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Analysis of the Interaction between the Eukaryotic Chaperonin CCT and Its Substrates Actin and Tubulin

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Two mechanisms have thus far been characterized for the assistance by chaperonins of the folding of other proteins. The first and best described is that of the prokaryotic chaperonin GroEL, which interacts with a large spectrum of proteins. GroEL uses a nonspecific mechanism by which any conformation of practically any unfolded polypeptide interacts with it through exposed, hydrophobic residues. ATP binding liberates the substrate in the GroEL cavity where it is given a chance to fold. A second mechanism has been described for the eukaryotic chaperonin CCT, which interacts mainly with the cytoskeletal proteins actin and tubulin. Cryoelectron microscopy and biochemical studies have revealed that both of these proteins interact with CCT in quasi-native, defined conformations. Here we have performed a detailed study of the docking of the actin and tubulin molecules extracted from their corresponding CCT:substrate complexes obtained from cryoelectron microscopy and image processing to localize certain regions in actin and tubulin that are involved in the interaction with CCT. These regions of actin and tubulin, which are not present in their prokaryotic counterparts FtsA and FtsZ, are involved in the polymerization of the two cytoskeletal proteins. These findings suggest coevolution of CCT with actin and tubulin in order to counteract the folding problems associated with the generation in these two cytoskeletal protein families of new domains involved in their polymerization. **© 2001 Academic Press**

Key Words: **chaperonin; CCT; actin; tubulin; electron microscopy; docking; cytoskeleton evolution.**

INTRODUCTION

Proteins may require, at some periods in the course of their existence, the help of other proteins to overcome kinetic barriers that prevent them from reaching their native conformations (Bukau and Horwich, 1998). Such helper proteins are grouped in an ever-increasing family of proteins termed molecular chaperones, of which the chaperonins are perhaps the most well studied members (Ellis, 1996). The chaperonins are ATPases of 60 kDa that form multimeric complexes built up by one or two rings, placed in a back-to-back orientation. The ring is the functional unit where protein folding takes place and can be composed of seven to nine subunits, depending on the type of chaperonin. Chaperonins have been categorized in two groups. Group I encompasses all those from eubacteria and endosymbiotic organelles (Bukau and Horwich, 1998; Ellis and Hartl, 1999), whereas all the chaperonins found in archaebacteria and in the eukaryotic cytosol (Willison, 1999; Gutsche *et al.*, 1999) are included in Group II. All Group I chaperonins have their rings made up of seven identical subunits, whereas the rings of Group II chaperonins may be 8- or 9-mers made of two, three, or even eight different subunits, as in the case of eukaryotic cytosolic chaperonin CCT or TriC (Liou and Willison, 1997; Marco *et al.*, 1994).

There is a large body of biochemical and structural information concerning Group I chaperonins, obtained mainly with chaperonin GroEL from *Escherichia coli*. X-ray studies have revealed the existence of three characteristic domains (Braig *et al.*, 1994). The equatorial domain holds most of the intra- and inter-subunit interactions and the binding site for ATP, whose binding and subsequent hydrolysis maintain the chaperonin functional cycle. The apical domain encompasses the entrance of the ring

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and holds all the hydrophobic residues involved in substrate binding. The intermediate domain lies between the equatorial and the apical domains and acts as a hinge for the upward and outward movements of the apical domain induced by the equatorial domain upon ATP binding and hydrolysis (Roseman *et al.*, 1996; Xu *et al.*, 1997; Llorca *et al.*, 1997). These movements allow the binding to GroEL of its cochaperonin GroES, a small heptamer present only in the Group I chaperonins (Hunt *et al.*, 1996) that caps the chaperonin cavity and induces a further movement of the apical domains. This step is critical in the mechanism of protein folding by GroEL because the unfolded polypeptide that had previously interacted with GroEL surfaces is now liberated into an isolated, hydrophilic, and much larger cavity where it is free to fold using the information encoded in its own amino acid sequence. The mechanism is not energetically passive, because the movements of the apical domains result in the stretching of the partially unfolded polypeptide, thus possibly lifting it from local energy minima (Shtilerman *et al.*, 1999). This mechanism is inefficient (several rounds of polypeptide encapsulation and liberation in GroEL may be needed before some proteins are fully folded; Weissman *et al.*, 1995), but the lack of efficiency is traded for utility since this chaperonin interacts with a large spectrum of proteins *in vivo* (Houry *et al.*, 1999).

Much less information has been obtained so far for the Group II chaperonins. The atomic structure of the thermosome (Ditzel *et al.*, 1998) and the electron microscopy studies carried out with several Group II chaperonins (Nitsch *et al.*, 1998; Llorca *et al.*, 1998, 1999a, 1999b, 2000; Gutsche *et al.*, 2000; Schoehn *et al.*, 2000a, b) have revealed an overall architecture that is similar to the Group I chaperonins, the main difference being the lack of a cochaperonin that is replaced in this family by an extra helical protrusion built in at the tip of the apical domain that serves as a cap of the chaperonin cavity during its functional cycle (Klummp *et al.*, 1997; Ditzel *et al.*, 1998; Nitsch *et al.*, 1998; Llorca *et al.*, 1999a). The two main conformers found for GroEL, the open, substrate-receptive conformation and the closed conformation where substrate is folded, seem to exist also in the Group II chaperonins (Nitsch *et al.*, 1998; Llorca *et al.*, 1999a, 1999b, 2000; Schoehn *et al.*, 2000a, b). However, other important differences are found between the two groups of chaperonins, especially when GroEL is compared with the cytosolic chaperonin CCT (also termed TRiC). Unlike GroEL, CCT rings consist of eight different subunits positioned in a precise arrangement (Liou and Willison, 1997; Willison, 1999). The differences in the sequence among the CCT subunits are found mainly in the substrate-binding domain (apical domain; Kim *et al.*, 1994), which taken together with the fact that this chaperonin seems to assist in the folding of a very small number of proteins, mainly the cytoskeletal proteins actin and tubulin, has suggested some kind of specificity of the CCT subunits toward substrate binding (Kubota *et al.*, 1994, 1995). The evolution of the chaperonin toward a specificity in the folding of actin and tubulin is reinforced by the parallel evolution of prefoldin, a CCT cofactor involved in delivering substrates to CCT (Vainberg *et al.*, 1998). Archaeal prefoldin is made up of two types of subunits and it is able to interact with a wide range of substrates (some of them artificial; Leroux *et al.*, 1999), but eukaryotic prefoldin has diverged into six distinct species and seems to interact only with actin and tubulin (Hansen *et al.*, 1999). All these data have prompted the notion that CCT may function in assisting in the folding of the proteins using a different mechanism from that described for GroEL. This idea has been confirmed recently by the demonstration that both actin and tubulin bind to CCT in quasi-native conformations and interact with specific subunits of the chaperonin (Tian *et al.*, 1995b; Llorca *et al.*, 1999b, 2000).

In this study, a further and more elaborate analysis of the docking between the actin and the tubulin molecules bound to CCT and their atomic counterparts (Llorca *et al.*, 2000) has been performed to locate some putative domains of the two cytoskeletal proteins involved in CCT binding. We thus compared these regions with the same regions in their prokaryotic homologues, FtsA and FtsZ, which are presumably more like their precursor structures, using primary sequence alignment techniques.

It is remarkable that the majority of the CCT-binding sites found in the modern actins and tubulins are demonstrably absent in FtsA and FtsZ proteins. We discuss the functional significance of these observations in terms of polymerization properties of the more functionally sophisticated actins and tubulins.

MATERIALS AND METHODS

Three-dimensional reconstructions of the CCT:α-actin and CCT:tubulin complexes. Recombinant β-tubulin was prepared by insertion of human β-tubulin cDNA into pET21a vector (Novagen) and expression in Epicurian Coli BL21 (DE3) competent cells (Stratagene). Either β-tubulin or bovine brain tubulin (from Cytoskeleton) was denatured in 7 M guanidinium chloride and diluted 100-fold in 5 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5 (final concentration), containing 0.4 μM murine CCT, purified as described by Liou and Willison (1997). The three-dimensional reconstructions of the CCT:α-actin and CCT:tubulin complexes were obtained as described in Llorca *et al.* (1999b) and Llorca *et al.* (2000), respectively. Briefly, complexes of CCT and each of the two cytoskeletal proteins were prepared by incubation of the chemically denatured proteins with CCT. Images were recorded

at a 20° tilt in a Jeol 1200EX-II electron microscope equipped with a Gatan cold stage operated at 120 kV and recorded on Kodak SO-163 film at a 60 000× nominal magnification and approximately 1.5-μm underfocus. Top views were selected and a two-dimensional processing step was carried out to separate the substrate-bound from the substrate-free particles and the first group was subjected to a three-dimensional reconstruction procedure using angular refinement algorithms provided by SPIDER (Frank *et al.*, 1996). The volumes were generated using ART (algebraic reconstruction techniques) with blobs (Marabini *et al.*, 1998). The final resolution was calculated by Fourier ring correlation of two independent reconstructions and the value obtained was used in low-pass filtering the volumes (30 Å for the CCT:α-actin complex and 25 Å for the CCT:tubulin complex).

Docking of the cryoelectron microscopy structures of actin and tubulin bound to CCT with their atomic counterparts. Docking was performed using SITUS (Wriggers *et al.*, 1999), as described in Llorca *et al.* (2000). The visualization of the docking was carried out using VMD (Humphrey *et al.*, 1996). After docking of both actin and tubulin, the domains of both proteins putatively involved in CCT binding were localized by visual inspection of the docking.

Sequence alignment of actin and tubulin with their prokaryotic homologues FtsA and FtsZ. Representative sequences of the FtsA, FtsZ, actin, and tubulin families of proteins were obtained from Pfam (Bateman *et al.*, 2000; http://www.sanger.ac.uk/Software/Pfam) and HSSP (Sander and Schneider, 1991; ftp://ftp.embl-heidelberg.de/pub/databases/protein_extras/hssp). The three-dimensional structures of β-tubulin (entry 1TUB-B), yeast actin (entry 1YAG-A), FtsZ from *Methanococcus jannaschii* (entry 1FSZ), and FtsA from *Thermotoga maritima* (entry 1E4F-T) were obtained from the Protein Data Bank. The secondary structure elements of 1YAG-A, 1FSZ, and 1E4F-T were extracted from the DSSP database (Kabsch and Sander, 1983; ftp://ftp.embl-heidelberg.de/pub/databases/protein_extras/dssp). The secondary structure of 1TUB-B was obtained from the original PDB file as reported by the authors (Nogales *et al.*, 1998a). The structure-based sequence alignments of actin versus FtsA and tubulin versus FtsZ were performed using DALI (Holm and Sander, 1996; http://www2.ebi.ac.uk/dali). The alignments were visualized and colored using Belvu 2.8 (http://www.sanger.ac.uk/~esr/Belvu.html).

RESULTS

Analysis of the Actin and Tubulin Residues Involved in Binding to CCT

It has already been shown that the cytosolic chaperonin CCT is stringent for the complete folding of actin and tubulin *in vitro* (Rommelaere *et al.*, 1993) and *in vivo* (Miklos *et al.*, 1994; Chen *et al.*, 1993; Vinh and Drubin, 1994; Willison, 1999; Leroux and Hartl, 2000) and that, although GroEL is capable of recognizing unfolded forms of these proteins, it is not able to fold them (Tian *et al.*, 1995a). This indicates a folding mechanism for actin and tubulin different from that for the proteins folded by GroEL, one in which many different unfolded conformations are able to interact with the prokaryotic chaperonin using a nonspecific recognition mechanism based on hydrophobic interactions (Bukau and Horwich, 1998; Chen and Sigler, 1999; Shtilerman *et al.*, 1999; Farr *et al.*, 2000). When the nucleotide-free, receptive form of CCT is left to interact with chemically denatured actin or tubulin, stable complexes of these two proteins are slowly formed with the cytosolic chaperonin that are amenable to three-dimensional reconstruction using electron microscopy and image processing techniques (Llorca *et al.*, 1999b, 2000). CCT:substrate complexes formed this way are functional as they are able to renature actin and tubulin after ATP addition (Melki and Cowan, 1994).

In an attempt to characterize some of the CCT-binding sites of actin and tubulin when bound to the cytosolic chaperonin, we performed a detailed analysis of the interaction between the two cytoskeletal proteins and CCT. The three-dimensional reconstructions of the nucleotide-free CCT complexed to actin or to tubulin shown in Fig. 1 clearly reveal that the two cytosolic proteins bind to the chaperonin in a defined conformation that withstands all the averaging procedures carried out during the three-dimensional reconstruction process. These conformations actually represent each one of two possible conformations that cannot be distinguished because of the low resolution of the two three-dimensional reconstructions and which have been characterized by immunomicroscopy (see Llorca *et al.*, 1999b, 2000). It cannot be ruled out in any case that one of the two possible arrangements is more populated under our *in vitro* conditions, so that the final reconstruction represents mainly that conformation. The solid and the reticulate surfaces represent the complex between CCT and its substrate (actin or tubulin) obtained by cryoelectron microscopy, and even at this low resolution (30 Å for actin and 25 Å for tubulin) the views obtained are very suggestive of the two cytoskeletal proteins being arranged in a mostly folded but open conformation in which two domains of these two proteins interact with opposite sides of CCT. To test this, a docking procedure was carried out using the cryoelectron microscopy structures of actin and tubulin extracted from their complex with CCT and their corresponding atomic structures (Llorca *et al.*, 2000). In the case of actin, the best solution was found after cutting its atomic structure into the two topological domains (small and large domains; Kabsch *et al.*, 1990) so that the molecule fits very well into the rod-shaped electron microscopy structure of actin (Fig. 1A). Other solutions were either almost identical or unacceptable because they placed the cut ends facing the CCT subunits and not each other. The docking chosen places the tips of the small and large domains (left and right end of the actin molecule, respectively) interacting with one CCT subunit each. Immunomicroscopy experiments with CCT:α-actin complexes labeled with monoclonal antibodies specific for dif-

ferent CCT subunits have demonstrated that the small domain binds to CCTδ and the large domain to either CCTβ or CCTε (Llorca *et al.*, 1999b).

Based on the docking performed, a visual recognition of the actin regions involved in CCT binding was carried out, allowing us to determine two regions at the tip of the small domain of actin (R37-D51 and R62-T66) that seem to interact with the base of the CCTδ apical domain. This corroborates the biochemical results by Hynes and Willison (2000), who have found, using a β-actin peptide array to screen for CCT-binding sequences, two regions (A26-K50 and D56-P70) implicated in the interaction with CCT (sites Ii and Iii, respectively). On the other side of the actin molecule, the tip of the large domain contains two other sequences, E195-R206 and T229-I250, that seem to interact with one CCT subunit (either CCTβ or CCTε). Almost the same sequences (R196-A220 and A231-G245) have been found, using the β-actin peptide array referred to above, to be involved in CCT binding (sites IIi and IIii; Hynes and Willison, 2000). Other biochemical experiments carried out with actin and actin peptides have shown that the fragment D244-C285 competes with denatured actin for CCT binding (Rommelaere *et al.*, 1999). Binding of this region to CCT seems indeed to be strong because a chimera, constructed by linking residues L178-F262 of human β-actin to residues 1–168 of human Ha-Ras, interacts avidly with CCT and, unlike β-actin, which is actively folded by CCT, is maintained in reticulocyte lysate in equilibrium between a free, unbound population and a CCT-bound population (Llorca *et al.*, 1999b). The interaction of these two fragments of the large domain with CCTβ and CCTε is very strong and withstands immunoprecipitation of the CCT:β-actin complexes using mixed micelle detergent buffers, which cause complete disruption of CCT into its constituent monomers (Hynes and Willison, 2000). Using these kind of experiments, it has been found that the two strongest interactions between CCT and β-actin occur through CCTβ or CCTε, suggesting that these two subunits and the actin sequences encompassing residues E195-R206 and T229-I250 may have a leading role in the interaction between CCT and the actin molecule to be folded. In an accompanying study (McCormack *et al.*, 2001a) it is shown by point mutagenesis of β-actin that two residues in Site II critical for CCT binding are T203 and D244. The putative binding sites described here in some cases accord with those identified by Rommelaere *et al.* (1999) using competition experiments or those found by Hynes and Willison (2000) using peptide scans. There are, however, other putative binding sites described in the last two papers that have not been found in this docking analysis. This discrepancy may partially be explained by the fact that this work has been performed with the CCT:actin complex in the absence of nucleotide, and it is possible that in the subsequent steps of the folding cycle other interactions between CCT and actin occur.

A more complex picture is observed during the docking analysis of tubulin bound to CCT (Fig. 1B). The tubulin molecule is divided into two large domains, connected by a small linker, and interacts with opposing regions of the cytosolic chaperonin. The docking analysis has found that the N-terminal region of the tubulin molecule (left domain in Fig. 1B) has the shape of a truncated cone and interacts with two CCT subunits, while the C-terminal region occupies a more spread-out structure and interacts with three CCT subunits. All in all, the two domains interact with opposite regions of the cytosolic chaperonin and, if the fitting between the electron microscopy structure of tubulin and its atomic counterpart is indeed correct, it follows that the tubulin molecule is recognized by CCT in an open, quasi-folded conformation. Immunomicroscopy experiments carried out with CCT:tubulin complexes and several monoclonal antibodies specific for different CCT subunits have allowed the determination of two modes of interaction (Llorca *et al.*, 2000). The first involves the interaction of the N-terminal domain with CCTη and CCTα and of the C-terminal domain with CCTβ, CCTγ, and CCTθ. The second mode of interaction implicates subunits CCTδ and CCTθ in the interaction with the N-terminal region of tubulin and subunits CCTε, CCTζ, and CCTβ with the C-terminal region of tubulin (Llorca *et al.*, 2000). In using these two ways of binding, tubulin interacts with all eight CCT subunits and this fact suggests the possibility of early coevolution of both tubulin and CCT (see below). The interaction between tubulin and CCT involves not only more CCT subunits than in the case of actin, but also a larger region of each of the CCT subunits (not shown here but see Llorca *et al.*, 1999b, 2000), encompassing this time the helical protrusion and the base of the apical domains, and could explain the differences in CCT conformation observed in Figs. 1A and 1B. Thus the fact that CCTβ and CCTθ are involved in the two modes of interaction could be explained by the two CCT subunits using, in the two cases, different regions of their apical domains.

The docking performed between the electron microscopy structure complexed to CCT and the modified atomic structure allowed us to make a putative assignment of regions of tubulin interacting with certain CCT subunits. The assignments were proved to be correct by immunomicroscopy experiments carried out with complexes formed between CCT and

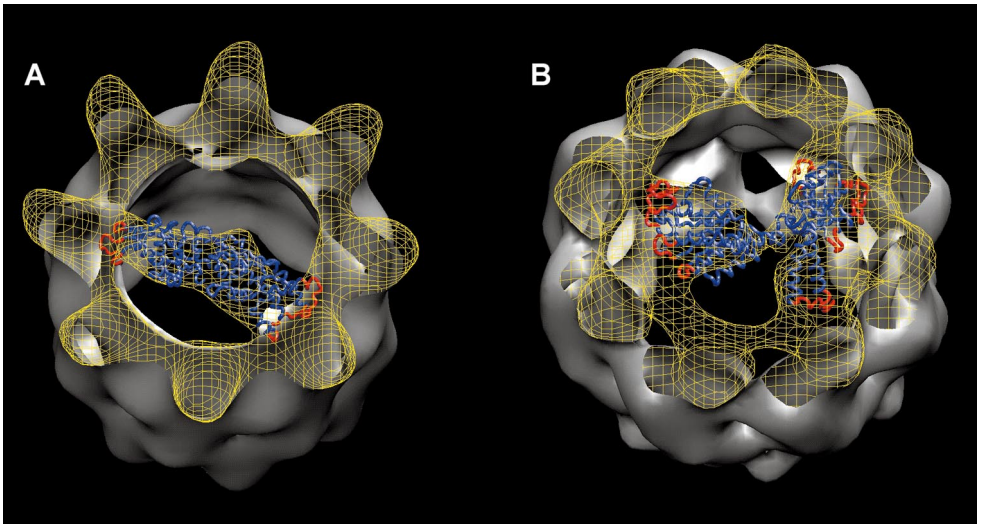

FIG. 1. Images of the CCT:α-actin and CCT:tubulin complexes. A view of the three-dimensional reconstruction of the (A) CCT:α-actin and (B) CCT:tubulin complex generated by cryoelectron microscopy and image reconstruction, to which the atomic, open structure of actin (A) and tubulin (B) has been fitted. The yellow and reticulated region of the CCT has been drawn to show the interaction between the cytoskeletal proteins and the chaperonin. The regions of actin and tubulin pictured in red correspond to the regions suggested to interact with CCT.

several chimeric proteins constructed with the residues 1–168 of human Ha-ras and the regions of the β-tubulin molecule previously assigned to interact with specific CCT subunits (Llorca *et al.*, 2000). Consequently, certain residues of the fragment D205-L265 (unless stated otherwise, the numbering corresponds to the sequence of the pig β-tubulin as described by Nogales *et al.*, 1998a) interact with CCTζ or CCTγ subunits, others in the fragment P263-I384 interact with CCTϵ or CCTβ subunits, and other residues of the fragment I384-A455 interact with CCTβ or CCTθ subunits. According to these results, the N-terminal domain, which includes residues M1-D205 of β-tubulin, would bind to CCTθ/δ or to CCTη/α subunits. The N-terminal domain forms a Rossmann fold and binds nucleotide, and the docking performed is consistent with the domain being already folded when interacting with CCT (see left domain in Fig. 1B). Experiments measuring CCT binding following *in vitro* translation with a chimeric protein containing the N-terminal domain suggest that this domain is able to fold in the absence of the rest of the molecule (see the discrete species in Fig. 6A in Llorca *et al.*, 2000).

A more detailed analysis of the docking shown in Fig. 1B allows the recognition of several regions of tubulin involved in CCT binding. The N-terminal domain has three regions, a large region encompassing residues T33-A57 and two small regions that include S126-Q133 and E160-R164. The first region seems to be interacting, according to the docking analysis performed by Llorca *et al.* (2000), with CCTδ or CCTα, whereas the other two regions interact with CCTθ or CCTη. In the C-terminal domain the situation is more complex, since there are five regions of the tubulin sequence interacting with three different CCT subunits. Fragments T239-K254 and P261-H266 interact with CCTζ or CCTγ, fragments S277-V288 and V355-P359 interact with CCTϵ and CCTβ, and fragment W407-E417 interacts with CCTβ or CCTθ. There is biochemical confirmation of some of these findings. Ritco-Vonsovici and Willison (2000), using a peptide array covering the complete sequence of α-, β-, and γ-tubulin to screen for CCT-binding sites, have found various fragments that interact with CCT. In the case of β-tubulin, the so-called site B (R48-P72) partly overlaps with the large fragment T33-A57 localized by the docking analysis. The same occurs with another fragment placed in the N-terminal domain, E160-R164, which is encompassed by fragment L153-V177 (site D), found to interact with CCT. Two other CCT-binding regions localized by the docking analysis, T239-K254 and P261-H266, can be found within the large binding site R243-F267 (site F). The region S277-V288 overlaps with the core sequence of the CCT-binding site P263-T292 (site G) and the region V355-P359 is encompassed by the large binding site K (T353-R390). The C-terminal fragment W407-E417 assigned by the docking analysis is encompassed by binding site L (E386-E420), a large fragment found to interact with CCT (Ritco-Vonsovici and Willison, 2000). Thus, all the regions predicted by the present analysis, except S126-Q133, have

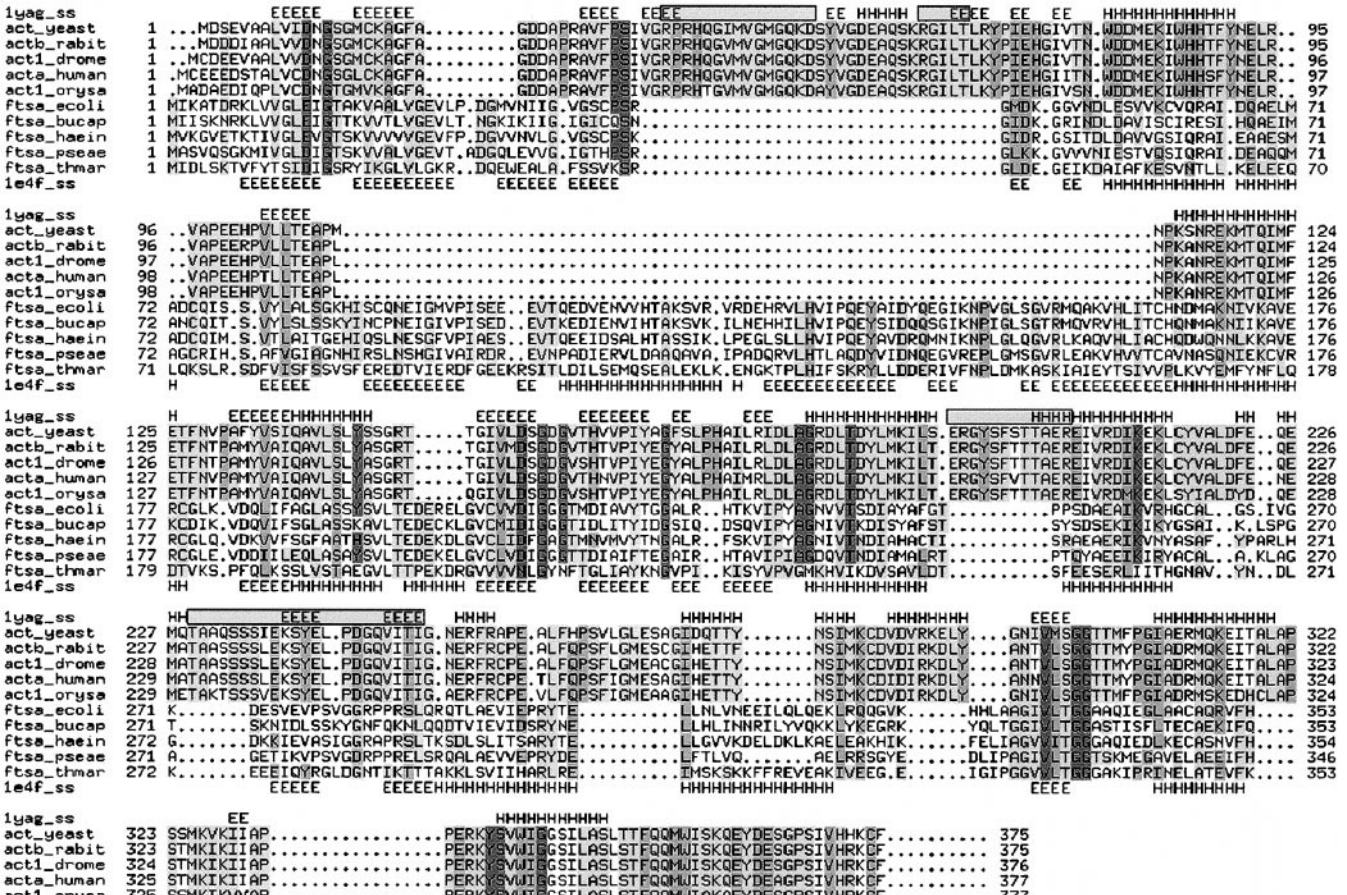

FIG. 2. Structure-based alignment of five representative sequences of actin (yeast, rabbit, *Drosophila*, human, and rice) and five FtsA proteins from different bacteria and archaeabacteria species. Residues are colored by average BLOSUM62 score using Belvu v.2.8 (Erik Sonnhammer, http://www.sanger.ac.uk/~esr/Belvu.html-). The secondary structures of actin from yeast and FtsA protein from *Thermotoga maritima*, both extracted from the DSSP database (Kabsch and Sander, 1983), are also depicted in the alignment (E, β-sheet; H, α-helix). The CCT-binding motifs of the actin sequence are marked as gray boxes. Sequences legend: act_yeast (actin from yeast (*Saccharomyces cerevisiae*)), actb_rabit (cytoplasmic β-actin from rabbit (*Oryctolagus cuniculus*)), act1_drome (actin-5C from vinegar fly (*Drosophila melanogaster*)), acta_human (α-actin 2, aortic smooth muscle (*Homo sapiens*)), act1_orysa (actin 1 from rice (*Oryza sativa*)), ftsa_ecoli (cell division protein FtsA (*Escherichia coli*)), ftsa_bucap (cell division protein FtsA (*Buchnera aphidicola*)), ftsa_haein (cell division protein FtsA (*Haemophilus influenzae*)), ftsa_pseae (cell division protein FtsA (*Pseudomonas aeruginosa*)), and ftsa_thmar (cell division protein FtsA (*T. maritima*)).

been identified in the previous screening (Ritco-Vonsovici and Willison, 2000) as potential CCT-binding sites.

The most interesting fragment of tubulin regarding CCT binding is the fragment P263-I384, which binds to CCTβ or CCTϵ and when linked to Ha-Ras binds CCT almost as tightly as β-tubulin upon *in vitro* translation (Llorca *et al.*, 2000). The strong affinity of the P263-I384 fragment for CCTβ or CCTϵ is confirmed by experiments in which, after immunoprecipitation of CCT:β-tubulin complexes treated with mixed micelle detergent buffers, the complexes between tubulin and CCTβ or CCTϵ are the strongest found (Llorca *et al.*, 2000). These results and the fact that other parts of the tubulin molecule have a much lower binding affinity suggest that this region is very important in the interaction between CCT and tubulin. There is indeed in the literature evidence that this is the case. Dobrzynski *et al.* (1996), using several deletion mutants of β-tubulin, have suggested that the region between residues 150 and 350 is involved in CCT binding, and this region has been narrowed by Rommelaere *et al.* (1999) to the fragment between residues 224 and

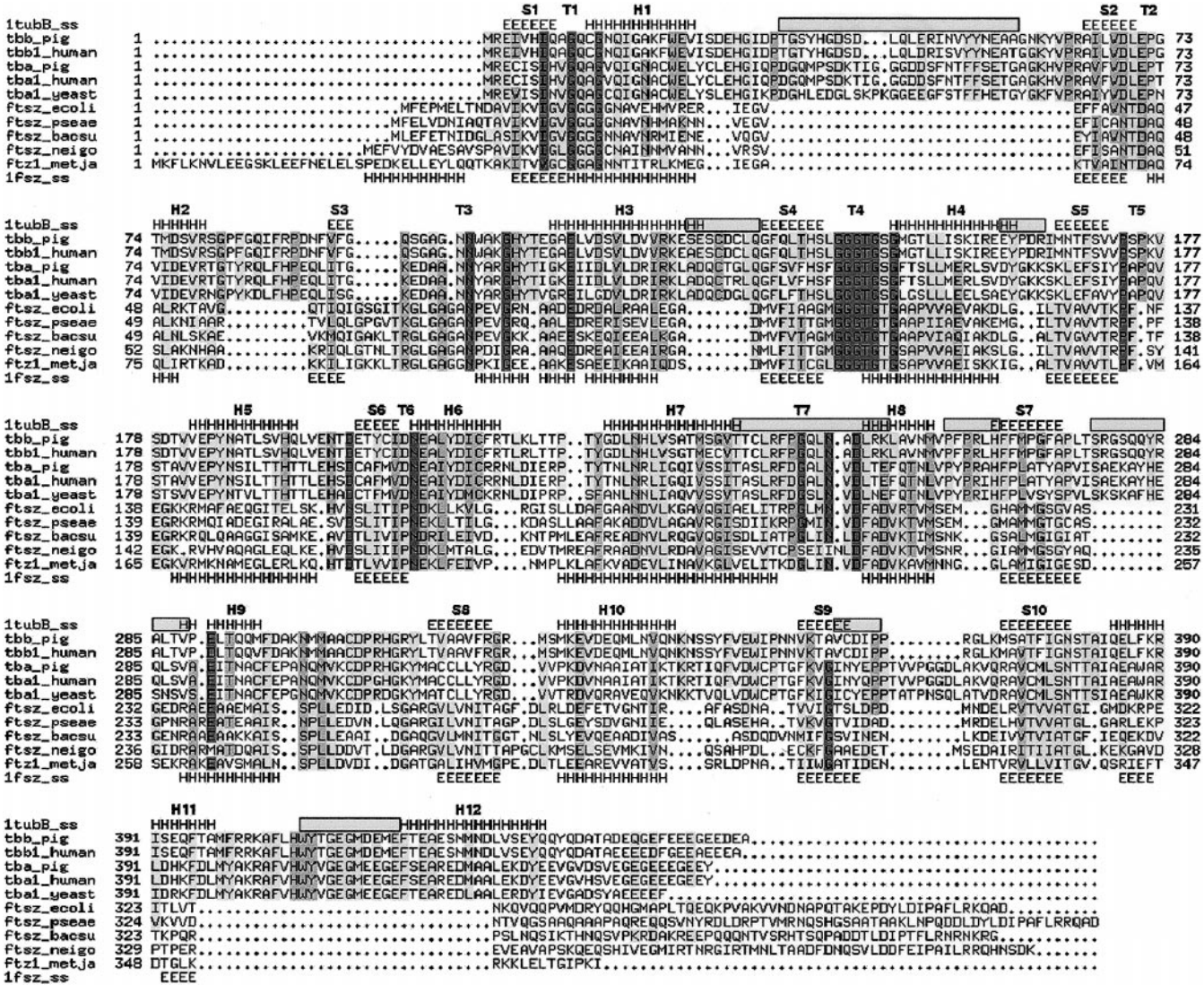

FIG. 3. Structure-based alignment of five representative sequences of α- and β-tubulins (pig, human, and yeast) and five FtsZ proteins from different bacteria and archaeabacteria species. All the symbols are as in Fig. 2. The numbering of the pig β-tubulin corresponds to that described by Nogales *et al.* (1998a). Sequences legend: tba_pig (α-tubulin from porcine brain (*Sus scrofa*)), tba1_human (tubulin α-1 chain, brain specific (*Homo sapiens*)), tbb_pig (β-tubulin from porcine brain (*S. scrofa*)), tbb1_human (tubulin beta-1 chain, brain specific (*H. sapiens*)), tba1_yeast (tubulin α-1 chain (*Saccharomyces cerevisiae*)), ftsz_ecoli (cell division protein FtsZ (*Escherichia coli*)), ftsz_pseae (cell division protein FtsZ (*Pseudomonas aeruginosa*)), ftsz_bacsu (cell division protein FtsZ (*Bacillus subtilis*)), ftsz_neigo (cell division protein ftsZ (*Neisseria gonorrhoeae*)), and ftsz_metja (cell division protein FtsZ (*Methanococcus janaschii*)).

323 as the one with the information required for CCT binding. Ritco-Vonsovici and Willison (2000) have also found the region between residues 263 and 384 to be the main tubulin-binding region. Finally, Dobrzynski *et al.* (2000) have localized a "hot spot" in CCT binding in the segment between residues 261 and 274. Our docking analysis localizes two CCT-binding fragments (S277-V288 and V355-P359) in this area. These two fragments have been confirmed as CCT-binding sites by Ritco-Vonsovici and Willison (2000), who have found two CCT-binding sites, site G (P263-T292) and site K (T353-R390), each one encompassing one of the two fragments described above.

Localization of the CCT-Binding Sites in the Sequence Alignment of Actin and Tubulin with Their Prokaryotic Homologues FtsA and FtsZ

A visual inspection of the docking (Fig. 1) quickly reveals that the CCT-binding sites of actin and tubulin are located mostly in loops between stretches of secondary structure. As explained earlier, the fact that CCT is involved mainly in the folding of actin and tubulin leads us to think that CCT may have

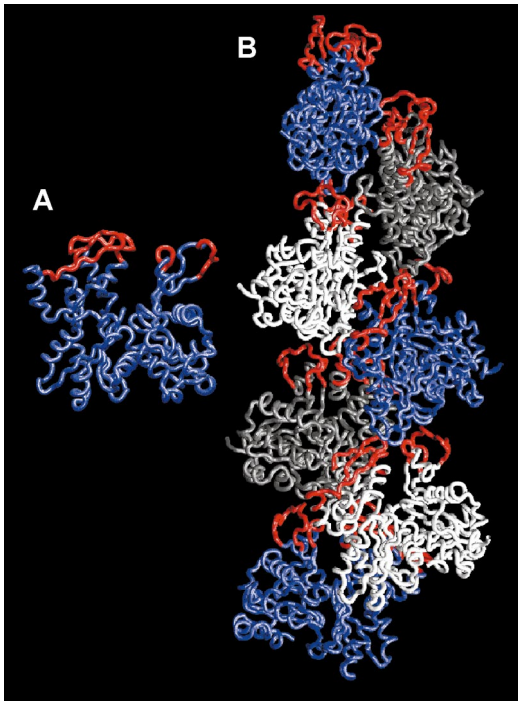

FIG. 4. Localization of the actin CCT-binding motifs in the F-actin filament. (A) Atomic structure of α-actin (Kabsch *et al.*, 1990) with the CCT-binding domains colored red. (B) Atomic model of the F-actin filament (Holmes *et al.*, 1990).

evolved from a primordial chaperonin to deal with specific folding problems of the two cytoskeletal proteins that appeared after the two proteins had begin to evolve from their predecessors. We therefore decided to analyze the CCT-binding sites of actin and tubulin in the context of a primary sequence alignment of the two proteins with their prokaryotic homologues, FtsA and FtsZ.

Figure 2 shows the alignment of 5 sequences of actin with 5 sequences of FtsA (an alignment with 40 sequences of the actin family and 32 sequences of the FtsA family has also been performed, and the results were essentially identical). Despite the absence of a high degree of similarity in the primary sequence, the atomic structure of FtsA (van den Ent and Löwe, 2000) shows an architecture similar to that of actin in the core domain and in the nucleotide-binding site (Kabsch *et al.*, 1990). Figure 2 shows indeed a similar distribution of the secondary structure elements, the main difference being in FtsA the lack of the so-called subdomain 2 of actin and the presence of a new subdomain, absent in actin, that is located on the opposite side of subdomain 2 of actin. Other differences reside in small stretches of residues that are present in one of the proteins and absent in the other. When the CCT-binding sites of actin were compared with the sequence alignment of actin and FtsA, it was surprising to see that the four binding sites were localized mostly in segments of the actin sequence absent in FtsA. The two first fragments are positioned very close in the primary sequence of actin and located at the tip of its subdomain 2, absent in FtsA. The first segment (R37-D51) is placed in a large loop between

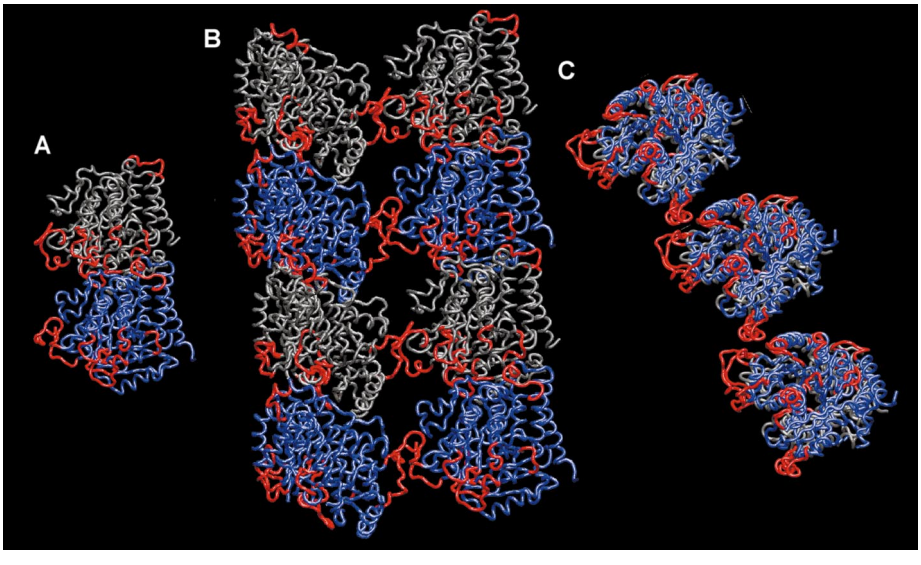

FIG. 5. Localization of the tubulin CCT-binding motifs in the microtubules. (A) Atomic structure of the α,β-tubulin dimer (Nogales *et al.*, 1998a) with β-tubulin molecule colored silver and the α-tubulin colored blue. The loops implicated in CCT binding are colored red. (B) Longitudinal view of two protofilaments showing the longitudinal and lateral interactions that generate the protofilaments and the microtubules, respectively. (C) End-on view from the plus end of three protofilaments, showing the arrangement of a microtubule, as in Nogales *et al.* (1999). The color code is as in (A).

two small β-strands and the second segment (R62-T66), much smaller, is located mainly between an α-helix and a β-strand. The other two CCT-binding sites are located in subdomain 4 of actin, which has a counterpart in FtsA. However, the segment E195-R206 is placed in the actin loop located between two large helices that is absent in the prokaryotic homologue. The situation is less clear in the fourth CCT-binding site, T229-I250, since it encompasses not only a loop in the actin sequence that is absent in FtsA, but also a large segment of secondary structure that is also present in the prokaryotic protein.

A similar situation occurs between tubulin and its prokaryotic homologue FtsZ. Despite the limited sequence identity between the two proteins, there is, however, a high degree of structural similarity (Nogales *et al.*, 1998a; Löwe and Amos, 1998). Both proteins have a common core that includes two domains, each of them formed by a β-sheet surrounded by α-helices, with a GTP-binding site that is also similar (Nogales *et al.*, 1998b). Figure 3 shows an alignment between 5 sequences of tubulin and 5 sequences of FtsZ (an alignment has also been performed with 32 sequences of the tubulin family and 41 sequences of the FtsZ with similar results), where the similarities in the secondary structure between the two proteins can be observed. Some differences are also present. The main difference is the presence of two large helices at the C-terminus of the tubulin molecule that are not present in FtsZ. Other differences are related in the case of the eukaryotic protein to the existence of larger loops linking the segments of secondary structure, and these are the places where the tubulin CCT-binding sites are located (Fig. 3). Out of 8 CCT-binding sites, 6 of them are located in segments of the tubulin molecule that are not present in FtsZ.

As described above, there are three CCT-binding sites in the N-terminal domain of tubulin and all three are localized in regions that have no FtsZ counterpart. The first CCT-binding segment (T33-A57) is localized within the large loop between α-helix 1 and β-strand 2 (hereafter denoted H1 and S2, as in Nogales *et al.*, 1998b). The second binding site (S126-Q133) is placed in a loop between H3 and S4, and the third (E160-R164) encompasses a very small loop between H4 and S5 and part of H4. There is also a large loop between H2 and S3 in the N-terminal domain of tubulin (P82-D90) that is absent in FtsZ and that has not been assigned by the docking analysis as a possible CCT-binding domain. However, in our docking analysis this region is interacting so closely with CCT that we cannot rule this to be the case.

There are five CCT-binding sites in the C-terminal region of tubulin, and three of them are located in loops not present in the FtsZ structure. CCT-binding sites P261-H266 and S277-V288 are contiguous and separated by a β-strand, and segment W407-E417 is localized in a large loop of the C-terminal region, between H11 and H12 (Nogales *et al.*, 1998a). Two of the CCT-binding sites occupy regions of the tubulin molecule that are also present in FtsZ. The first, T239-K254, is located within the so-called T7 loop (between H7 and H8), one of the most conserved regions in the tubulin and FtsZ molecule, and its role will be discussed later. The other CCT-binding site, V355-P359, is placed near a region of the tubulin molecule that has no prokaryotic counterpart (between S9 and S10) and is not present in most of the β- and γ-tubulin sequences.

Role of the CCT-Binding Sites in the Polymerization of Actin and Tubulin

In the search for the functional differences between actin and tubulin and their prokaryotic homologues that may have driven the evolution of CCT from a primordial chaperonin, we reasoned that this may be the capacity of these molecules to polymerize. In this regard, little is known about the function of FtsA, although there is evidence that FtsA and FtsZ interact *in vivo* and that this interaction is stringent for bacterial cell survival (Addinall and Lutkenhaus, 1996). No polymeric structure has yet been found to be formed by FtsA. Despite its structural similarity to FtsA, actin is capable of forming the large filaments that are utterly fundamental to cytokinesis and cell motility. As described earlier, the atomic structure of actin is known (Kabsch *et al.*, 1990) and there is also an atomic model of the actin filament F-actin (Holmes *et al.*, 1990). These have allowed us to locate the CCT-binding sites of actin within F-actin (Fig. 4). According to the model, a large part of the interactions generating the filament occur through residues that form part of the CCT-binding sites involving, among others, residues 40–45, 63–64, 195–197, 202–204, and 243–245 (for a more detailed description, see Holmes *et al.* (1990)). Figure 4 shows explicitly this fact since it reveals quite clearly that the CCT-binding sites of actin (red loops in Fig. 4B) are involved in the sites of formation of the actin filament. There are other residues, located mainly in the C-terminal region, that are involved in actin polymerization and that do not form part of the CCT-binding sites assigned by the docking analysis. Some of them have, however, been found by peptide array experiments with β-actin to be part of a CCT-binding site (site III (residues 301–345); Hynes and Willison, 2000; McCormack *et al.*, 2001a) that is not detected in the docking we have performed so far. A possible explanation for this apparent discrepancy is the fact that

our docking analysis has been performed with only one of the actin conformers in the CCT folding cycle, the one that is recognized by apo-CCT, and it may be the case that later on in the CCT cycle other actin regions interact with the cytosolic chaperonin.

A different case is that of FtsZ and tubulin. Both proteins are involved in cytokinesis, although forming polymers of different nature. FtsZ polymerizes *in vivo* forming a structure named Z-ring, which is part of the septum that constricts the cell and generates two daughter cells (Bi and Lutkenhaus, 1991). Each FtsZ monomer can contribute a 22° step to the ring and the filament consists of a linear string of monomers (see Erickson, 2001). In the case of tubulin, dimers of α,β-tubulin polymerize longitudinally and laterally to form microtubules, structures that are involved in cell motility, chromosome segregation, and vesicle movement (Nogales, 2000). Both proteins are also able to polymerize *in vitro* into various types of structures, which demonstrates their tendency to aggregate. The atomic structures of FtsZ (Löwe and Amos, 1998) and the α,β-tubulin dimer (Nogales *et al.*, 1998a) have been docked respectively into the FtsZ filaments reconstructed from two-dimensional crystals (Löwe and Amos, 1999) and into the three-dimensional reconstruction of a microtubule (Nogales *et al.*, 1999). From this docking, some of the longitudinal interactions among the monomers forming the filaments and the lateral interactions between filaments have been inferred (Fig. 5). The filaments generated by these two proteins are stabilized by longitudinal interactions between regions that are homologous in the two proteins. Among them, loop T7 plays a very important role. Loop T7 is one of the tubulin CCT-binding sites found in our docking analysis, and one of the two CCT-binding domains that has a FtsZ counterpart. In fact, the T7 loop is one of the most conserved regions when the two proteins are compared. The T7 loop is placed very close to the GTP-binding site of the monomer downstream in the filament and has been implicated in the GTPase-dependent formation of the filaments in both proteins, which speaks of its conservation throughout evolution. Other sequences are involved in filament stabilization in both proteins. Two of them are present only in tubulin. These correspond to the regions encompassing residues M398, A403, F404, H406, and W407, belonging to the CCT-binding domain located between helices H11 and H12, and the sequence encompassing residues C131 and Q133, located between H3 and S4 (Fig. 4) (Nogales *et al.*, 1999).

There are further clear differences between FtsZ and tubulin regarding the lateral interactions occurring between filaments (Figs. 5B and 5C). The docking of the atomic structure of FtsZ into the three-dimensional reconstruction of FtsZ filaments suggests that the lateral interactions between filaments occur through two types of contacts: a large one between the strands S3 of two FtsZ molecules that stabilize the two parallel filaments that form one thick filament, and a limited one between the C-terminal domains (residues 357–372) of the FtsZ molecules that stabilize two thick filaments (Löwe and Amos, 1999). It is, however, not clear whether these interactions occur *in vivo* or are just generated by the crystallization conditions. There is no evidence that FtsZ forms dimeric or higher order polymeric filaments (see Erickson, 2001). On the other hand, the atomic structure of the α,β-tubulin dimer has been docked into the three-dimensional reconstruction of a microtubule and therefore it is very likely that the interactions described correlate with those occurring *in vivo* (Nogales *et al.*, 1999). Among the lateral interactions described, the most important one occurs between three of the CCT-binding sites assigned by our docking analysis, three sequences that are not present in FtsZ. This interaction occurs between the M loop (located between S7 and H9; Nogales *et al.*, 1998b), which correlates with the CCT-binding site encompassing residues S277-V288, H3, and the loop between H3 and S4, which partly coincides with the CCT-binding site encompassing residues S126-Q133, and part of the loop between H1 and S2 (residues 51–55), which coincides with the CCT-binding site encompassing residues T33-A57. There is yet another interaction between the M loop and a region of the tubulin monomer opposed laterally. This region corresponds to the loop between H2 and S3, a stretch of sequence that is not present in FtsZ (see Fig. 3) and although it is not interacting with CCT in our docking analysis, it is so close to doing so that it may also be another CCT-binding site.

DISCUSSION

Chaperonins are a group of proteins involved in providing assistance in the folding of other proteins. The mechanism by which they do so has been characterized for the prokaryotic chaperonin GroEL and was thought previously to be general for all the chaperonins. Its function involves the recognition of hydrophobic residues of unfolded polypeptides. The chaperonin then undergoes a series of conformational changes driven by the binding and hydrolysis of ATP, which, with the help of a cochaperonin ring, stretches and liberates the unfolded protein within the cavity of the chaperonin, in an environment in which the protein may fold by itself. This mechanism is not very efficient but serves to aid a large number of proteins.

However, there are exceptional proteins like actin and tubulin, which when chemically unfolded are able to interact with GroEL, but are unable to be folded by it (Tian *et al.*, 1995a). The activity of the cytosolic chaperonin CCT is stringent for the folding of these substrates, and the structural and biochemical analyses carried out so far indicate that the mechanism by which CCT folds proteins is of a different nature. Actin and tubulin both seem to reach a quasi-native conformation before interacting with CCT (Rommelaere *et al.*, 1993; Melki and Cowan, 1994; Llorca *et al.*, 1999b, 2000). Both cytoskeletal proteins interact with CCT in a defined way involving specific subunits of CCT. This chaperonin is built up from eight different, albeit homologous proteins placed in a defined arrangement in each ring (Liou and Willison, 1997), which also speaks of a specialized role of CCT in protein folding.

The actin and tubulin molecules bound to the nucleotide-free, open conformations of CCT are therefore stabilized in quasi-native conformations. By quasi-native we assume that a high degree of secondary and tertiary structure is found in these CCT-bound molecules. Nevertheless, these CCT-bound intermediates are highly susceptible to proteolysis (Grantham *et al.*, 2000). The docking performed with the modified atomic structures of actin and tubulin into their corresponding volumes of the three-dimensional reconstruction of the CCT:substrate complexes shows in both cases that the two cytoskeletal proteins are maintained in an open conformation in which their N- and C-terminal domains interact with opposing regions of the CCT ring (Llorca *et al.*, 2000). The importance of the opening of the molecules in their interaction with CCT is corroborated in the case of actin by a mutant, G150P, located in the putative hinge linking the small and large domains, which seems to block the opening of the actin molecule and its interaction with the two sides of the CCT cavity, thus preventing its folding (McCormack *et al.*, 2001b). In the case of actin, the N-terminal domain interacts with CCTδ and the C-terminal domain with CCTβ or CCTϵ. In the case of tubulin, the N-terminal domain interacts with CCTδ/θ or CCTη/α and the C-terminal domain with CCT$\epsilon/\zeta/\beta$ or CCT$\beta/\gamma/\theta$, respectively (Llorca *et al.*, 1999a, 2000). Immunomicroscopy experiments with complexes made up of CCT and fragments of actin and tubulin have allowed us to correlate specific segments of the sequence of the two proteins with the CCT subunit with which they are interacting. Independent biochemical analyses with fragments of these substrates have revealed that for both actin and tubulin molecules, the strongest binding affinity toward CCT occurs through the segments of both proteins that interact with CCTβ or CCTϵ. This allows the suggestion that these two subunits are the ones driving the high-affinity interaction between CCT and the two cytoskeletal proteins (Llorca *et al.*, 2000), and this explains the competition between the two proteins for binding to CCT (Melki *et al.*, 1993).

In this work we have proceeded further with the analysis of the docking, which has allowed us to localize more precisely the fragments of the two cytoskeletal proteins that seem to be involved in CCT binding. According to our docking analysis, there are four such CCT-binding sites in actin and eight in tubulin. The nature of the residues forming part of the CCT-binding sites is different from that of those residues involved in the interaction with GroEL. Whereas in the latter case, they are hydrophobic residues hidden within the structure of the native protein, those that are recognized by the same type of residue in the chaperonin (Braig *et al.*, 1994; Fenton *et al.*, 1994; Buckle *et al.*, 1997), the CCT-binding sites assigned by this docking analysis encompass a majority of charged and polar residues, which again strengthens the idea of a specific interaction between CCT and its natural substrates (Hynes and Willison, 2000).

Another interesting observation is the fact that when the sequences of actin and tubulin are aligned with those of their prokaryotic homologues FtsA and FtsZ, almost all the CCT-binding sites are located in loops, in stretches of sequences that do not exist in FtsA and FtsZ. Furthermore, these CCT-binding sites localize in domains involved in the polymerization of the two cytoskeletal proteins, and this raises important questions about the evolution of the two modern proteins. No oligomeric structure is known to be formed by FtsA, and FtsZ forms cylindrical structures thus far poorly characterized that are generated in certain periods of the bacterial cycle. However, it is well known that actin and tubulin form large and complex structures ever present in the cell, which are implicated in various cellular processes such as cell division, chromosome aggregation and segmentation, muscle contraction, amoeboid movement, and processes of endocytosis and exocytosis. Both proteins have also evolved to interact with motor proteins (Kreis and Vale, 1999), and in the case of tubulin, such interactions occur through a region (the C-terminal domain) that is again not present in FtsZ. All this strongly suggests that these regions have appeared in actin and tubulin subsequent to their initial duplication from their respective ancestors, to generate more complex structures capable of playing roles other than those related to cell division. It is generally believed that it is the development of the cytoskeleton that gave rise to the appearance of the eukaryotes (Doolittle,

1995). It has already been suggested that CCT may have evolved to completion during the early stages of the evolution of eukaryotes (Kubota *et al.*, 1994, 1995) at the same time as actin and tubulin (Willison and Horwich, 1996; Willison, 1999; Llorca *et al.*, 2000). There are two types of results that support this view. As shown by Llorca *et al.* (2000), tubulin interacts with all five CCT subunits in two arrangements that use all eight CCT subunits, which suggests that CCT may have evolved specifically to cope with the folding problems of the newly evolved tubulin and later actin came to interact with CCT, using the latest evolving pair of CCT subunits (CCTδ and CCTϵ; see Archibald *et al.*, 2000; Hynes and Willison, 2000; Llorca *et al.*, 2000). Other proteins came later, like the clearly opportunistic EBNA-3 from the Epstein–Barr virus (Kashuba *et al.*, 1999). A second piece of evidence comes from the fact that the most of the CCT-binding sites of actin and tubulin are located in stretches of the sequence that do not exist in their prokaryotic homologues FtsA and FtsZ, proteins that one assumes are more similar than actin and tubulin to their respective ancestors.

There is therefore a clear relationship between the new domains in actin and tubulin that have provided these two proteins with their new and important properties and these new domains interact with the specific chaperonin CCT. But what is the purpose of such interactions during the folding process? Why do both actin and tubulin interact with CCT in an open and quasi-native conformation? It seems clear that the folding of the actin and tubulin domains is chaperonin-independent, the quasi-native conformation probably being acquired before interacting with CCT, occurring either by themselves or with the help of a cofactor (prefoldin). It has been hypothesized for actin that CCT may be involved in nucleotide binding or loading into the interdomain cleft during the folding of the protein (Llorca *et al.*, 2000). For tubulin, biochemical studies carried out with CCT and the cytoskeletal protein clearly indicate that at least one function of GTP binding is to stabilize the quasi-native tubulin molecule during the CCT-facilitated folding (Tian *et al.*, 1995b). Other hypotheses are also plausible, like the protection of these CCT-binding sites from unwanted interactions before the complete folding of the cytoskeletal molecules has occurred. More experiments await before answers to these exciting questions can be obtained.

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REFERENCES

Addinall, S. G., and Lutkenhaus, J. (1996) FtsZ ring formation in *fts* mutants. *J. Bacteriol.* **178,** 3877–3884.

Archibald, J. M., Logsdon, J. M., and Doolittle, W. F. (2000) Origin and evolution of eukaryotic chaperonins: Phylogenetic evidence for ancient duplications in CCT genes. *Mol. Biol. Evol.* **17,** 1456–1466.

Bateman, A., Birney, B., Durbin, R., Eddy, S. R., Howe, K. L., and Sonnhammer, E. L. (2000) The PFAM protein families database. *Nucleic Acids Res.* **28,** 263–266.

Bi, E. F., and Lutkenhaus, J. (1991) FtsZ ring structure associated with division in *Escherichia coli. Nature* **353,** 161–164.

Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature* **371,** 578–586.

Buckle, A. M., Zahn, R., and Fersht, A. R. (1997) A structural model for GroEL–polypeptide interaction. *Proc. Natl. Acad. Sci. USA* **94,** 3571–3575.

Bukau, B., and Horwich, A. L. (1998) The hsp70 and hsp60 chaperone machines. *Cell* **92,** 351–366.

Chen, L., and Sigler, P. B. (1999) The crystal structure of a GroEL/peptide complex: Plasticity as a basis for substrate diversity. *Cell* **99,** 757–768.

Chen, X., Sullivan, D. S., and Huffaker, T. C. (1993) Two yeast genes with similarity to TCP-1 are required for microtubule and actin function *in vivo. Proc. Natl. Acad. Sci. USA* **91,** 9111–9115.

Ditzel, L., Löwe, J., Stock, D., Stetter, K. O., Huber, H., Huber, R., and Steinbacher, S. (1998) Crystal structure of the thermosome, the archaeal chaperonin and homologue of CCT. *Cell* **93,** 125–138.

Dobrzynski, J. K., Sternlicht, M. L., Farr, G. W., and Sternlicht, H. (1996) Newly-synthesized β-tubulin demonstrates domain-specific interactions with the cytosolic chaperonin. *Biochemistry* **35,** 15 870–15 882.

Dobrzynski, J. K., Sternlicht, M. L., Peng, I., Farr, G. W., and Sternlicht, H. (2000) Evidence that β-tubulin induces a conformation change in the cytosolic chaperonin which stabilizes binding: Implications for the mechanism of action. *Biochemistry* **39,** 3988–4003.

Doolittle, R. F. (1995) The origins and evolution of eukaryotic proteins. *Philos. Trans. R. Soc. London B* **349,** 235–240.

Ellis, R. J. (1996) Chaperonins: introductory perspective. *in* Ellis, R. J. (Ed.), The Chaperonins, pp. 1–25, Academic Press, San Diego.

Ellis, R. J., and Hartl, F. U. (1999) Principles of protein folding in the cellular environment. *Curr. Opin. Struct. Biol.* **9,** 102–110.

Erickson, H. P. (2001) The FtsZ protofilament and attachment of ZipA—Structural constraints on the FtsZ power stroke. *Curr. Opin. Cell. Biol.* **13,** 55–60.

Farr, G. W., Furtak, K. Rowland, M. B., Ranson, N. A., Saibil, H. R., Kirchausen, T., and Horwich, A. L. (2000) Multivalent binding of nonnative substrate proteins by the chaperonin GroEL. *Cell* **100,** 561–573.

Fenton, W. A., Kashi, Y., Furtak, K., and Horwich, A. L. (1994)

Residues in chaperonin GroEL required for polypeptide binding and release. *Nature* **371,** 614–619.

Frank, J., Radermacher, M., Penczek, P., Zhu, J., Li, Y., Ladjadj, J., and Leith, A. (1996) SPIDER and WEB: Processing and visualisation of images in 3D electron microscopy and related fields. *J. Struct. Biol.* **116,** 190–199.

Grantham, J., Llorca, O., Valpuesta, J. M., and Willison, K. R. (2000) Partial occlusion of both cavities of CCT with antibody has no effect upon the rates of β-actin or α-tubulin folding. *J. Biol. Chem.* **275,** 4587–4591.

Gutsche, I., Essen, L. O., and Baumeister, W. (1999) Group II chaperonins: New TRIC(k)s and turns of a protein folding machine. *J. Mol. Biol.* **293,** 295–312.

Gutsche, I., Holzinger, J., Röble, M., Heumann, H., Baumeister, W., and May, R. P. (2000) Conformational rearrangements of an archaeal chaperonin upon ATPase cycling. *Curr. Biol.* **10,** 405–408.

Hansen, W. J., Cowan, N. J., and Welch, W. J. (1999) Prefoldin-nascent chain complexes in the folding of cytoskeletal proteins. *J. Cell. Biol.* **145,** 265–277.

Holm, L., and Sander, C. (1996) Mapping the protein universe. *Science* **273,** 595–602.

Holmes, K. C., Popp, D., Gebhard, W., and Kabsch, W. (1990) Atomic model of the actin filament. *Nature* **347,** 44–49.

Houry, W. A., Frishman, D., Eckerskorn, C., Lottspeich, F., and Hartl, F. U. (1999) Identification of *in vivo* substrates of the chaperonin GroEL. *Nature* **402,** 147–154.

Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD—Visual molecular dynamics. *J. Mol. Graphics* **14,** 33–38.

Hunt, J. F., Weaver, A. J., Landry, S. J., Gierasch, L., and Deisenhofer, J. (1996) The crystal structure of the GroES co-chaperonin at 2.8 Å resolution. *Nature* **379,** 37–45.

Hynes, G. M., and Willison, K. R. (2000) Individual subunits of the chaperonin containing TCP-1 (CCT) mediate interactions with binding sites located on subdomains of β-actin. *J. Biol. Chem.* **275,** 18 985–18 994.

Kabsch, W., and Sander, C. (1983) Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22,** 2577–2637.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., and Holmes, K. C. (1990) Atomic structure of the actin:DNase I complex. *Nature* **347,** 37–44.

Kashuba, E., Pokrovskaja, K., Klein, G., and Szekely, L. (1999) Epstein-Barr virus-encoded nuclear protein EBNA-3 interacts with the ϵ-subunit of the t-complex protein 1 chaperonin complex. *J. Hum. Virol.* **2,** 33–36.

Kim, S., Willison, K. R., and Horwich, A. L. (1994) Cytosolic chaperonin subunits have a conserved ATPase domain but diverged polypeptide-binding domains. *Trends Biochem. Sci.* **19,** 543–548.

Klumpp, M., Baumeister, W., and Essen, L. O. (1997) Structure of the substrate binding domain of the thermosome, an archaeal group II chaperonin. *Cell* **91,** 263–270.

Kreis, T., and Vale, R. (1999) Guidebook to the Cytoskeleton and Motor Proteins, Oxford Univ. Press, London/New York.

Kubota, H., Hynes, G., Carne, A., Asworth, A., and Willison, K. (1994) Identification of six TCP-1 related genes encoding divergent subunits of the TCP-1 containing chaperonin. *Curr. Biol.* **4,** 89–99.

Kubota, H., Hynes, G., and Willison, K. (1995) The chaperonin containing t-complex polypeptide 1 (TCP-1). Multisubunit machinery assisting in protein folding and assembly in the eukaryotic cytosol. *Eur. J. Biochem.* **230,** 3–16.

Leroux, M. R., Fändrich, M., Klunker, D., Siegers, K., Lupas, A. N., Brown, J. R., Schiebel, E., Dobson, C. M., and Hartl, F. U. (1999) MtGimC, a novel archaeal chaperone related to the eukaryotic chaperonin cofactor Gim/prefoldin. *EMBO J.* **18,** 6730–6743.

Leroux, M. R., and Hartl, F. U. (2000) Protein folding: Versatility of the cytosolic chaperonin TRiC/CCT. *Curr. Biol.* **10,** R260–R264.

Liou, A. K. F., and Willison, K. R. (1997) Elucidation of the subunit orientation in CCT (chaperonin containing TCP1) from the subunit composition of micro-complexes. *EMBO J.* **16,** 4311–4316.

Llorca, O., Marco, S., Carrascosa , J. L., and Valpuesta, J. M. (1997) Conformational changes in the GroEL oligomer during the functional cycle. *J. Struct. Biol.* **118,** 31–42.

Llorca, O., Smyth, M. G., Marco, S., Carrascosa, J. L., Willison, K. R., and Valpuesta, J. M. (1998) ATP binding induces large conformational changes in the apical and equatorial domains of the eukaryotic cytoplasmic chaperonin (CCT). *J. Biol. Chem.* **273,** 10 091–10 094.

Llorca, O., Smyth, M. G., Carrascosa, J. L., Willison, K. R., Radermacher, M., Steinbacher, S., and Valpuesta, J. M. (1999a) 3D reconstruction of the ATP-bound form of CCT reveals the asymmetric folding conformation of a type II chaperonin. *Nat. Struct. Biol.* **6,** 639–642.

Llorca, O., McCormack, E. Hynes, G., Grantham, J., Cordell, J., Carrascosa, J. L., Willison, K. R., Fernández, J. J., and Valpuesta, J. M. (1999b) Eukaryotic type II chaperonin CCT interacts with actin through specific subunits. *Nature* **402,** 693–696.

Llorca, O., Martín-Benito, J., Ritco-Vonsovici, M., Grantham, J., Hynes, G., Willison, K. R., Carrascosa, J. L., and Valpuesta, J. M. (2000) Eukaryotic chaperonin CCT stabilizes actin and tubulin folding intermediates in open quasi-native conformations. *EMBO J.* **19,** 5971–5979.

Löwe, J., and Amos, L. A. (1998) Crystal structure of the bacterial cell-division protein FtsZ complexed with GDP. *Nature* **391,** 203–206.

Löwe, J., and Amos, L. A. (1999) Tubulin-like protofilaments in Ca^{2+}-induced FtsZ sheets. *EMBO J.* **18,** 2364–2371.

Marabini, R., Herman, G. T., and Carazo, J. M. (1998) 3D reconstruction in electron microscopy using ART with smooth spherically symmetric volume elements (blobs). *Ultramicroscopy* **72,** 53–65.

Marco, S., Carrascosa, J. L., and Valpuesta, J. M. (1994) Reversible interaction of β-actin along the channel of the TCP-1 cytoplasmic chaperonin. *Biophys. J.* **67,** 364–368.

McCormack, E., Rohman, M., and Willison, K. R. (2001a) Mutational screen identifies critical amino acid residues of β-actin mediating interaction between folding intermediates and cytosolic chaperonin TCP-1. *J. Struct. Biol.* **135,** 185–197.

McCormack, E., Llorca, O., Carrascosa, J. L., Valpuesta, J. M., and Willison, K. R. (2001b) Point mutations in a hinge linking the small and large domains of β-actin result in trapped folding intermediates bound to cytosolic chaperonin containing TCP-1. *J. Struct. Biol.* **135,** 198–204.

Melki, R., Vainberg, I. E., Chow, R. L., and Cowan, N. J. (1993) Chaperonin-mediated folding of vertebrate actin-related protein and gamma-tubulin. *J. Cell Biol.* **122,** 1301–1310.

Melki, R., and Cowan, N. J. (1994) Facilitated folding of actins and tubulins occurs via a nucleotide-dependent interaction between cytoplasmic chaperonin and distinctive folding intermediates. *Mol. Cell. Biol.* **14,** 2895–2904.

Miklos, D., Caplan, S., Martens, D., Hynes, G., Pitluk, Z., Brown,

C., Barrell, B., Horwich, A. L., and Willison, K. R. (1994) Primary structure and function of a second essential member of heterooligomeric TCP1 chaperonin complex of yeast. TCP1β, *Proc. Natl. Acad. Sci USA* **91,** 2743–2747.

Nitsch, M., Walz, J., Typke, D., Klumpp, M., Essen, L. O., and Baumeister, W. (1998) Group II chaperonin in an open conformation examined by electron tomography. *Nat. Struct. Biol.* **5,** 855–857.

Nogales, E. (2000) Structural insights into microtubule function. *Annu. Rev. Biochem.* **69,** 277–302.

Nogales, E., Wolf, S. G., and Downing, K. H. (1998a) Structure of the $\alpha\beta$-tubulin dimer by electron crystallography. *Nature* **391,** 199–203.

Nogales, E., Downing, K. H., Amos, L. A., and Löwe, J. (1998b) Tubulin and FtsZ form a distinct family of GTPases. *Nat. Struct. Biol.,* **5,** 451–458.

Nogales, E., Whittaker, M., Milligan, R. A., and Downing, K. H. (1999) High-resolution model of the microtubule. *Cell* **96,** 79–88.

Ritco-Vonsovici, M., and Willison, K. R. (2000) Defining the eukaryotic chaperonin-binding sites in human tubulins. *J. Mol. Biol.* **304,** 81–98.

Rommelaere, H., Van Troys, M., Gao, Y., Melki, R., Cowan, N. J., Vandekerckhove, J., and Ampe, C. (1993) Eukaryotic cytosolic chaperonin contains t-complex polypeptide 1 and seven related subunits. *Proc. Natl. Acad. Sci. USA* **90,** 11 975–11 979.

Rommelaere, H., De Neve, M., Melki, R., Vandekerckhove, J., and Ampe, C. (1999) The cytosolic class II chaperonin CCT recognizes delineated hydrophobic sequences in its target proteins. *Biochemistry* **38,** 3246–3257.

Roseman, A. M., Chen, S., White, H., Braig, K., and Saibil, H. R. (1996) The chaperonin ATPase cycle: Mechanism of allosteric switching and movements of substrate-binding domains in GroEL. *Cell* **87,** 241–251.

Sander, C., and Schneider, R. (1991) Database of homology derived protein structures and the structural meaning of sequence alignment. *Proteins* **9,** 56–68.

Schoehn, G., Quaite-Randall, E., Jimenez, J. L., Joachimiak, A., and Saibil, H. R. (2000a) Three conformations of an archaeal chaperonin, TF55 from *Sulfolobus shibatae. J. Mol. Biol.* **296,** 813–819.

Schoehn, G., Hayes, M., Cliff, M., Clarke, A. R., and Saibil, H. R. (2000b) Domain rotations between open, closed and bullet-shaped forms of the Thermosome, an archael chaperonin. *J. Mol. Biol.* **301,** 323–332.

Shtilerman, M., Lorimer, G. H., and Englander, S. W. (1999) Chaperonin function: Folding by forced unfolding. *Science* **284,** 822–825.

Tian, G., Vainberg, I. E., Tap, W. D., Lewis, S. A., and Cowan, N. J. (1995a) Specificity in chaperonin-mediated protein folding. *Nature* **375,** 250–253.

Tian, G., Vainberg, I. E., Tap, W. D., Lewis, S. A., and Cowan, N. J. (1995b) Quasi-native chaperonin-bound intermediates in facilitated protein folding. *J. Biol. Chem.* **270,** 23 910–23 913.

Vainberg, I. E., Lewis, S. A., Rommelaere, H., Ampe, C., Vandejerchove, J., Klein, H. L., and Cowan, N. J. (1998) Prefoldin, a chaperone that delivers unfolded proteins to cytosolic chaperonin. *Cell* **93,** 863–873.

van den Ent, F., and Löwe, J. (2000) Crystal structure of the cell division protein FtsA from *Thermotoga maritima. EMBO J.* **19,** 5300–5307.

Vinh, D. B., and Drubin, D. G. (1994) A yeast TCP-1-like protein is required for actin function in vivo. *Proc. Natl. Acad. Sci. USA* **91,** 9116–9120.

Weissman, J. S., Hohl, C. M., Kovalenko, O., Kashi, Y., Chen, S., Braig, K., Saibil, H. R., Fenton, W. A., and Horwich, A. L. (1995) Mechanism of GroEL action: Productive release of polypeptide from a sequestered position under GroES. *Cell* **83,** 577–587.

Willison, K. R. (1999) Composition and function of the eukaryotic cytosolic chaperonin-containing TCP1. *in* Bukau, B. (Ed.), Molecular Chaperones and Folding Catalysts, pp. 555–571, Harwood Academic, Amsterdam.

Willison, K. R., and Horwich, A. L. (1996) Structure and function of chaperonins. *in* Ellis, R. J. (Ed.), The Chaperonins, pp. 107–135, Academic Press, San Diego.

Wriggers, W., Milligan, R. A., and McCammon, J. A. (1999) Situs: A package for docking crystal structures into low-resolution maps from electron microscopy. *J. Struct. Biol.* **125,** 185–195.

Xu, Z., Horwich, A. L., and Sigler, P. B. (1997) The crystal structure of the asymmetric GroEL-GroES-$(ADP)_7$ chaperonin complex. *Nature* **388,** 741–750.