Individual Subunits of the Eukaryotic Cytosolic Chaperonin Mediate Interactions with Binding Sites Located on Subdomains of β-Actin

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The chaperonin containing TCP-1 (CCT) of eukaryotic cytosol is composed of eight different subunit species that are proposed to have independent functions in folding its in vivo substrates, the actins and tubulins. CCT has been loaded with 35S-β-actin by in vitro translation in reticulocyte lysate and then subjected to immunoprecipitation with all eight anti-CCT subunit antibodies in mixed micelle buffers, conditions that disrupt CCT into its constituent monomers. Interactions between 35S-β-actin and isolated CCTα, CCTβ, CCTε, or CCTθ subunits are observed, suggesting that polar and electrostatic interactions may mediate actin binding to these four CCT subunits. Additionally, a β-actin peptide array was screened for CCT-binding sequences. Three regions rich in charged and polar amino acid residues, which map to the surface of native β-actin, are implicated in interactions between actin and CCT. Several of these biochemical results are consistent with the recent cryo-electron microscopy three-dimensional structure of apo-CCT-α-actin, in which α-actin is bound by the apical domains of specific CCT subunits. A model is proposed in which actin interacts with several CCT subunits during its CCT-mediated refolding cycle.

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1 The abbreviations used are: CCT, chaperonin containing TCP-1; PAGE, polyacrylamide gel electrophoresis; PBSA, Dulbecco’s phosphate-buffered saline solution A; mAb, monoclonal antibody.
actin and CCT during folding. We propose that some critical interactions are mediated between specific CCT subunits and actin folding intermediates and that hydrophilic loops and strands, which in native actin are found located on the surface, are components of the binding sites recognized by CCT.

**EXPERIMENTAL PROCEDURES**

cDNA Plasmids and in Vitro Protein Expression—Full-length β-actin cDNA (residues M1-F375 of human β-actin) was cloned into pCITE vector (Novagen). Ha-Ras-β-actin subdomain 4 fusion cDNA, abbreviated as β-actin.sub4 (residues 1–168 of human Ha-Ras linked by Ser-1 to residues Leu215→Phe218 of human β-actin in pBlueScript SK− vector; Stratagene), was described previously by Llorca et al. (12). All eight constitutively expressed mouse CCT subunit cDNAs in pBlueScript SK− were used as described by Liou et al. (13). In vitro transcription/translation reactions were carried out in the TNT™ rabbit reticulocyte lysate (Promega) in the presence of 40 μCi (40 pmol) of [35S]methionine (Amersham Pharmacia Biotech)/50 μl of volume as described (13). Individual translation reactions were performed at 30 °C for 22 min (β-actin), 60 min (CCTα, CCTβ, CCTγ, CCTε, CCTη, CCTθ, or CCT-1 subunits), or 45 min (β-actin.sub4).

Immunoprecipitation of β-Actin and β-Actin.sub4 Following In Vitro Translation—[35S]-Labeled full-length β-actin and β-actin.sub4 protein expression was achieved in rabbit reticulocyte lysate using monoclonal antibodies directed against β-Actin by immunoprecipitation with anti-CCT subunit antibodies (14, 15). Followings in vitro translation and sucrose gradient fractionation of rabbit reticulocyte lysate, three different types of immunoprecipitation experiment were performed.

In the first set of reactions (see Fig. 1), 114-μl aliquots of the fractions containing the hexadecameric CCT peak (20–23% sucrose) (16) were made up to a volume of 500 μl with either Nonidet P-40 immunoprecipitation buffer (50 mM HEPES, pH 7.2, 90 mM KCl, 0.5% Nonidet P-40 (final detergent concentration)) or mixed micelle immunoprecipitation buffer (50 mM HEPES, pH 7.2, 100 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS (final detergent concentrations)) and incubated with 5 μl of antibody for 2 h on ice. Protein A-Sepharose beads (Sigma) in either Nonidet P-40 buffer or mixed micelle buffer (packed volume, 50 μl) were added, and reactions were mixed for 2 h at 4 °C. The beads were then washed three times with 500 μl of Nonidet P-40 buffer or mixed micelle buffer.

In the second set of reactions (see Fig. 2C), fractions containing unlabeled hexadecameric CCT peak (20–23% sucrose) (16) were mixed with the fraction containing [35S]-labeled β-actin monomers (13% sucrose). The protein samples (total volume, 94 μl) were made up to a volume of 500 μl with either Nonidet P-40 or mixed micelle immunoprecipitation buffer and incubated with 5 μl of antibody for 2 h on ice. Protein A-Sepharose beads (Sigma) in either Nonidet P-40 buffer or mixed micelle buffer (packed volume, 50 μl) were added, and reactions were mixed for 2 h at 4 °C. The beads were then washed three times with 500 μl of Nonidet P-40 buffer or mixed micelle buffer.

In the third set of reactions (see Fig. 3), 130-μl aliquots of the sucrose fractions containing free CCT subunits and β-actin monomers (13–17% sucrose) were made up to a volume of 1 ml with Nonidet P-40 immunoprecipitation buffer and incubated with 10 μl of antibody for 2 h on ice. Protein A-Sepharose beads in Nonidet P-40 buffer (packed volume, 100 μl) were added, and reactions were mixed for 2 h at 4 °C. The beads were then divided into two equal samples, and one sample was washed three times with 500 μl of Nonidet P-40 buffer, whereas the other sample was washed three times with 500 μl of mixed micelle buffer.

Beads were resuspended in Laemmli loading buffer for analysis by SDS-PAGE on 8% (full-length β-actin) or 12.5% (β-actin.sub4) gels. [35S]-Labeled protein bands were visualized by autoradiography and quantified by PhosphorImager analysis on a Storm 860 (Molecular Dynamics).

**Purification of Hsc/Hsp70 Proteins—**Actin and CCT during folding. We propose that some critical interactions are mediated between specific CCT subunits and actin folding intermediates and that hydrophilic loops and strands, which in native actin are found located on the surface, are components of the binding sites recognized by CCT.

**RESULTS**

Differential Interaction between CCT Subunits and Subdomains of β-Actin—CCT in complex with [35S]-labeled β-actin was prepared under approximate physiological conditions by in vitro translation in rabbit reticulocyte lysate followed by sucrose gradient fractionation to separate actin in complex with hexadecameric CCT away from actin monomers and free CCT.
subunits (13). Initially, to follow CCT subunit recovery during immunoprecipitation, each of the eight CCT subunits was 35S-labeled individually and incorporated into rabbit CCT in reticulocyte lysate using the single-ring mediated assembly cycle discovered by Liou et al. (13). Two CCT preparations each having 35S-labeled β-actin and four 35S-labeled CCT subunit types were prepared by sucrose gradient fractionation of five pooled in vitro translation reactions (set 1 contained 35S-labeled β-actin and CCTα, β, δ, and ζ-1, whereas set 2 contained 35S-labeled β-actin and CCTγ, ε, η, and θ). These two sets were mixed after fractionation but prior to immunoprecipitation to produce CCT mixtures labeled in each of the eight subunits (Fig. 1A, lane S). Thus, in the following experiments it should be borne in mind that no CCT complex contains more than one 35S-labeled component, be it specific CCT subunit or the actin substrate. Immunoprecipitation of the pooled 35S-labeled CCT-β-actin mixtures with monoclonal anti-CCTα antibody 23C (16, 17) in 0.5% Nonidet P-40 recovered all eight CCT subunits and actin (Fig. 1A, lane N). This is to be expected, because this nonionic detergent does not disrupt CCT-substrate complexes (8, 16). Immunoprecipitation of 35S-labeled CCT-β-actin mixtures with monoclonal anti-CCTα antibody 23C in mixed micelle buffer recovered only the CCTα subunit but none of the other seven CCT subunit types (Fig. 1A, lane α), consistent with the fact that incubation in mixed micelle buffers causes disruption of CCT complex into its constituent subunits (16). However, some actin remains bound to the CCTα subunit dissociated from holo-CCT by detergent (Fig. 1A, lane α). Immunoprecipitation of the 35S-labeled CCT-β-actin mixtures with the other seven anti-CCT C-terminal epitope antibodies (14) in mixed micelle buffer found that each antibody recovered its cognate CCT subunit and various amounts of 35S-β-actin (Fig. 1A, lanes β, γ, δ, ε, η, θ, and ζ-1).

Recovery of each CCT subunit by immunoprecipitation with its cognate antibody under mixed micelle conditions was quantitated and is shown graphically in Fig. 1B. There is a 10-fold range in recovery that is probably a consequence of several variables, including antibody affinity and accessibility of the C-terminal epitopes of individual CCT subunits. These immunoprecipitations were repeated with unlabeled CCT-35S-β-actin complexes (Fig. 1C), and the data were quantitated to show the relative amount of actin recovered by each anti-CCT subunit antibody (Fig. 1D). This experiment was repeated several times with similar results, and the conclusion is that 35S-β-actin is recoverable in complex with CCTα, β, ε, and θ under conditions that cause disruption of holo-CCT into its constituent monomers. Certain CCT subunits are well recovered by immunoprecipitation under mixed micelle conditions, such as CCTγ (Fig. 1B), but do not appear complexed with actin under these stringent conditions. It is, of course, possible that different and weaker interactions are occurring between some CCT subunits and β-actin, but these cannot be discerned under milder conditions, because such conditions do not disrupt holo-CCT.

Overall, when these data are compared with the CCTα-actin complex model (12), some aspects are consistent, such as the substantial β-actin signals recovered with anti-CCTβ and anti-CCTε antibodies, whereas others are not accountable, such as the substantial β-actin signals recovered with CCTθ and CCTα antibodies. Furthermore, an important interaction seen between CCTθ and actin subdomain 2 in the three-dimensional
structure is only weakly discernible under these immunoprecipitation conditions (Fig. 1, C and D). Llorca et al. (12) also determined a structure of apo-CCT bound to recombinant 35S-β-actin subdomain 4 fused to Ha-Ras, named 35S-β-actin.sub4, in which the hybrid protein appears to contact either CCTβ or CCTe but not CCTeβ. 35S-β-actin.sub4 interacts with CCT upon in vitro translation in rabbit reticulocyte lysate, and the interaction is mediated through actin subdomain 4 of the chimeric protein, because Ha-Ras does not interact with CCT. Therefore, a similar immunoprecipitation analysis to the experiment with 35S-β-actin was carried out on unlabelled CCT complexed to 35S-β-actin.sub4 (Fig. 1, E and F). The data are again consistent with the three-dimensional reconstruction; these are the substantial 35S-β-actin signal recovered with anti-CCTβ and anti-CCTe antibodies and the absence of any 35S-β-actin.sub4 signal with CCTβ antibody. Again, CCTα and CCTθ produce positive interactions with 35S-β-actin.sub4, indicating a contribution of these CCT subunits to associations with the large domain of actin.

A number of control experiments were performed to confirm that the interactions between newly synthesized 35S-β-actin and CCT subunits are productive (Fig. 2). β-Actin is actively folded and processed to a non-CCT interacting monomer conformation upon in vitro translation in rabbit reticulocyte lysate (12), indicating that interaction between newly synthesized 35S-β-actin and CCT is transient. The 20 S sucrose peak containing CCT-35S-β-actin complexes was incubated with Mg-ATP to induce release of bound substrate. Quantitation of 35S-β-actin recovery upon immunoprecipitation of CCT indicates that a significant amount of 35S-β-actin is released from CCT subunits in response to Mg-ATP and that the released β-actin is not re-bound by CCT nor does it adhere nonspecifically to the protein A-Sepharose beads (Fig. 2A).

β-Actin monomers, produced through the action of CCT during the course of the in vitro translation reaction, sediment in the top fractions of the sucrose gradient (13–17% sucrose). A DNase I shift assay indicates that the β-actin monomers have been folded to a native conformation (Fig. 2B), presumably as a result of their interaction with CCT, and that they retain their native conformation during fractionation. These native 35S-β-actin monomers were mixed with unlabelled CCT complex, and immunoprecipitation reactions similar to those described in Fig. 1 were performed (Fig. 2C). No interaction between 35S-β-actin and CCT subunits was detected in either 0.5% Nonidet P-40 or mixed micelle buffers. This indicates that native β-actin is not bound by CCT complex, despite the input 35S-β-actin monomer counts being in 10-fold excess compared with the 35S-β-actin counts preloaded on CCT in in vitro translation under physiological conditions (Fig. 1). This demonstrates that the specific interactions detected between newly synthesized β-actin and CCT subunits upon in vitro translation do not occur upon mixing CCT and folded β-actin monomers. Furthermore, nonspecific interactions between CCT subunits and β-actin do not occur either in Nonidet P-40 or in mixed micelle buffers during the course of the immunoprecipitation experiments.

**Dissociated CCTα and CCTθ Interact with Native β-Actin Monomers in Nonionic Detergent**—In rabbit reticulocyte lysate and the cytosol of other eukaryotic cells, it has been demonstrated that CCT subunits can exist as populations of free monomers and microcomplexes as well as components of the 900-kDa hexadecameric CCT complex (5, 13, 19–21); however, the function of these free CCT subunits and microcomplexes within cells is not yet fully understood.

Free CCT subunits dissociated from the holo-chaperonin in lysate co-sediment with the native β-actin monomers in sucrose gradients at 13–17% sucrose. Further immunoprecipitation experiments were conducted to investigate whether any free CCT subunits could interact with the monomeric folded actin. An interaction preserved in nonionic detergent was observed between dissociated CCTα and CCTθ and native β-actin monomers. Fig. 3 shows that 35S-β-actin is recovered upon immunoprecipitation with anti-CCTα and anti-CCTθ antibodies in 0.5% Nonidet P-40. This is presumably a qualitatively different interaction to the one observed when immunoprecipitating from the fractions containing holo-CCT-β-actin, because these interactions between β-actin and CCTα or CCTθ are not preserved under mixed micelle conditions. Conversely, the mixed micelle-resistant interactions between β-actin and CCTα or CCTθ derived from holo-chaperonin are not observed in mixtures of free CCT monomers and native β-actin monomers. Although these results demonstrate that the interaction between folded actin monomers and dissociated CCTα and CCTθ subunits can be maintained under these experimental conditions, the physiological relevance of these associations is unclear. They could reflect aspects of the CCT disassembly cycle.
immunoprecipitations from the mixture of 35S-labeled CCT and 32P-β-actin taken from the sucrose gradient fractions containing dissociated CCT subunits and native β-actin monomers (13–17% sucrose) were used as the starting sample for immunoprecipitation with the set of anti-CCT subunit antibodies. Autoradiograms show recovery of individual CCT subunits (asterisk) and associated β-actin (arrow) in 0.5% Nonidet P-40 (lanes N) or mixed micelle (lanes M) buffers. Left panel shows immunoprecipitations from the mixture of 35S-labeled CCTα, CCTβ, CCTδ, CCTɛ-1, and β-actin, and the right panel shows immunoprecipitations from the mixture of 32P-labeled CCTγ, CCTε, CCTθ, and β-actin. In each panel, lane S indicates starting sample for immunoprecipitation, and lane B indicates background signal obtained by incubation of starting sample with beads alone in 0.5% Nonidet P-40 buffer. Immunoprecipitates were resolved on 8% SDS-PAGE gels. Molecular mass markers are indicated on the right-hand side of each autoradiogram.

Fig. 3. Immunoprecipitation of dissociated CCT subunit-β-actin monomer complexes. Two mixtures of four 35S-labeled CCT subunits and 32P-β-actin were resolved on 8% SDS-PAGE gels. Molecular mass markers are indicated on the right-hand side of each autoradiogram.

(13) in which β-actin departs from holo-CCT still bound by some subunit(s). Alternatively, there could be a role for these two CCT subunits, when dissociated from holo-CCT in the stabulization of actin monomers (21). Holo-CCT does not interact with native β-actin monomers (Fig. 2C). The observation that two CCT subunits, which are able to interact strongly with actin in holo-CCT, CCTβ, and CCTε-1, do not appear to participate in associations with native conformers of actin supports the recent structural model of apo-CCT-α-actin (12).

Solid Phase β-Actin Peptide Array Screen for CCT Binding Sequences in β-Actin—We also took a completely separate approach to investigate specific interactions between CCT and defined regions of actin. A set of 73 immobilized peptides scanning the primary structure of mouse β-actin (375 residues) was screened for interaction with mouse testis CCT. Each peptide was 15 residues in length, and subsequent peptides were offset by 5 residues. The assay to detect the interaction of CCT with individual peptides involved sequential incubation with mouse testis CCT, a monoclonal antibody, 91A, to the CCTα subunit (14, 15, 17), and a secondary antibody conjugated to alkaline phosphatase. Immune conjugates were then detected in an enzyme-linked immunosorbent assay. Fig. 4A shows that CCT interacts with high affinity with 16 peptides distributed throughout the primary structure of β-actin. Previous studies have demonstrated that CCT can be induced to release bound substrates in the presence of ATP (22). Interaction of the peptides with CCT followed by incubation in 1 mM ATP for 2 h at 37 °C reduced the bound CCT signal on 11 of the peptides (Fig. 4B). One signal (number 74) represents interaction between the antibody probe and a peptide containing the 91A mAb epitope sequence (mouse CCTα, A454VAKLRA460) (15) and is seen with antibody incubation alone (Fig. 4C) but is not affected by incubation with ATP (Fig. 4B). This signal thus represents the maximum obtainable in this assay. During the course of these experiments, the β-actin peptide array was reprobed again 15 times, which involved stripping of bound protein and regeneration of peptides. It concerned us that the negatively binding peptides might reflect poor synthesis on a particular pin or chemical damage to peptides after repeated probing and regeneration. Therefore the peptide array was probed (probing number 16) with anti-actin monoclonal antibody AC-40 (Sigma), which recognizes an epitope located at the C terminus of β-actin residues 365–375. Peptide number 73 reacted strongly with the mAb despite never having previously supported a signal above background (giving a signal of approximately 63% of the maximum possible; data not shown).

We also investigated which sequences in the β-actin peptide array were recognized by members of different eukaryotic cytosolic molecular chaperone family. The peptides were screened for interaction with mouse testis Hsc/Hsp70 proteins, which are homologues of bacterial DnaK. Fewer β-actin peptides are bound by Hsc/Hsp70 than CCT, despite using a 9-fold molar excess of Hsc/Hsp70 compared with CCT to screen the array (Fig. 4D). Some of the peptides are recognized by both chaperones (Fig. 4, A and D). Different Hsp70 family members are known to display different peptide binding specificities; however, the common feature recognized is defined as a stretch of at least seven residues that includes large hydrophobic and basic amino acids but few or no acidic residues (23). Several of the actin peptides bound by Hsc/Hsp70 are enriched in large aliphatic residues, particularly leucine, isoleucine, and valine, and the strongest reacting peptide, number 35 (LPHAIRLDLAGRDL), contains several leucines and basic residues compatible with the DnaK binding motif as defined by Rudiger et al. (24, 25). Furthermore, this peptide sequence is located as a buried β-strand in native actin. We conclude from probing the β-actin peptide array with Hsc/Hsp70 and CCT isolated from the same cell type that their recognition patterns are different, despite overlapping to some degree, which may reflect their functions in recognizing early and late stage protein folding intermediates, respectively (26).

Definition of the β-Actin Sites—A number of three-dimensional crystal structures of native actin molecules have been determined (27–29). Firstly we note, obviously, that these three-dimensional native actin structures cannot inform us accurately of the structure(s) of actin folding intermediate(s) recognized by CCT, because native actin cannot be bound by CCT; nevertheless the native structures give us views of secondary structural elements that may already be formed in the folding intermediates.

The CCT-binding 15-mer peptide segments were mapped onto the native actin monomer structures. Table I shows that the peptides that interact with CCT are distributed throughout the primary structure of β-actin. However, these CCT interacting sites are grouped in only three main locations in β-actin in three-dimensional space, as determined by examination of the x-ray structures of α-actin-DNase I complex (27) and β-actin-profilin complex (28). These interaction sites were therefore named β-actin sites I, II, and III, with each site having two components, i and ii (Table I). Only the 11 peptide signals that diminished in response to ATP incubation were classified as β-actin sites; nevertheless, other positively reacting peptides (e.g. peptides 26, 28, 35, 39, and 70) (Table I) may indeed contain bona fide CCT interacting motifs.

A Van der Waals’ surface diagram of monomeric actin, highlighting residues contained within β-actin sites I–III (Fig. 5A), indicates that these amino acids are predominantly exposed on the surface of the native molecule in subdomains 2, 3, and 4. This was unexpected, because type I chaperonins recognize hydrophobic residues that are buried in native proteins (1), and
β-Actin Interactions with CCT

A. Interaction of CCT with the actin peptide array

B. Interaction of CCT with the actin peptide array followed by incubation with ATP at 37°C

C. Interaction of Hsc70 with the actin peptide array

D. Interaction of Hsc70 with the actin peptide array

Absorbance (410nm)

Peptide number
two groups have proposed that hydrophobicity is important for CCT-recognition of tubulin (30) and actin (31). The sites are predominately located on the front surface of native G-actin (standard view) (Fig. 5, A and B), further indicating the specific nature of the interaction between CCT and actin. We analyzed the distribution of surface charge and hydrophobicity on actin

### Table I

Summary of the interactions of CCT with the β-actin peptides

| Peptide (number in array) | Peptide sequence | β-Actin site | CCT signal | ATP-dependent reduction of CCT signal | Properties of amino acid side chains |
|--------------------------|-----------------|--------------|------------|--------------------------------------|------------------------------------|
| 6                        | 26APRAVFPSSIVGRPRB | Site I i     | +          | +                                    | APRAVFESIVGRPRH                     |
| 7                        | 31FPSSIVGRPRHQGMV | Site I i     | +          | +                                    | FPSSIVGRPRHQGMV                      |
| 8                        | 36GRPRHQGMVQGQCK | Site I i     | +          | +                                    | GRPRHQGMVQGQCK                      |
| 12                       | 5DEAQSKRGILTLKYP | Site I ii    | +          | +                                    | DEAQSKRGILTLKYP                      |
| 26                       | 126TFNTPAMYVAIQAVL | Site II i    | +          | −                                    | TFNTPAMYVAIQAVL                      |
| 28                       | 123IQAVLASYASRGTTG | Site II i    | +          | −                                    | IQAVLASYASRGTTG                      |
| 35                       | 171LFAIIALLDLAGRDL | Site II i    | +          | −                                    | LFAIIALLDLAGRDL                      |
| 39                       | 191KILTERGSPFTTTAE | Site II i    | +          | −                                    | KILTERGSPFTTTAE                      |
| 40                       | 196RGYSFTTAAREIVR | Site II i    | +          | −                                    | RGYSFTTAAREIVR                      |
| 42                       | 206REIVRDIKEKLYVA | Site II i    | +          | −                                    | REIVRDIKEKLYVA                      |
| 47                       | 231ASSSSLEKYGELPG | Site II i    | +          | −                                    | ASSSSLEKYGELPG                      |
| 51                       | 311GGITMFPGDHRRK | Site III i   | +          | −                                    | GGITMFPGDHRRK                       |
| 65                       | 321APSTMKIKIAPPERR | Site iii i   | +          | −                                    | APSTMKIKIAPPERR                     |
| 67                       | 331APPERKYSWIGGI345 | Site III ii  | +          | −                                    | APPERKYSWIGGI345                     |
| 70                       | 346LASLSTFQWMISKQ | Site III ii  | +          | −                                    | LASLSTFQWMISKQ                      |
| 74                       | 450STDVLAKLRAFHNEA | 91a epitope  | −          | −                                    | 91a epitope                         |

* Peptides are numbered according to mouse β-actin amino acid sequence (except peptide 74, which is numbered according to mouse CCTa sequence).
* +/− indicates small reduction of signal.
* Properties of amino acid side chains: normal text, basic; italic, acidic; underlined, nonpolar; double underlined, uncharged polar; bold, aromatic.

**Fig. 5. Model of CCT interaction with β-actin: interaction between sites on both sides of the nucleotide binding cleft of actin.** A, Van der Waals' surface diagram of native G-actin highlighting all the residues of the 11 peptide sequences used to define β-actin sites I, II, and III (see Table I). The front (left) and back (right) views of native actin structure are shown, indicating that the peptide sequences comprising β-actin site I (red), II (green), and III (blue) are exposed to the surface. Subdomains 1–4 of actin are numbered at the corners. B, Ribbon diagram of native G-actin showing the same front (left) and back (right) views. β-Actin sites I, II, and III and subdomains of actin are indicated as in A.
The ribbon diagrams depicting secondary structural elements of actin (27) (Fig. 5B) indicate that β-actin sites I–III are located predominantly in loop and β-strand regions. The peptide sequences corresponding to β-actin sites I–III share no consensus sequence or any obvious similarities in amino acid sequence or side chain characteristics. This observation may suggest that CCT subunits recognize specific sequence motifs in actin (Table I). If surface-located loops and β-strands are indeed the main elements of actin folding intermediates recognized by CCT, it is understandable why the peptide screening assay yielded positive signals with 15-mer peptides.

An analysis of the domain motions in actin, using four available crystal structures, indicates that a number of conformationally variable loops are included within the β-core sequence (36GRPRH40) of β-actin (27) (Fig. 5A). According to the two published models of F-actin structure (32, 33), residues within all three β-actin sites would be involved in intersubunit contacts in the actin filament. Furthermore, residues contained within β-actin sites I and II contribute to the binding site for DNase I (Fig. 6A) (27), an actin monomer binding protein that inhibits filament assembly.

**Mutation of β-Actin Site II**—We have focused our attention on β-actin site Ii, a high affinity site that occupies three overlapping peptides and spans amino acid residues 26–50 of β-actin subdomain 2 (Table I and Fig. 6A). We demonstrated the interaction between CCT and N-terminally biotinylated peptide in solution. CCT and peptide corresponding to β-actin site Ii (Table I, peptide 8) were incubated together, and the reactions were then electrophoresed on native PAGE gels, electrotransferred to nitrocellulose membrane, and probed with streptavidin-horseradish peroxidase conjugate to detect the biotinylated peptide (Fig. 6B, lanes 1 and 2). A biotin signal was detectable within a 10-fold concentration range of peptide (1.33–13.3 μM) and fixed concentration of CCT (70 nM). Five alanine scan point mutations across the core sequence (36GRPRH40) of β-actin site Ii were screened for effects on interaction with CCT (Fig. 6B, lanes 3–7). The mutant peptides showed equivalent (lane 6, 36GRPRH40), reduced (lane 4, 36GAPRH40), or enhanced (lane 3, 36ARPRA40; lane 5, 36GRARH40) binding to CCT. Replacement of all five residues of the GRPRH core sequence by five alanines resulted in complete abrogation of binding (lane 8, 36AAAAA40).

**DISCUSSION**

**Chaperonin Interactions with Polypeptide Substrates**—It is a tenet in the field of chaperonin research that interactions between chaperonin and substrates are mediated predominantly through hydrophobic interactions (1, 35). This view has its strongest direct support via mutagenesis of hydrophobic residues in the apical polypeptide chain binding domains of the type I chaperonin of *Escherichia coli*, GroEL; when several hydrophobic residues are changed to charged residues, substrate binding is abrogated (36). An x-ray structure of the GroEL apical domain in isolation reveals a direct interaction between an artificial N-terminal peptide tag and residues located in α-helices 8 and 9 of the apical domain (37).

Most recently, a crystal structure of the complex formed between a model peptide and GroEL tetradecamer has been solved (38). The interaction of the model peptide with GroEL is proposed to mimic the binding of a substrate. The peptide is bound in a hydrophobic groove between α-helices 8 and 9 in the apical domain, notably the same site occupied by the N-terminal peptide tag (37) and GroES mobile loop in the structure of the GroEL-GroES complex (39). Thus, the GroEL substrate binding site appears to be capable of accommodating many different amino acid sequences. The interactions between the bound peptide ligands and apical domain surface are a mixture of hydrophobic and polar contacts. In each case, hydrophobic side chains from the peptide are buried in hydrophobic pockets in the binding site, and a number of hydrogen bonds are formed between polar side chains in the apical domain and the peptide...
backbone. Recently, Hartl and colleagues (40) have examined the spectrum of substrate proteins that interact with GroEL in vivo and have found several hundred GroEL interacting proteins, some of which seem to share the common feature of domains with αβ-folds. It may be the hydrophobic residues in the buried β-sheets of these types of secondary structural elements that are preferentially bound by GroEL.

The type II chaperonins of archaea and eukaryotic cytosol are much less well characterized than GroEL in most respects. X-ray structural analyses of the thermosome indicate significant differences compared with GroEL in the structure of its apical domains (41, 42). Particularly surprising is the absence of hydrophobic residues in the region of the thermosome apical domain corresponding to the substrate binding site of GroEL, suggesting to us that the physico-chemical nature of interaction between substrate and chaperonin could be significantly different in the thermosome (10); however, others have proposed that the substrate interaction sites are located elsewhere on the apical domains, such as in the helical protrusions (41).

Presently, there is very little known about the natural substrates of archaeabacterial type II chaperonins (43) but, in the case of CCT, there is a large body of data that indicates actins and tubulins as major in vivo substrates (10). It is the nature of the interactions between CCT and its natural substrates that we are interested in elucidating.

It has been suggested in previous studies that both actins (41, 44) and tubulins (30) interact with CCT through defined hydrophobic regions and that CCT functions exactly like GroEL (45). However, the type I chaperonin GroEL can bind chemically denatured actin, presumably through hydrophobic interactions, and yet actin cannot be productively folded by GroEL (46). This is puzzling because it indicates that GroEL is unable to provide some aspect of specificity to the folding of actin that is imparted by CCT.

Recently, the first structure of any chaperonin bound with substrate was obtained when Llorca and colleagues (12) determined a structure of α-actin bound to nucleotide-free CCT by cryo-electron microscopy. This complex probably resembles an early conformation in the presumably complicated series of interactions that occur sequentially between CCT and actin during the complete, but presently ill defined, folding cycle (12). Although the apo-CCT-α-actin structure represents a “snapshot” of the interaction between CCT and actin, it seems that, at least at one stage, the interaction is both geometry-specific and subunit-specific. This structure is immediately suggestive of a model in which the interaction between actin and CCT is sequence-specific with respect to both the chaperonin and substrate components. One can imagine that α-actin is opened up across the nucleotide binding cleft when bound to apo-CCT and that CCT is holding α-actin via the tips of the arms of the small and large domains (12). The biochemical studies reported in this paper strongly support the structural model because we find that 15-mer peptide sequences derived from the tips of the small and large domains of actin react strongly with CCT. In addition, the apo-CCT-α-actin structure shows that CCTβ interacts with subdomain 2 and that either CCTβ or CCTε can interact with subdomain 4, and once more, in this study immunoprecipitation of mixed micelle detergent disrupted CCT-β-actin complexes with all eight anti-CCT subunit antibodies directly supports this model. We should emphasize that the CCT-β-actin complexes that we have analyzed biochemically were formed under the physiological conditions of in vitro translation in rabbit reticulocyte lysate. Because the CCT-β-actin complexes are derived under physiological conditions, we presume that there exists a mixture of CCT-actin complexes recovered in different stages of the reaction cycle, and this may account for the additional interactions observed between actin and CCT subunits, such as CCTα and CCTβ.

Model of Interaction between CCT and Actin—A previous approach to investigate which regions of actin are bound by CCT involved the study of interaction of truncated actin molecules to CCT upon in vitro translation in rabbit reticulocyte lysate (31). In the context of the new structure and our biochemical data, reinterpretation of some of the data of Rommelare et al. (31) is now possible. In their study, they constructed a large series of N- and C-terminal truncation mutants and found evidence for several specific regions of actin involved in CCT interaction, some of which overlap regions that we now implicate in binding. However, their study did not take into account the native structure of β-actin, nor were they able to distinguish between deletions that enhanced or reduced binding from those that might have affected processing and release. We have shown that a fusion protein of the Ha-Ras small GTP-binding protein linked to subdomain 4 of β-actin can bind to the CCTβ and CCTε subunits by structural analysis (12) and here directly by immunoprecipitation. This fusion protein migrates as a discrete species in native PAGE and exists in equilibrium between an unbound and a CCT-bound in time course analysis upon in vitro translation in rabbit reticulocyte lysate (12). We surmise that in this fusion protein the β-actin subdomain 4 component is in a conformation that resembles the conformation of this domain in the β-actin folding intermediate, which is the normal substrate for CCT, and thus enables its capture by CCT. Furthermore, the existence of a stable CCT-binding subdomain of actin can be understood in the context of our model because we suggest that actin binds to CCT through regions that are finally found exposed in the native actin structure rather than transiently exposed hydrophobic residues normally found buried in the core of the native protein.

There are aspects of the association of β-actin with CCT that we do not yet understand. In particular, why some interactions between actin subdomains and individual CCT subunits in the holo-chaperonin are resistant to mixed micelle detergent conditions (e.g. interactions mediated by CCTα, CCTβ, CCTε, and CCTθ), whereas others appear susceptible (e.g. interactions mediated by CCTδ). Furthermore, why two types of interaction occur between β-actin and CCTα, and CCTε, dependent upon whether these subunits are components of the holo-chaperonin or populations of dissociated monomers. Thus, it is likely that a series of reactions are occurring during actin folding on CCT. We have no structural information on the nature of ATP-CCT-β-actin complexes, but we already know that the structure of ATP-CCT (6) has a hemispherical folding cavity in a very different conformation to the α-actin bound ring in the apo-CCT-α-actin structure (12). Biochemical studies demonstrate that CCT can be induced to release its bound actin in the presence of Mg-ATP (this study) (22). No doubt further three-dimensional structures and biochemical studies of the interaction of actin with CCT will help in understanding its maturation pathway on CCT, which takes several minutes to complete in vivo in mammalian cells at 37 °C (47).

In conclusion, we believe that the mode of interaction between CCT and actin is likely to be between residues exposed on the surface of a compact quasi-native folding intermediate and specific CCT subunits. The new structure of apo-CCT-α-actin is also consistent with this model because the actin binds well below the helical protrusions of the apical domains to regions enriched in charged rather than hydrophobic residues (41). It may be time to give serious consideration to the possibility that the substrate recognition mechanisms of GroEL and CCT are very different from one another.
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