β-tubulin requires the presence of two additional protein cofactors (Gao et al., 1993). Since γ-tubulin shares ~35% amino acid sequence identity with α- and β-tubulin

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(Oakley and Oakley, 1989), we expected the successful generation of in vitro folded γ-tubulin to be dependent on one or both of these cofactors. However, no protein cofactors were required in addition to chaperonin itself for the generation of either actin-RPV or γ-tubulin in in vitro folding reactions (Fig. 3, E and F). Thus, the requirement for cofactors in α- and β-tubulin folding reactions may be related to the fact that these polypeptides associate to form the tubulin heterodimer.

The relative yields of binary complex and monomer generated in our experiments differed depending on whether the experiment involved in vitro translation or chaperonin-mediated folding (Figs. 2 and 3). This observation is not surprising, since translation involves the vectorial extrusion of polypeptides from ribosomes in the presence of vast numbers of proteins which may influence the efficiency of production of native proteins. In contrast, in vitro dilution experiments involve the abrupt exposure of a full-length and completely unfolded polypeptide to a buffer solution that is devoid of all protein with the exception of chaperonin.

To investigate the properties of in vitro folded actin-RPV, we tested its ability to copolymerize with native brain actin, since several regions of actin that are thought to be involved in actin–actin interactions in the polymer (Holmes et al., 1990) are conserved in actin-RPV (Lees-Miller et al., 1992). Actin-RPV translated or folded by homogeneous chaperonin in vitro cocycled efficiently with native actin (Fig. 4, A and B), though at the very low concentrations of material generated in our in vitro assays, actin-RPV did not polymerize on its own (Fig. 4 C). This observation does not preclude the possibility that actin-RPV can form homopolymers above a critical concentration akin to that of globular actin. Actin-RPV does not appear to be part of the actin stress fiber network in cultured cells (Clark and Meyer, 1992). However, the fact that actin addition is required for the sedimentation of radiochemical amounts of actin-RPV demonstrates that actin-RPV either copolymerizes with globular actin into mixed filaments, or interacts with actin filaments as a capping or bundling protein. Furthermore, the low abundance of actin-RPV and its diffuse cytoplasmic localization make it hard to rule out its presence in actin polymers in vivo. We also measured the ability of actin-RPV to bind to immobilized nucleotide; we found that actin-RPV, like β-actin, showed no marked preference for the binding of ATP relative to GTP (Table I).

The generation of soluble, monomeric γ-tubulin allowed us to investigate the properties of this low abundance protein. Given the relatedness of γ-, α-, and β-tubulin, we first attempted to incorporate β-tubulin into native α/β-tubulin dimers by exchange, but these experiments failed to yield any labeled heterodimers. We therefore tested the ability of monomeric γ-tubulin to interact with preassembled microtubules, since the role of γ-tubulin in nucleating microtubule assembly (Joshi et al., 1992) suggests that it can interact with microtubule ends. In these experiments, a significant proportion of the labeled γ-tubulin from either in vitro translation or in vitro folding reactions was found in association with microtubules (Fig. 4, D and E). Moreover, when the concentration of microtubule ends was varied, the binding of γ-tubulin to polymers varied in proportion to the number of available microtubule ends (Fig. 5). These data reinforce the notion that γ-tubulin acts as a microtubule nucleating factor.

In experiments in which in vitro folded γ-tubulin was tested for its ability to bind to columns of immobilized nucleotide, γ-tubulin showed some preference for GTP binding (Table I). However, the presence of GTP is not strictly required in order to correctly fold γ-tubulin: in vitro folding reactions done in the presence of ATP alone resulted in a product indistinguishable from that of reactions done in the presence of both ATP and GTP, although the yield of monomeric γ-tubulin in reactions done with ATP alone was much diminished (Fig. 3 F). This result contrasts with the requirements of α- and β-tubulin folding reactions, which depend on the presence of both ATP and GTP (Gao, Y., and N. J. Cowan, unpublished observations).

We previously showed that β-actin and α- and β-tubulin compete for interaction with cytoplasmic chaperonin. To see whether actin-RPV and γ-tubulin are recognized by the same sites on cytoplasmic chaperonin (and indeed whether these same sites recognize actin and α- and β-tubulin), we tested the ability of actin-RPV and γ-tubulin to compete with one another and with actin and α-tubulin for the formation of binary complexes. All these cytoskeletal proteins competed efficiently with one another for binary complex formation (Fig. 6). These data strongly suggest the existence of one or more common structural motif(s) that must be recognized by the cytoplasmic chaperonin. Such structural motif(s) may reflect some aspects of secondary or tertiary structure that are common to folding intermediates in actins, tubulins and actin- or tubulin-like proteins.

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