Newly-Synthesized β-Tubulin Demonstrates Domain-Specific Interactions with the Cytosolic Chaperonin†

Jane K. Dobrzynski, Mona L. Sternlicht, George W. Farr,‡ and Himan Sternlicht*

Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106

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ABSTRACT: Tubulin folding requires two chaperone systems, i.e., the 900 kDa cytosolic chaperonin referred to as the TCP-1 complex or TRiC which facilitates folding of the α- and β-tubulin subunits and a *ca*. 180 kDa complex which facilitates further assembly into heterodimer. β-Tubulin mutants were expressed in rabbit reticulocyte lysates, and the effect of C-terminal, N-terminal, and internal deletions on the binding of β-tubulin polypeptides to the 900 and 180 kDa complexes was ascertained. Proteolytic studies of chaperonin-bound β-tubulin were also implemented. These studies support the concept of *quasi-native* chaperonin-bound intermediates [Tian et al. *J. Biol. Chem.* (1995) *270*, 1−4]. Three "domains" similar in size to the domains in the native protein were implicated in facilitated folding: i.e., an internal or "M-domain" composed of residues ∼140−260 which binds to TRiC; a "C-domain" composed of residues ∼300−445 which interacts less strongly with TRiC and may contain regulatory sequences for tubulin release from the chaperonin; and an "N-domain" composed of residues ∼1−140 which apparently does not interact with TRiC but does interact with the 180 kDa complex. The major TRiC-interacting region, residues ∼150−350 (the "interactive core"), overlapped portions of the M- and C-domains and included a putative hydrophobic-rich interdomain segment which may be a preferential site of interaction with TRiC. This segment may also be important for microtubule assembly and/or tubulin dimer formation. Removal of two residues from the N-terminal end or *ca*. 27 residues from the C-terminal end caused the polypeptide to arrest on TRiC. It is proposed that N- and C-terminal regions of β-tubulin structurally interact with TRiC-binding region ∼150−350 to inhibit binding to TRiC.

Tubulin and actin are major proteins of the eukaryote and are essential for a variety of cellular processes including mitosis, motility, and maintenance of cell shape. To achieve a biologically active conformation these proteins require the assistance of a cytosolic chaperonin (TRiC)[1] (also referred to as the TCP1 complex, CCT, or Ct-cpn60) (Chen et al., 1994; Frydman et al., 1992; Gao et al., 1992, 1993; Miklos et al., 1994; Sternlicht et al., 1993; Vinh & Drubin, 1994; Yaffe et al., 1992). This chaperonin is a 950 kDa heterooligomer consisting of t-complex polypeptide-1 (TCP1) and seven or eight related polypeptides ranging in M_r from 52 to 62 kDa (Frydman et al., 1992; Kim et al., 1994; Kubota et al., 1994; Lewis et al., 1992). Like other chaperonins, TRiC is toroidal in shape (Lewis et al., 1992; Saibil & Wood, 1993), requires ATP (Frydman et al., 1992; Gao et al., 1992; Melki & Cowan, 1994; Yaffe et al., 1992), and works with cofactors or co-chaperonins (Gao et al., 1993, 1994). However, unlike GroEL and mitochondrial cpn60, TRiC appears to have a limited range of substrates confined primarily to tubulin, actin, and related proteins (Chen et al., 1994; Melki et al., 1993; Sternlicht et al., 1993; Vinh & Drubin, 1994; Melki & Cowan, 1994). Furthermore, its putative co-chaperonins Cofactor A and Cofactor B are distinct from GroES and cpn10, the respective co-chaperonins of GroEL and mitochondrial cpn60 (Gao et al., 1993, 1994). Although considerable progress has been made in elucidating the mechanism of action of GroEL and GroES (Jackson et al., 1993; Martin et al., 1993; Todd et al., 1994; Weissman et al., 1994, 1996; Mayhew et al., 1996), the mechanism of action of TRiC and the basis for its limited range of substrates remain unclear (Burns & Surridge, 1994; Tian et al., 1995). The situation is further complicated in the case of tubulin by the requirement for an additional as yet poorly understood chaperone system, i.e., the 180 kDa complex (also referred to as the 300 kDa complex) thought to participate with TRiC in the formation of tubulin heterodimer (αβ-tubulin) (Yaffe et al., 1988; Campo et al., 1994; Paciucci, 1994; Tian et al., 1996). Additional chaperones and proteins are also postulated to facilitate tubulin subunit folding via interactions with the nascent polypeptides [cf. Frydman et al. (1994) and Hansen et al. (1994)].

We implemented a proteolytic and mutational study of β-tubulin folding in rabbit reticulocyte lysate to address the basis for TRiC's selectivity for substrate and to probe its mechanism of action. The majority, if not all, of the substrates of TRiC are nucleotide-binding proteins. TRiC substrates are also involved in self-assembly (e.g., tubulin

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* Corresponding author. Tel: (216) 368-3387. FAX: (216) 368-3395. E-mail: hxs3@po.cwru.edu.

‡ Current address: Howard Hughes Institute, Boyer Center for Molecular Medicine, 295 Congress Ave., Yale School of Medicine, New Haven, CT 06510.

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[1] Abbreviations: TRiC, TCP1 ring complex; β−R, β−TRiC, β−500, β−180, complexes of β-tubulin or β-tubulin polypeptide fragments with, respectively, the ribosome, TRiC, and the 500 and 180 kDa chaperones (β symbol omitted in the Figures for simplicity); DHFR, dihydrofolate reductase; N-, M-, and C-domains, three major proteolytically defined compact regions in native β-tubulin encompassing, respectively, the *N*-terminal ∼1−140, the *M*iddle, ∼140−260; the *C*-terminal, ∼300−445 residues; N- and C-fragments, N- and C-terminal chymotryptic fragments of β-tubulin with apparent M_rs of *ca*. 35 and 22 kDa, respectively. (N- and C-fragments should not be confused with the N- and C-domains although they do share sequences with these domains).

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and actin) or participate in extensive protein−protein interactions (e.g., γ-tubulin and centractin). We wondered whether they contained structural elements related to these functions that require a chaperonin during the course of folding.

TRiC, unlike mitochondrial cpn60 or GroEL, has a high affinity in vitro for slow-folding intermediates of tubulin and actin (Melki & Cowan, 1994). α-Tubulin folding has been recently shown to involve a "quasi-native" phase characterized by the presence of quasi-native chaperonin-bound intermediates (Tian et al., 1995). Proteolytic studies of chaperonin-bound β-tubulin which indicated intermediates with native-like domains (Yaffe et al., 1992) are consistent with a quasi-native phase. The ability of TRiC to facilitate folding during this phase is apparently unique since its substitution by GroEL or mitochondrial cpn60 leads to futile cycles of binding and release of incorrectly-folded tubulin and actin (Melki & Cowan, 1994). As one possibility Tian et al. proposed that TRiC may actively fold its substrates during this phase (Tian et al., 1995).

β-Tubulin contains a nucleotide-binding domain (residues ~1−300) which binds MgGTP exchangeably and a C-terminal regulatory domain (C-domain) essential for microtubule assembly (residues 300−445) (Farr & Sternlicht, 1992; Jayaram & Haley, 1994; Kirchner & Mandelkow, 1985; Mandelkow et al., 1987; Shivanna et al., 1993). Proteolytic studies showed that the nucleotide-binding domain is further comprised of two domains, i.e., N and M[1] (Angeles et al., 1990; de la Vina et al., 1988), consistent with X-ray fiber diffraction data which indicated a three domain structure for the subunits (Beese et al., 1987). Proteolytic studies were implemented to determine if newly-synthesized β-tubulin interacted with TRiC via quasi-native domains and to clarify the role these domains might play in folding. Bound polypeptide was found to have a domain organization/structure similar to that of the native protein and to interact with TRiC apparently via a hydrophobic and proline-rich sequence that interconnected the M- and C-domains, as well as with adjacent residues in the M-domain. Mutational studies implicated a larger region of interaction with TRiC (the "interactive core") that centered approximately on the interconnecting sequence and included portions of the C-domain. The functional significance of this sequence is not known. By analogy with actin (Kabsch et al., 1990; Holmes et al., 1990), we suggest that it represents a site important for microtubule assembly or tubulin dimer formation.

MATERIALS AND METHODS

High-Pressure Liquid Chromatography. Size-exclusion (Superose 6) and anion-exchange (Mono Q) chromatography were performed at 4 °C as previously described (Farr & Sternlight, 1992; Yaffe et al., 1992). 200 and 500 μL fractions were collected at 0.4 and 1.0 mL/min flow rates (Superose 6 and Mono Q columns, respectively) and analyzed by denaturing PAGE fluorography or phosphorimagery. Superose 6 chromatograms displayed a large radioactive peak at *ca*. 35 kDa due to the [^{35}S]methionyl-tRNA complex (designated S′) which overlapped the tubulin monomer peak (Yaffe et al., 1988, 1992) (cf. Figure 5). Estimates of monomer levels in this region were obtained by fluorography.

Denaturing PAGE. Translation reactions were electrophoresed on SDS−PAGE or tricine−SDS−PAGE gels (Schagger & Jagow, 1987). Size-exclusion column fractions were generally concentrated 10−20-fold by chloroform/methanol/water precipitation prior to electrophoresis. Because of its exceptionally high concentration in rabbit reticulocyte lysate, hemoglobin was often observed to "aggregate" and run as multimers or denaturing gels (increasing the sample SDS concentration mitigated but did not eliminate this problem). These forms were radioactive due to residual hemoglobin synthesis in the lysate and caused anomolous bands to be present in a number of the fluorograms (e.g., Figure 4).

Non-Denaturing PAGE. Non-denaturing PAGE was performed at 4 °C in the presence of GTP (supplemented to 0.5 mM in the gel and to 0.1 mM in the buffer) as previously described (Gao et al., 1992). The gels were fixed in 10% acetic acid and 25% isopropanol (v/v) for 30 min and further treated with Amplify (Amersham Corp) for fluorography or direct quantitation of radioactivity via a PhosphorImager (Molecular Dynamics).

Immunoprecipitations with Anti-TCP1α Antibody 23C. Immunoprecipitations with Ab 23C were performed as previously described (Sternlicht et al., 1993).

Construction of pALTERβ. pTZ19Rβ (Farr & Sternlicht, 1992) was digested with *Hin*dIII and *Sal*I to generate the 1.5 kb chicken βII-tubulin cDNA (cβII) (Valenzuela et al., 1981). This 1.5 kb fragment was ligated into pALTER1 (Promega). The resulting plasmid, pALTERβ, contains the β-tubulin cDNA 3′ to a SP6 RNA polymerase promoter and will direct the synthesis of full-length β-tubulin mRNA.

Site-Directed Mutagenesis/C-Terminal Truncations. pALTERβ was mutagenized by the double-stranded procedure described in the Altered Sites II Technical Manual (Promega), which allows multiple site-directed mutations to be simultaneously introduced into the DNA insert. Mutagenic oligos were designed to introduce either a new restriction site or to destroy a preexisting site in the cDNA sequence. Positive clones were identified by restriction enzyme digestion analysis of mini-prepared plasmids and confirmed by double-stranded DNA sequence analysis (Core facility, Cleveland Clinic). Internal and N-terminal deletions were constructed with the aid of new restriction sites introduced adjacent to the regions to be deleted. Molecular weights of internal deletion mutants Δ85−144, Δ86−251, and Δ85−381 and N-terminal deletion mutants Δ2−145 and Δ2−346 determined by SDS−PAGE were ~5−8 kDa greater than the predicted weights based on the observed DNA sequence and mRNA size (data not shown). Numbers following the Δ symbol denote the deleted region. Mutants with stop codons at the *Bam*HI and *Avi*II restriction sites (i.e, Δ^S344−445 and Δ^S251−445) were constructed as controls. C-terminal truncations were generated from pTZ19Rβ by exploitation of preexisting restriction sites. Δ2−251/Δ344−445 was generated by restriction digestion of Δ2−251 with *Bam*HI.

Translations. Wild type (wt) and mutant β-tubulin mRNAs were translated in micrococcal nuclease-treated rabbit reticulocyte lysate for various times at 30 °C and prepared for further analysis as previously described (Yaffe et al., 1992). In pulse−chase studies, translations were performed for the indicated times in the presence of [^{35}S]methionine (pulse) followed by the addition of unlabeled methionine to

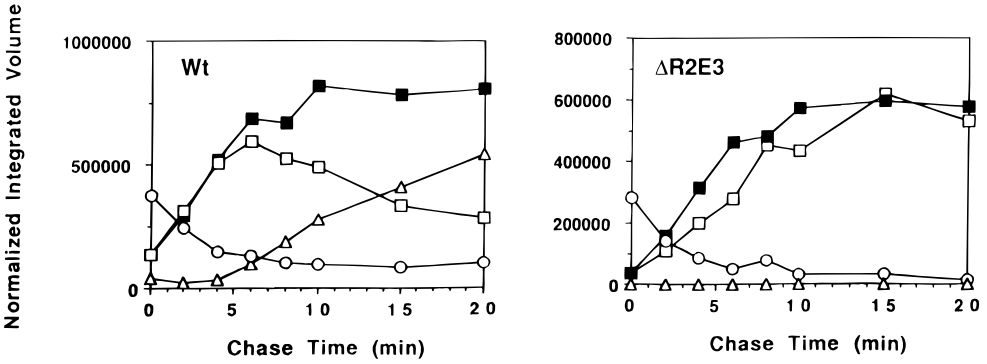

FIGURE 1: Early folding events of wt and ΔR2E3 β-tubulin. Pulse−chase studies of the synthesis reactions. Wt or ΔR2E3 β-tubulin mRNAs were translated (30 °C) in rabbit reticulocyte lysate for 8 min in the presence of [^{35}S]methionine following Yaffe et al. (1988). Aliquots were supplemented with unlabeled methionine, chased for the indicated times, and analyzed by non-denaturing and denaturing PAGE. β−TRiC (□), β−180 (○), and monomer (△) levels by non-denaturing PAGE; fully-synthesized (■) levels by denaturing PAGE. Radioactivity (expressed as integrated volume) was estimated with a PhosphorImager (Molecular Dynamics) and normalized to equivalent loads. Integrating efficiencies for denaturing and non-denaturing gels were taken to be identical. The results shown were confirmed by three independent studies.

1 mM (chase) and incubation continued at 30 °C for the specified times.

Formic Acid Digestion. Procedures for formic acid digestion were modified from Serrano et al. (1984). Briefly, recombinant [^{35}S]β-tubulin from *Escherichia coli* BL21DE3 obtained by overexpressing a chicken βII-tubulin cDNA inserted into pET11C (Phadtare et al., 1994; Valenzuela et al., 1981) was solubilized in 8 M urea. Growth of *E. coli* BL21DE3, induction of β-tubulin mRNA with IPTG, and isolation of inclusion bodies enriched in [^{35}S]β-tubulin were as described by Gao et al. (1992). 100 μL of solubilized protein (5×10^6 cpm/μL) was combined with 25 μL of unlabeled carrier chicken β-tubulin (at *ca*. 10-fold higher protein concentration), supplemented with 750 μL of 88% formic acid and digested for 20 or 40 h at 37 °C. 700 μL of the digested samples was extensively dialyzed in a Slide-A-lyzer cassette (3 mL capacity, 10 kDa cutoff; Pierce) against a pH 7.4 neutralization buffer (0.4 M Hepes, 1 M urea, 1 mM EGTA, 10 mM DTT). Aliquots of the dialyzed materials ($\sim 2 \times 10^5$ dpm/μL) were diluted 1:25 into rabbit reticulocyte lysate, incubated for various times at 30 °C, and analyzed by size-exclusion chromatography.

Chymotrypsin Digestion. Digestions with α-Chymotrypsin (TLCK-treated, Worthington Biochemicals) were performed either at 25 °C (Figure 2) or on ice (Figure 3) as indicated. Aliquots removed for denaturing PAGE were quenched by the addition of SDS−PAGE sample buffer and boiling (Figures 2 and 3). Samples analyzed by size-exclusion chromatography were treated with a 5-fold molar excess of soybean trypsin−chymotrypsin inhibitor immediately following digestion (Bowman-Birk Inhibitor; Sigma, T9777) (Figure 3). Fluorography or phosphorimagery was performed with tricine gels which allowed detection of fragments with molecular weights as low as 3 kDa (Schagger & von Jagow, 1987).

Secondary Structure Predictions. Secondary structure predictions for α-helix, β-sheet, coil and turn were performed using the Chou−Fasman and Garnier-Osguthorpe-Robson algorithms (Lasergene Software, DNAstar Inc.) (Chou & Fasman, 1978; Garnier et al., 1978).

RESULTS

Chaperonin-Bound β-Tubulin. The N-terminal region of β-tubulin either contains the binding site for the guanine

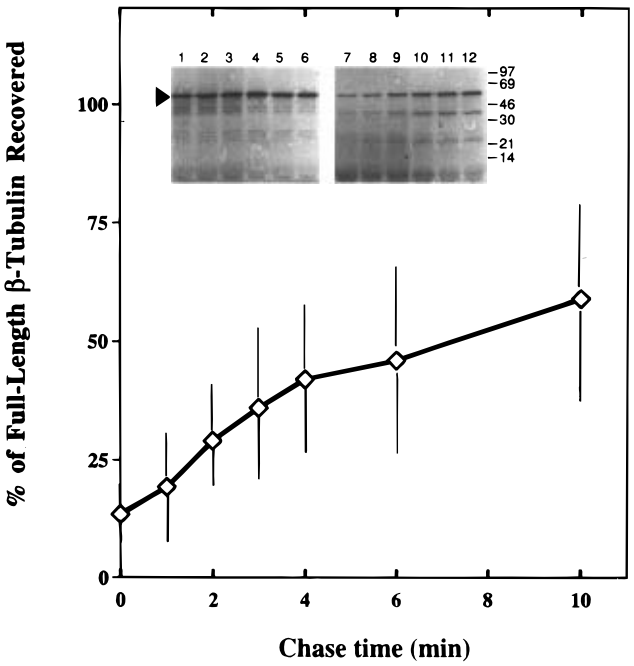

FIGURE 2: Maturation of TRiC-associated β-tubulin. β-tubulin was translated in rabbit reticulocyte lysate for 8 min and chased in the presence of unlabeled methionine for 0 (lanes 1 and 7), 1 (lanes 2 and 8), 2 (lanes 3 and 9), 3 (not shown), 4 (lanes 4 and 10), 6 (lanes 5 and 11), and 10 min (lanes 6 and 12). Aliquots were removed at the indicated times, incubated for 5 min in the presence (lanes 7−12) or absence (lanes 1−6) of chymotrypsin (20 μg/mL) and analyzed by denaturing PAGE fluorography/phosphoimagery. Arrowhead denotes the position of full-length β-tubulin. The percent of full-length β-tubulin recovered (i.e., lanes 7−12 relative to lanes 1−6, respectively), plotted in the graph as a function of time, was taken as a measure of the protease resistance of newly-synthesized β-tubulin (text). The results shown represent one of four experiments. In all cases a monotonic increase in the percent of full-length β-tubulin recovered was observed as a function of chase time. Error bars indicate the range of variability between experiments.

moiety of GTP (i.e., ∼residues 3−35) or contributes significantly to this site (Jayaram & Haley, 1994; Shivanna et al., 1993). In vitro, chemical modification of the N-terminal methionine drastically perturbs tubulin function (Levison et al., 1989). In vivo, yeast and mammalian tubulin mutants that lack N-terminal residues are compromised in microtubule function (Gu & Cowan, 1989; Reijo et al., 1994). Figure 1 shows a comparison of pulse−chase studies of wild-

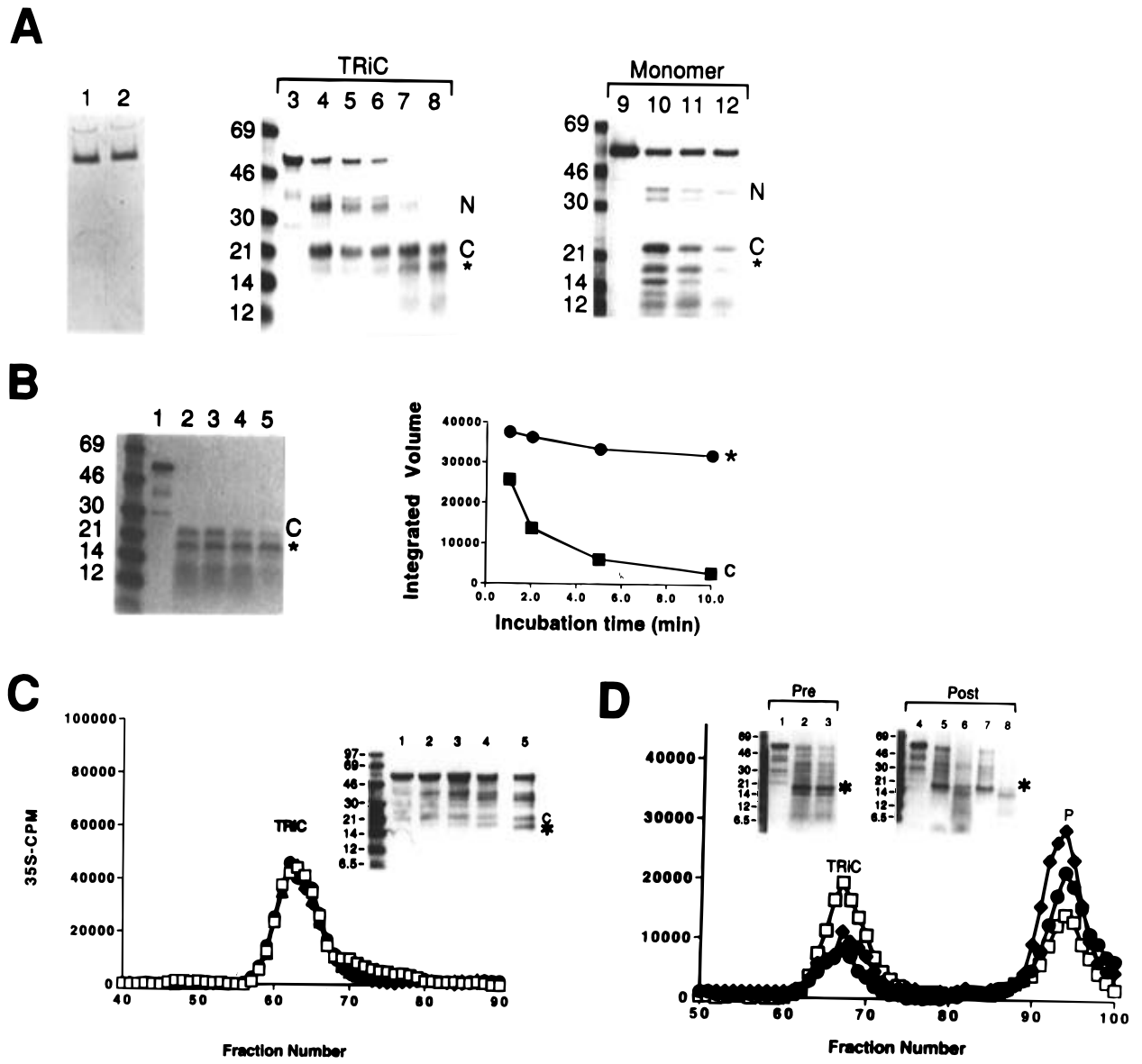

FIGURE 3: Chymotrypsin digestion of β-tubulin−TRiC complex generates a resistant, TRiC-associated 18 kDa fragment. (A) Concentration and time studies. β-Tubulin−TRiC complexes were isolated from an 8 min synthesis, 1 min chase (lanes 3−8), and incubated in the presence of 0 (lane 3), 6 (lane 4), 12 (lane 5), 25 (lane 6), 50 (lane 7), and 100 μg/mL (lane 8) chymotrypsin for 1 min. Native monomers (lane 9) isolated from 60 min syntheses were incubated in the presence of 6 μg/mL chymotrypsin for 0.5 (lane 10), 1 (lane 11), and 2 min (lane 12). Reactions were analyzed by tricine-denaturing PAGE/phosphorimagery. "N" indicates the N-terminal (34−37 kDa) fragments of β-tubulin. "C" indicates the C-terminal (22 kDa) fragment of β-tubulin. "*" denoates an 18 kDa breakdown product attributed to the N-terminal fragments (see text, also panel B). Purified chaperonin isolated from bovine testis as a control was treated with 1 or 100 μg/mL chymotrypsin for 1 min, electrophoresed on a non-denaturing gel, and stained with Coomassie blue (lanes 1 and 2, respectively). Denaturing gels (not shown) also failed to detect significant proteolysis of the cytosolic chaperonin. (B) The 18 kDa fragment is resistant to proteolysis in the presence of TRiC. β-Tubulin was synthesized for 8 min and chased for 1.5 min. β−TRiC was isolated, incubated for 0.5, 1, 2, and 5 min in the presence of 100 μg/mL chymotrypsin, subjected to tricine−SDS−PAGE/fluorography (lanes 2−5, respectively) or quantitated by phosphorimagery (1−10 min incubations) (●, 18 kDa fragment; ■, C-fragment). An undigested β−TRiC aliquot is shown in lane 1. (C) The 18, 22, 34, and 37 kDa fragments are recovered in association with TRiC following partial digestion. β-Tubulin−TRiC was isolated from a 12 min synthesis, 2 min chase. Aliquots were supplemented to 6 μg/mL chymotrypsin and incubated for 4, 15, and 30 min. Reactions were quenched and analyzed by size-exclusion chromatography (□, no chymotrypsin control, ▲, 4 min; ●, 15 min; ◆, 30 min digest) followed by tricine−SDS−PAGE fluorography (inset: lanes 1−4, respectively). Lane 5 shows a 30 min digestion quenched and analyzed directly by tricine−SDS−PAGE. (D) The 18 kDa fragment is recovered in association with TRiC following extensive digestion. β-Tubulin−TRiC isolated as in C was digested for 10 or 20 min in the presence of 100 mg/mL chymotrypsin. Aliquots were either analyzed directly by tricine−SDS−PAGE/fluorography (Pre: no chymotrypsin control, lane 1; 10 min, lane 2; 20 min, lane 3) or eluted on Superose 6 (□, no chymotrypsin control; ◆, 10 min; ●, 20 min) followed by tricine−SDS−PAGE of β−TRiC (Post: lane 4, no chymotrypsin control; lane 5, 10 min; lane 7, 20 min) and P peaks (inset: lane 6, 10 min; lane 8, 20 min).

type (wt) and N-terminal deletion mutant ΔR2E3 β-tubulin in rabbit reticulocyte lysate. mRNAs were translated in the presence of [^{35}S]methionine ("pulse"), chased for various times in the presence of a large excess of unlabeled methionine, and analyzed by non-denaturing and denaturing PAGE. During the first 4−5 min of the chase incompletely synthesized [^{35}S]β-tubulin polypeptides labeled during the "pulse" were extended into fully-synthesized polypeptide. After this initial period very little further synthesis of radiolabeled polypeptide could be detected. Greater than 80% of translated wt polypeptide eventually folded into monomer (data not shown). β-Tubulin was recovered as complexes with TRiC (β−TRiC), the 180 kDa chaperone (β−180), and as monomers ("M") (wt only). At the start of

the chase the majority of the radiolabel was in the peptidyl−tRNA−ribosomal complex. This complex, which was not quantitatively recovered, was detected at the top of the non-denaturing gels. Small amounts of tubulin dimers or dimer aggregates were also detected but were not plotted in Figure 1. The β−180 radioactivity seen at early times arose mainly from incompletely-synthesized [^{35}S]polypeptides (<16 kDa) (see below) which prematurely released from the ribosome (180 integrated volumes at the start of the chase, for example, were several-fold greater than those of the fully-synthesized polypeptides as determined by SDS−PAGE). Radiolabel in β−180 (wt) was observed to increase at later times due to the binding of monomers released from the chaperonin.

Figure 1 shows the radioactivity in *fully-synthesized* wt or ΔR2E3 as a function of chase time determined from SDS−PAGE (■) compared with the radioactivity in β−TRiC determined from non-denaturing PAGE (□). The radioactivities in the case of wt were superimposable at early times (<5 min chase) which suggested that wt β-tubulin polypeptide had either rapidly associated with TRiC (within 1 min or less) following its release from the ribosome or had been released from the ribosome as a preformed complex with TRiC [cf. Frydman et al. (1994)]. Taken together with previous studies of chaperonin-mediated folding of β-tubulin that showed a binding requirement for folding intermediates distinct from the molten globule state (Melki & Cowan, 1994), this finding implied that wt β-tubulin was *partially folded* at the time of its release from the ribosome.

A 3−4 min lag in the appearance of monomer relative to the binding of β-tubulin to TRiC was observed (Figure 1). This lag period was not seen with ΔR2E3 which instead arrested on TRiC. (The term "TRiC-arrest" or "arrest" should not be construed to exclude the possibility that mutant polypeptides cycle on-and-off the chaperonin [cf. Weissman et al. (1994)]. In contrast to wt, the arrested ΔR2E3 appeared to be derived from two distinct populations of polypeptide, i.e., a rapidly associating fraction (∼60−70% of total ΔR2E3) similar to that observed with wt, and a slowly associating fraction (∼30−40%) attributed to free polypeptides (Figure 1). Alternatively, the arrested form may have arisen from a single population which partially associated with TRiC while ribosomal-bound and completed association upon release from the ribosome. The free polypeptide pool deduced from a material balance was not detected on native gels presumably due to non-specific losses. ΔR2E3/Δ83−445, a C-terminal truncated variant of ΔR2E3 which lacked the M- and C-domains but largely retained the N-domain of β-tubulin (de la Vina et al., 1988) behaved like Δ83−445 (truncated wt control) in that it did not arrest on TRiC (data not shown). This result demonstrated a requirement *for the middle and/or C-terminal portions* of the polypeptide for expression of the arrest phenotype, and suggested that arrest was a secondary effect arising from deletion of the N-terminal residues.

Proteolytic Studies. Proteolytic studies were implemented to investigate the lag period observed with wt β-tubulin. β−Tubulin was synthesized for 8 min and chased for 0−10 min. Aliquots with and without chymotrypsin were incubated briefly at room temperature and analyzed by SDS−PAGE fluorography or phosphorimagery (Figure 2). Earlier studies (Figure 1 above) established that β−TRiC was the major β-tubulin form. A modest increase in resistance of the full-length polypeptide to chymotrypsin was observed with increasing chase time (Figure 2). Fragmentation patterns, on the other hand, were similar and appeared independent of chase time (inset: lanes 7−12). The major fragments at ∼34 and 22 kDa were assigned to the N- and C-terminal portions of β-tubulin, respectively, on the basis of their similarity in size to the ∼34 and 22 kDa chymotryptic fragments of the native β-tubulin monomer and reconstituted heterodimer as determined by Yaffe et al. (1988, 1992; also Figure 3A below) and the ∼31 and 20 kDa fragments of the tubulin heterodimer as determined by Mandelkow et al. in their SDS−PAGE system (Mandelkow et al., 1987). [These latter fragments corresponded to residues ∼1−280 and ∼281−450, respectively, and arose from a cleavage at a chymotrypsin sensitive site between the M- and C-domains (Mandelkow et al., 1987)]. Similar sized fragments were also observed with trypsin digests of β−TRiC (data not shown). These results supported the notion of quasi-native forms as chaperonin-bound folding intermediates (Tian et al., 1995).

To identify potential regions of β-tubulin that interact with the chaperonin, we subjected isolated β−TRiC to extensive proteolysis with chymotrypsin. We reasoned that such interactive regions might be protected by virtue of their association with the chaperonin. β−TRiC (isolated from translation reactions) and purified cytosolic chaperonin (isolated from bovine testes as a control) were digested with increasing concentrations of chymotrypsin and subjected to PAGE analysis (Figure 3A). Cytosolic chaperonin was resistant to chymotrypsin and persisted intact as a 900 kDa particle (lanes 1 and 2). β−TRiC on the other hand, was degraded with increasing chymotrypsin concentrations (lanes 3−8). Native monomer and dimer gave similar sized fragments as β−TRiC although important differences, e.g., susceptibility to digestion, were observed (Figure 3A, lanes 9−12; dimer data not shown). On the basis of their similarity to the fragments seen with native monomer and dimer (discussed more fully above), the 34 and 37 kDa fragments evident at low concentrations of chymotrypsin (N-fragments, lanes 4−6), were assigned to the N-terminal portion of β-tubulin, whereas the more resistant *ca*. 22 kDa fragment (C-fragment) was assigned to the C-terminal portion of β-tubulin.[1] The 34 and 37 kDa fragments were degraded at the higher chymotrypsin concentrations and apparently yielded a *ca*. 18 kDa fragment (lanes 7 and 8). Assignment of this product to the 34 and 37 kDa fragments was supported by a study done with longer digestion times (Figure 3B). The 18 kDa fragment was highly resistant to chymotrypsin in the presence of the chaperonin. Its integrated volume determined by SDS−PAGE/phosphorimagery remained essentially constant as digestion time increased, a result consistent with the notion that the 18 kDa product was derived from the N-fragments. In contrast, the integrated volume of the C-fragment, which initially was present at a comparable level as the 18 kDa fragment, decreased by a factor of ∼10 with increasing digestion time. The corresponding 18 kDa fragments generated from native monomer and dimer were not resistant to chymotrypsin and could only be detected at very short incubation times (data not shown) or at low concentrations of chymotrypsin (e.g., Figure 3A, lanes 10−12). These results suggested that the 18 kDa fragment seen with β−TRiC was protected from degradation by virtue of its association with the chaperonin.

To test this hypothesis, β−TRiC isolated from a 12 min pulse and 2 min chase reaction was partially or extensively digested with chymotrypsin, supplemented with protease

inhibitor, and analyzed by size-exclusion chromatography followed by denaturing PAGE/phosphorimagery. The 18 kDa fragment, as well as the 37, 34, and 22 kDa fragments, were quantitatively recovered in apparent association with the chaperonin following partial digestion (Figure 3C). The 18 kDa fragment, however, was the predominant associated product when β−TRiC was extensively digested (Figure 3D). In the latter study, approximately 50% of the initial radioactivity was recovered in association with the chaperonin following chromatography, with the radioactivity in the 18 kDa fragment representing *ca*. 45% of the total recovered (inset: lanes 4−6). Interestingly, smaller digestion products (M_r of ∼8−14 kDa) failed to be detected in association with TRiC (compare lanes 2 and 3 with 5 and 7). In contrast, a number of medium- to large-sized fragments ($18 < M_r < 50$ kDa) were found in association with TRiC (lanes 5 and 7). These fragments may correspond to C-terminal truncations of full-length β-tubulin by chymotrypsin, i.e., residual amounts of the 34, 37, and 22 kDa fragments as well as other fragments. Alternatively, they may be derived from incompletely synthesized polypeptides bound to the chaperonin.

A mutational study of β-tubulin was implemented. This study complemented the proteolytic studies and provided further insights into the 18 kDa fragment.

C-Terminal Deletions. C-terminally truncated sequences were generated by use of restriction sites within the coding sequence of β-tubulin and transcribed into mRNAs that lacked stop codons. The mRNAs were translated for various times, and the products were either immunoprecipitated with anti-TCP1α antibody 23C (Figures 4 and 5) or chromatographed on a Superose 6 size-exclusion column and analyzed by SDS-PAGE fluorography (Figure 4). The nascent polypeptides initially arrested on the ribosome as peptidyl-tRNA-ribosomal complex and in time were slowly released from the complex (cf. Figure 5). Chromatograms (Figure 5A) also show the level of resolution of the β-tubulin ribosomal, TRiC, 180, and monomer peaks obtained on the Superose 6 column in this study.

Truncation mutants Δ418−445, Δ344−445, and Δ251−445 (Figure 4, panels B, C, and D, respectively) slowly released from the ribosomal complex and accumulated either on TRiC (Δ418−445) or on TRiC and the 180 kDa complex (Δ344−445 and Δ251−445). Monomers were either not detected (Δ418−445) or were present at low levels (Δ344−445 and Δ251−445) compared to similar syntheses with full-length mRNA where monomers were the major product [Figure 4A; see also Yaffe et al. (1992)]. Nascent Δ144−445 (panel E) and Δ83−445 (panel F), on the other hand, were released rapidly from the ribosomal complex and accumulated mainly on the 180 kDa complex (Δ144−445) or as monomers (Δ83−445). These mutants either interacted weakly or did not interact with TRiC. The apparent inability of the latter polypeptides to interact with TRiC was not a consequence of the choice of synthesis times ($t \geq 10$ min in this example) since similar findings were obtained with short synthesis times ($t < 10$ min) (data not shown). Immunoprecipitations with Ab 23C confirmed significant binding between TRiC and mutants Δ251−445, Δ344−445, and Δ418−445 (Figures 4D and 5B; data for Δ344−445 not shown). Δ144−445 (Figure 4E) failed to immunoprecipitate. Additional immunoprecipitation studies with Δ418−445 using the isolated peptidyl-tRNA-ribosomal complex (peak R, Figure 5) indicated that TRiC associated with nascent C-terminally truncated polypeptides. This finding, and a similar finding with mutant Δ2−251/Δ344−445 (Figure 9B below), are in accord with Frydman et al.'s study of nascent C-terminally truncated firefly luciferase (Frydman et al., 1994). Immunoprecipitations at 14 min, where the major product was the peptidyl-tRNA-ribosome complex (R form), were less than that at 40 min, where this complex had chased into the 900 kDa form (Figure 5B, lanes 1 vs lane 4). This may indicate either weaker or partial association of TRiC with the nascent polypeptides or reduced accessibility of the immunoprecipitating antibody to TRiC when the latter is bound to the peptidyl-tRNA-ribosome complex.

Fontalba et al. recently showed that a minimum of ∼24 residues must be removed from the C-terminus to generate a "TRiC-arrest" phenotype (Fontalba et al., 1995). Additional studies in this laboratory (not shown) confirmed this conclusion. To test whether these residues might have a general "releasing-activity" a deletion mutant Δ344−420 which contained the penultimate 25 C-terminal residues was constructed (Materials and Methods). Δ344−420 gave only slightly higher levels of monomer than Δ344−445 but otherwise interacted similarly with TRiC and the 180 kDa complex (data not shown). To control for the possibility that normal processing of the nascent polypeptides on the ribosome might be perturbed in the case of the truncations, causing an artifactual accumulation of polypeptides on TRiC and the 180 kDa complex, stop codons were introduced at positions 251 and 344 to generate mutants Δ^S251−445 and Δ^S344−445. These behaved similarly to their counterparts generated by restriction digestion and either largely arrested on TRiC (Δ^S344−445) or on TRiC and to a larger extent on the 180 kDa complex (Δ^S251−445) (data not shown).

These findings were interpreted as evidence that a region starting between residues 144 and 250 and perhaps ending at residue ∼420 interacted with TRiC. The observation that the extent of association with TRiC correlated inversely with the size of the C-terminal truncation (Figure 4) suggested multiple binding sites within this region. When similar truncations were performed on ΔR2E3 to generate ΔR2E3/Δ144−445, ΔR2E3/Δ251−445, ΔR2E3/Δ344−445, and ΔR2E3/Δ418−445, the interaction with TRiC first became evident with ΔR2E3/Δ251−445 and progressively increased with ΔR3E3/Δ344−445 and ΔR2E3/Δ418−445, as was observed with wt (data not shown).

Formic Acid Digests. To test the hypothesis that region *ca*. 145−420 contained multiple sites of interaction with TRiC, [^{35}S]β-tubulin obtained by overexpression in *E. coli* was digested with formic acid (Methods). Formic acid cleaved the Asp-Pro bonds at positions 31 and 304 and generated four major fragments (Serrano et al., 1984) (Figure 6). Two of the fragments, $\beta_1 + \beta_2$ and β_2, closely approximated the nucleotide binding domain (β_2 presumably lacked the guanine-binding site) (Shivanna et al., 1993; Farr & Sternlicht, 1992; Jayaram & Haley, 1994; Kirchner & Mandelkow, 1985; Mandelkow et al., 1987; Shivanna et al., 1993), and a third, β_3, closely approximated the C-terminal domain (de la Vina et al., 1988). Formic acid also cleaved at a slow rate throughout the sequence which caused the relative amounts of these fragments to vary with time. After a 40 h digestion all of the major fragments were still present but the β_3 fragment was the predominant product (Figure 6A).

The digested material was diluted into rabbit reticulocyte lysate, incubated for 20 min and the mixture analyzed by

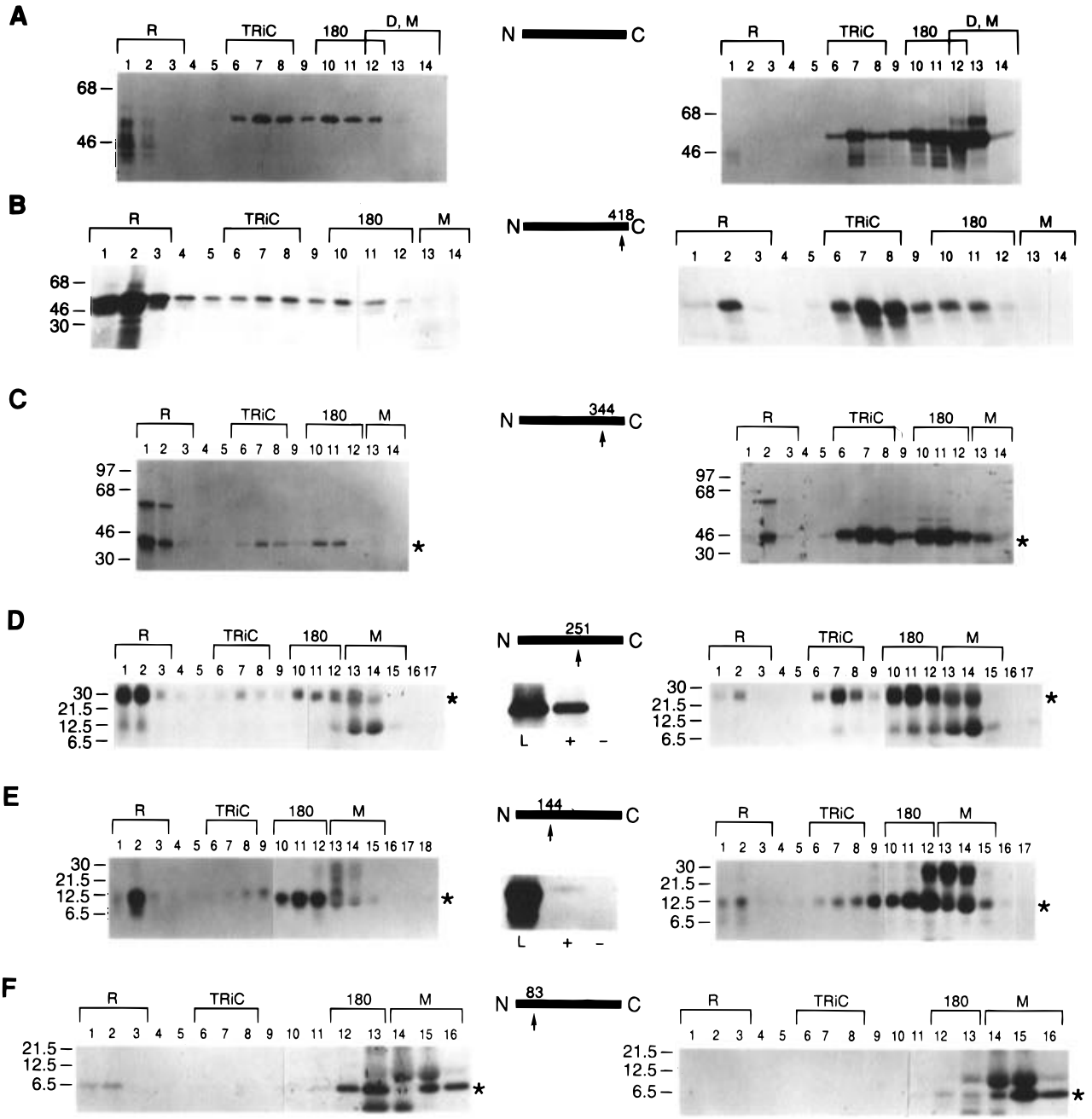

FIGURE 4: Inverse correlation between the size of the C-terminal truncation and the degree of association with TRiC suggests multiple binding sites. mRNAs coding for C-terminally truncated polypeptides Δ83−445, Δ144−445, Δ251−445, Δ344−445, or Δ418−445 were translated for the indicated times. The samples were chromatographed on a Superose 6 column. Even-numbered fractions were collected from the size-exclusion column and analyzed by SDS−PAGE fluorography (lane 1 = Fractions 40 + 42; lane 2 = Fractions 44 + 46 ··· lane 18 = fractions 108 + 110; etc.). "R" denotes the peptidyl-tRNA-ribosomal complex, which eluted in the void volume ($M_r \geq 30 \times 10^3$ kDa). "TRiC" and "180" denote complexes with TRiC and the 180 kD chaperone, respectively. "D" denotes tubulin dimer or dimer aggregates; "M" denotes free monomer or unbound polypeptide. (A) Full-length β-tubulin control. LHS, 10 min synthesis; RHS, 40 min synthesis. (B) Δ418−445. LHS, 10 min; RHS, 120 min. (C) Δ344−445. LHS, 14 min; RHS, 40 min. (D) Δ251−445. LHS, 10 min; RHS, 40 min. (E) Δ144−445. LHS, 10 min; RHS, 40 min. (F) Δ83−445. LHS, 14 min; RHS, 120 min. Radioactive bands from hemoglobin, evident at *ca*. 12.5 and 26 kD at late synthesis times, partly obscured the monomer region in some of the studies (panels D and E) (Methods). "*" denotes the major truncated polypeptide. (Center, D and E) Immunoprecipitates of 10 and 14 min translation reactions of Δ144−445 and Δ251−445, respectively, analyzed by SDS−PAGE fluorography. "+", with anti-TCP1α Ab 23C; "−", without Ab 23C. "L", initial translation reaction.

size-exclusion chromatography and fluorography. With the exception of fragment β_1, all fragments bound TRiC and the 180 kDa complex demonstrating there were multiple binding sites on the β-tubulin sequence for both classes of chaperones. However, fragments $\beta_1 + \beta_2$ and β_2 bound these complexes with significantly higher apparent affinity than did fragment β_3 as judged by SDS−PAGE fluorographs (Figure 6B). On the basis of their relative amounts in the initial digest versus in the final chaperone complexes, fragments $\beta_1 + \beta_2$ and β_2 bound TRiC and the 180 kDa complex, respectively, with *ca*. 3- and 8-fold higher apparent affinity than did β_3. Approximately 50−60% of fragment β_3 was lost. This missing fraction, which by material balance should have been in monomers, either bound non-specifically to the column or was slowly degraded in the lysate (peak B, Figure 6B). Our estimates of relative affinities assumed similar losses for all fragments. The proteolytic breakdown monitored by peak B was highly reproducible. This activity

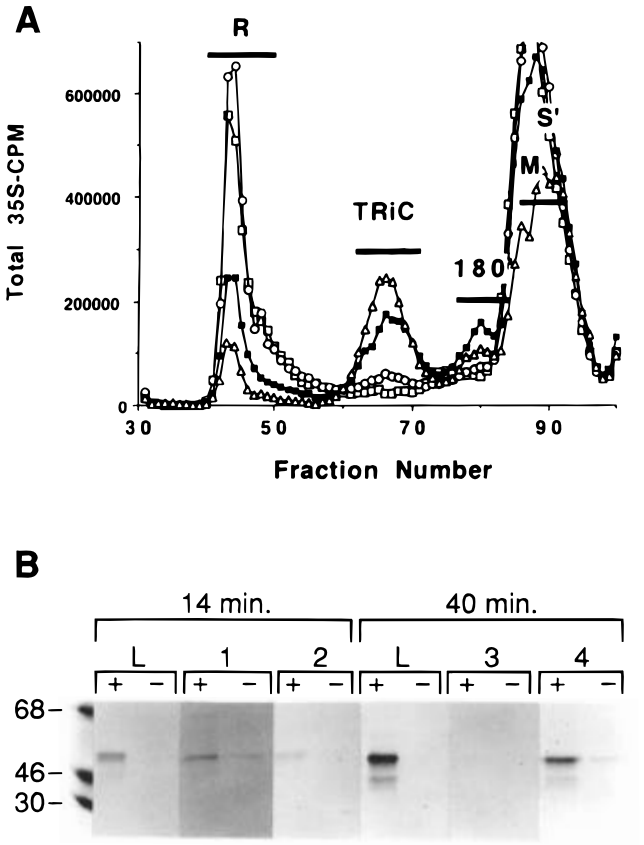

FIGURE 5: Ribosomal bound Δ418−445 is immunoprecipitated by anti-TCP1α antibody 23C. 10 (○), 14 (□), 40 (■), and 120 (△) min syntheses with Δ418−445 mRNA were chromatographed on a Superose 6 column. Fractions were pooled as indicated (A) and analyzed by SDS−PAGE fluorography (10 and 120 min fluorographs shown in Figure 4B above), or immunoprecipitated (±Ab 23C) followed by SDS−PAGE and fluorography (ribosomal peak "R", lanes 1, 3; "TRiC", lanes 2, 4; translation reaction, lane L) (B). (B) S′ in A denotes the elution position of the [^{35}S]methionyl-tRNA complex.

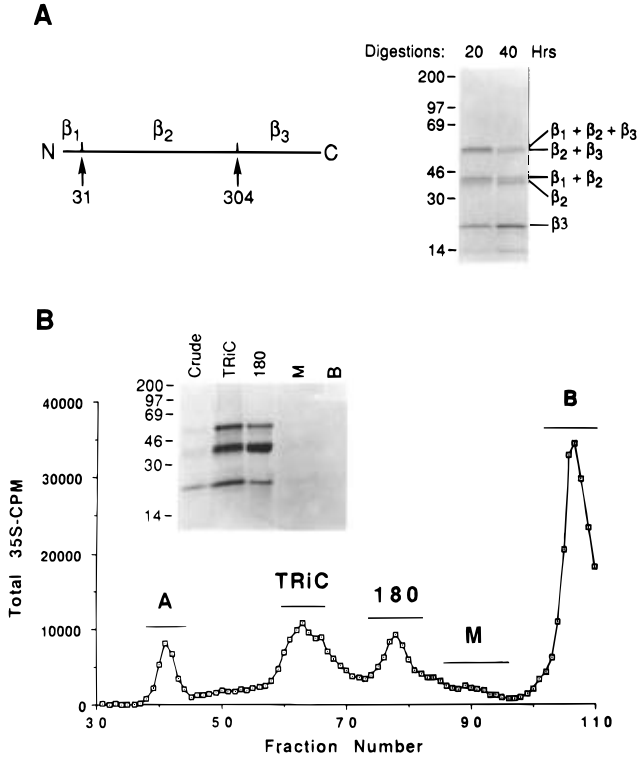

FIGURE 6: Relative apparent affinities of N-terminal sequences 1−304 and 31−304 and C-terminal sequence 304−445 for TRiC. (A) Formic acid digestion of urea-denatured [^{35}S]β-tubulin was performed for 20 or 40 h (Methods), and the products were analyzed by SDS−PAGE fluorography. Assignment and notation followed Serrano et al. (1984). (B) An aliquot from a 40 h digestion (9×10^5 dpm) was incubated for 20 min with rabbit reticulocyte lysate and chromatographed on a Superose 6 column. Peak A, aggregated material; peak B, proteolytic breakdown products. Fractions were pooled as indicated and analyzed by SDS−PAGE fluorography (inset). In the study shown samples were diluted out of 1 M urea. Similar results were obtained with samples diluted out of 8 M urea (data not shown).

may be intrinsic to the reticulocyte lysate (proteasomal?) (Frydman & Hartl, 1996) or may (less likely) reflect a protease contaminant introduced with the [^{35}S]β-tubulin digests.

N-Terminal and Internal Deletions. N-terminal deletion mutants Δ2−145, Δ2−251, and Δ2−346 were constructed to assess TRiC binding to the M- and C-domain regions (Figure 7). β−TRiC was not detected in the case of Δ2−346 although it was the major form in the case of Δ2−145 and Δ2−251. The 180 kDa form was diminished in these mutants. Instead, a *ca.* 400−500 kDa form was observed (Δ2−145 and Δ2−251), evident either as a distinct band in non-denaturing gels or as a shoulder (fractions 70−74) on the β−TRiC peak from the size-exclusion column (Figure 7).

Internal deletion mutants Δ85−144, Δ86−251, Δ85−306, Δ86−346, and Δ85−381 were constructed to assess TRiC binding to the C-domain region. Δ85−381 interacted weakly with TRiC (Figure 8A). Its monomer was the major product by non-denaturing PAGE and size-exclusion chromatography, with recovery significantly higher on the size-exclusion column (SDS−PAGE analysis not shown). In contrast, β−TRiC was the major form in the case of Δ85−144, Δ86−251, Δ85−306, and Δ86−346 (Figure 8A). A *ca.* 400−500 kDa form was also observed with these mutants (Figure 8). The 180 kDa form, on the other hand, was diminished (Figure 8A, chromatogram) which suggested that a common region ca. 85−144 missing in these mutants might be a major site of interaction with the 180 kDa chaperone. [Other explanations are also possible (Discussion).] Monomers were not detected by non-denaturing PAGE (Figure 8A, inset), although small (Δ85−144 and Δ86−251) or significant (Δ85−306 and Δ86−346) amounts were observed by size-exclusion chromatography followed by SDS−PAGE/fluorography (data not shown). Interestingly, Δ85−144 and Δ86−251 monomers *were* detected at significant levels by anion-exchange chromatography (Figure 8B). These monomers, which apparently dissociated from TRiC during chromatography, eluted as broad peaks centered at the wt monomer position. The elution position of wt protein is determined primarily by its highly negatively charged C-terminal end (Yaffe et al., 1988), i.e., residues *ca.* 420−445. Broad peaks may indicate that the C-terminal ends of the mutants were destabilized by the deletions and assumed a variety of conformations which interacted differently with the anion-exchange column (Discussion).

The Interactive Core. Region from *ca.* 200 ± 50 to 350 was deduced as the major TRiC-binding region in β-tubulin (Table 1). Mutant Δ2−251/Δ344−445 was constructed to test this assignment. Polypeptide 252−343 generated from this construct interacted strongly with TRiC as seen on non-denaturing gels (Figure 9A). Immunoprecipitation studies with Ab 23C of the 900 kDa complex (Figure 9B, lanes 1 and 3) as well as ribosomal fractions (Figure 9B, lanes 2

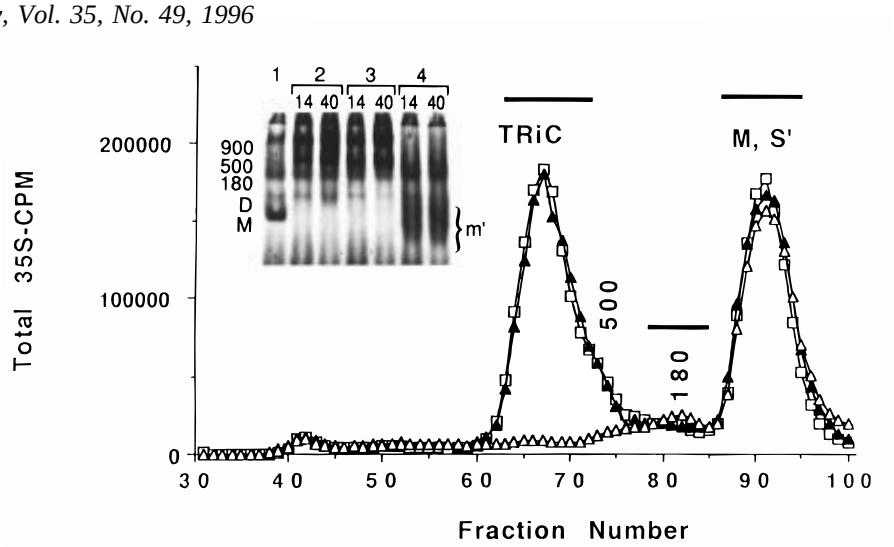

FIGURE 7: N-terminal deletions implicated region 250−340 as an interaction site(s) with TRiC. Δ2−145, Δ2−251, and Δ2−346 mRNAs were translated in reticulocyte lysate for 14 or 40 min and analyzed by non-denaturing PAGE (inset: 1, wt standard; 2, Δ2−145; 3, Δ2−251; 4, Δ2−346) or by size-exclusion chromatography (40 min syntheses): Δ2−145 (□); Δ2−251 (▲); Δ2−346 (△). "m′" denotes the Δ2−346 monomer. "M" denotes the wt β-tubulin monomer; "D" denotes the tubulin heterodimer or dimer aggregates.

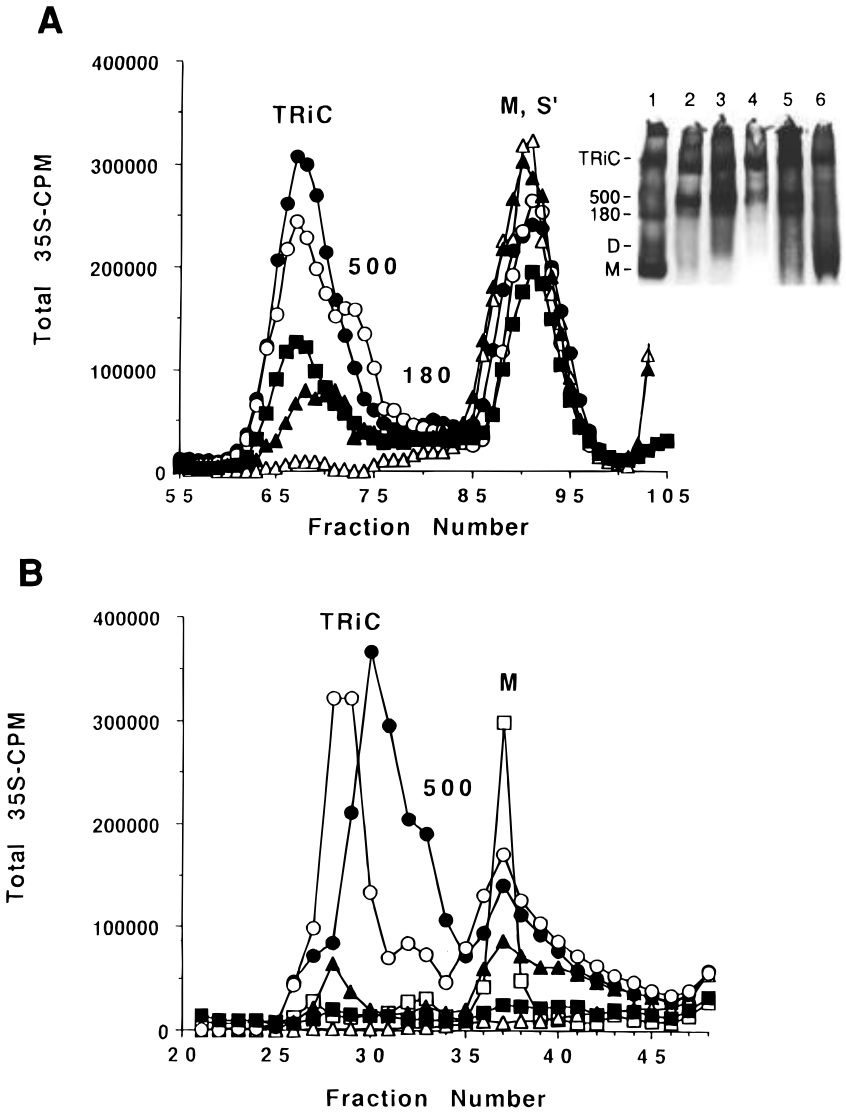

FIGURE 8: Internal deletions implicated region 340−380 as an interaction site(s) with TRiC. Δ85−144 to Δ85−381 mRNAs were translated for 40 min and analyzed by non-denaturing PAGE (inset panel A, lanes 1−6: wt, Δ85−144, Δ86−251, Δ85−306, Δ86−346, and Δ85−381, respectively) or by size-exclusion (A) and anion-exchange (B) chromatography [wt (□); Δ85−144 (●); Δ86−251 (○); Δ85−306 (▲); Δ86−346 (■); Δ85−381 (△)].

and 4) confirmed a significant association with the chaperonin. The latter immunoprecipitations were consistent with an initial association of TRiC with the nascent polypeptide (Figure 9B, lane 1 vs lane 3) (Frydman et al., 1994). A 500 kDa complex formed with Δ2−251/Δ344−445 (Figure 9A). This complex was resolved from TRiC on the size-exclusion

Table 1: Apparent TRiC-Binding Sites

mutations/digestions	interaction site (residues)	figure no.
C-terminal truncations	~150−445	4
ΔR2E3 truncations	~150−445	ns[a]
formic acid	30−300 ("major") 300−445 ("minor")	6
N-terminal truncations	250−350	7
internal deletions	350−380 (?)	8
"consensus" regions of interaction:	~200 ± 50 to 350 (major) 350−380 (minor)	

[a] Not shown.

column and could be immunoprecipitated by Ab 23C (Figure 9B, lane 5) (Discussion).

TRiC-binding region from 250 to 350 confirmed by mutant Δ2−251/Δ344−445 plus an additional *ca*. 50−100 binding residues N-terminal to residue 250 (Figure 4D,E) are referred to below as "the interactive core" of β-tubulin (Figure 9C).

DISCUSSION

In this study a variety of mutants were constructed to probe β-tubulin affinity for TRiC. This approach overall gave internally consistent findings (see Table 1) although there was an initial concern that this approach might profoundly perturb folding and yield artifactual or uninterpretable results. An unexpectedly large number of TRiC-arrested mutants were detected. Such arrest mutants could not be generated with a non-substrate control protein such as endolase (data not shown). A major effort was undertaken to identify common features in these mutants that could account for the arrest phenotype.

Removal of either two residues from the N-terminus or 27 residues from the C-terminus was sufficient to cause arrest on TRiC. More extensive deletions at either the N- or C-terminal ends, as well as simultaneous deletions at these ends, also produced the arrest phenotype. Only when the deletions were sufficiently large to disrupt a core region consisting of residues ~150−350 was the arrest phenotype lost. Furthermore, the N- and C-terminal regions, which appeared to be so essential for release, had little or no intrinsic affinity for TRiC. For example, C-terminal deletion mutant Δ83−445 showed no detectable interaction with the chaperonin and apparently folded independently of TRiC. Similarly, N-terminal deletion mutant Δ2−346 interacted only weakly with TRiC. In contrast, a *core region* ~150−350, consisting of multiple binding sites was identified by mutagenesis which interacted strongly with TRiC (Table 1 and Figure 9).

Intermediate Chaperone Forms. The 180 kDa forms were diminished, and "500" forms were observed with Δ2−251/Δ344−445 and a number of the N-terminal and internal deletion mutants. The latter form was also evident in wt β-tubulin at much lower concentrations (unpublished studies) and may have accumulated in the case of the mutants due to an increased stability of this form and/or a reduced ability of this form to interact with other cofactors/chaperones implicated in tubulin folding. This form may contain a single toroid of TRiC and could be relevant to the function of the cytosolic chaperonin. This interpretation is supported by immunoprecipitation studies with anti-TCP1α Ab 23C. ATP/K^+-dependent dissociation of tetradecameric cpn60 into single toroid heptamers has also been reported in the case of *Thermus thermophilus* cpn60 (Ishii et al., 1995). An alternative possibility is that the "500" forms represent a complex with cofactors D and E, two recently uncovered proteins which form a *ca*. 500 kDa complex with β-tubulin and which have been implicated at a late stage of β-tubulin folding following release from the chaperonin (Tian et al., 1996). Further studies will be required to distinguish between these possibilities.

Proteolytic Studies. Limit proteolysis of β−TRiC performed with chymotrypsin (Figures 2 and 3) yielded native-like digestion patterns consistent with the notion of quasi-native chaperonin-bound intermediates (Tian et al., 1995). These studies also revealed a highly-resistant 18 kDa fragment which was tentatively identified as a breakdown product of the 34 and 37 kDa fragments, i.e., the N-fragments encompassing residues ~1−280 and ~1−300, respectively. This residue assignment was based largely on the molecular weight equivalence of the quasi-native and native digestion products and made use of Mandelkow et al.'s previous determination of the initial chymotrypsin cleavage site in native protein (Figure 3A, Results). Confirmation of the assignment will ultimately require the isolation and sequencing of either the 18 kDa or 34 and 37 kDa fragments, a technically difficult undertaking because of the small amounts of material generated in translation studies. The 18 kDa product appeared resistant to chymotrypsin by *virtue* of its association with the chaperonin and is likely to represent a major region of interaction between TRiC and the quasi-native intermediates. Based on radioactivity content, the 18 kDa fragment contained a significant fraction of the methionyl residues in β-tubulin. This constraint limited the 18 kDa fragment to the later portion of the N-fragments (Valenzuela et al., 1981). On the basis of the assignment of the initial chymotrypsin cleavage site in β-tubulin to position 280 (Mandelkow et al., 1987), we suggest that the 18 kDa fragment encompasses core residues ~150−280 and possibly as much as an additional *ca*. 30−40 residues N-terminal to residue 150, which on their own probably do not interact significantly with TRiC (Figure 4D,E). [The precise number of additional residues depends on the true molecular weight of the fragment which at present is uncertain. In our study β-tubulin fragments often displayed anomalously high apparent molecular weights on denaturing gels (Materials and Methods) (below)]. Within the experimental uncertainties, the 18 kDa fragment encompassed a major portion of the M-domain, as well as the intervening region between this domain and the C-domain deduced previously from proteolytic studies of native dimer (de la Vina et al., 1988) (Figure 9C).

Limiting proteolysis revealed an apparent association of the C-terminal domain with TRiC ("C" band, Figure 3C). We suspect that this observed association reflects a continued association of the N-, M-, and C-domains following cleavage, with the M-domain and the intervening region providing the principal interaction with TRiC via residues ~150−300. This interpretation is consistent with Brown and Erickson's finding that the β-tubulin subunit in the native dimer remains intact following chymotrypsin cleavage (Brown & Erickson, 1983). It is also consistent with our failure to detect a resistant fragment which originated from the C-domain upon further proteolysis analogous to the 18 kDa fragment.

In situ studies of the full-length newly-synthesized polypeptide demonstrated a modest increase in chymotrypsin resistance at residue position ~280 with chase times suggestive

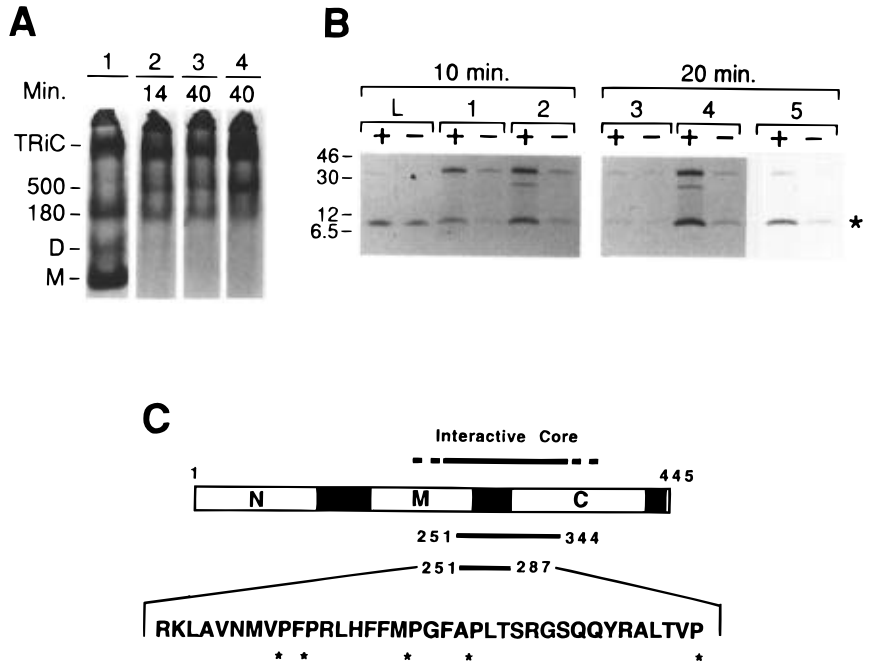

FIGURE 9: Interactive core encompasses portions of the M- and C-domains and an intervening interdomain sequence. (A, B) Polypeptide 251−344 interacts with TRiC. Translation reactions of mutants Δ2−251 and Δ2−251/Δ344−445 were analyzed by non-denaturing PAGE (wt standard, lane 1; Δ2−251/Δ344−445, lanes 2 and 3; Δ2−251, lane 4) (A) or chromatographed (Δ2−251/Δ344−445) on a size-exclusion column with peak aliquots subjected to immunoprecipitation (±Ab 23C) followed by SDS−PAGE and fluorography (ribosomal peak, lanes 1, 3; "TRiC", lanes 2, 4; translation reaction, lane L) or phosphorimagery ("500", lane 5) (B). Lanes 4 and 5 should not be directly compared as lanes 5 were enhanced on the PhosphorImager. By integration immunoprecipitated β−TRiC had a volume ∼5 times larger corrected for background than did immunoprecipitated "500", in good agreement with the ratio of their corrected peak heights (β−TRiC peak was ∼5−6-fold larger than "500" peak) in the chromatogram. "*" denotes the position of Δ2−251/Δ344−445. The upper band at *ca*. 32 kDa was assigned to the peptidyl-tRNA complex based on its sensitivity to RNase (data not shown). (C) The interactive core as defined by mutation analysis overlaps portions of the M- and C-domains and a protease-sensitive region, ∼260−290, which connects the two domains in the native protein [■, protease sensitive regions of β-tubulin; □, compact regions (de la Vina et al., 1988)]. A hydrophobic region, ∼253−287, rich in prolines and present in polypeptide 251−344 observed to bind TRiC (panels A and B) may be a major site of interaction (Discussion).

of a maturation of the polypeptide during the quasi-native phase (Figure 2). On the other hand, β−TRiC isolated during the quasi-native phase gave identical fragmentation patterns independent of chase times (Figure 2, data not shown). This finding is reminiscent of studies with GroEL where the GroEL-bound substrates similarly displayed invariant fragmentation patterns with time [cf. Weissman et al. (1994)]. This observation has been interpreted in terms of a mechanism whereby GroEL functions by carrying out multiple rounds of binding of aggregation-prone or kinetically trapped intermediates, maintaining them in an unfolded state, and releasing them in an ATP-dependent manner to attempt folding in solution (Jackson et al., 1993; Todd et al., 1994; Weissman et al., 1994). However, more recent studies indicate that the release of bound intermediates to solution is not essential for productive folding by GroEL and that the bulk of such folding, in the presence of GroES, is likely to occur in the GroEL cavity (Mayhew et al., 1996; Weissman et al., 1996). TRiC-mediated folding of α-tubulin is thought to occur on the chaperonin or within its central cavity (Tian et al., 1995). A similar process may apply to β-tubulin. The cytosolic chaperonin is heteromeric and more complex than GroEL. Increased resistance to chymotrypsin with chase time (Figure 2) characterized by invariant fragmentation patterns could reflect changes in the accessibility of the protease to the β-tubulin substrate due to changes in chaperonin conformation and/or in the sites of binding of substrate within the chaperonin. Alternatively, it could represent a subtle and progressive maturation of the β-tubulin polypeptide during the quasi-native phase [involving perhaps domain−domain contacts or other features that characterize the quasi-native state (see below)]. Cofactors/chaperones implicated in the release of β-tubulin from the chaperonin could also be contributing factors [Gao et al., 1994; Campo et al., 1994; Paciucci, 1994; see also Tian et al. (1996)].

The N-Terminus. Removal of Arg2 and Glu3 caused TRiC-arrest of the newly synthesized polypeptide, and apparently altered the rates of folding and/or association with the chaperonin (Figure 1). TRiC-arrest was absolished by removal of region 85−445 and significantly attenuated by removal of region 250−445 (data not shown). N-terminal region ∼3−35 either contains the binding site for the guanine moiety of GTP or contributes significantly to this site (Shivanna et al., 1993) and should be in close proximity to region 140−300, which is largely comprised of core residues and contains the other determinants of the nucleotide binding site (Jayaram & Haley, 1994; Shivanna et al., 1993). Deletion of Arg2 and Glu3 may have, therefore, perturbed the GTP-binding domain and caused residues in the core region to be exposed, i.e., to bind more tightly to TRiC. The possibility that removal of the N-terminal residues disrupted other chaperone processes which may be coupled to TRiC (Frydman et al., 1994; Tian et al., 1996) cannot be excluded

[2] Tian et al. (1996) recently characterized a 300 kDa folding intermediate of β-tubulin which appears to be identical to the "180" form of this study. This intermediate contains a newly discovered cofactor, i.e., cofactor D ($M_r \approx 120$ kDa), which like the 180 kDa chaperone of this study induces release of β-tubulin from the TRiC complex and stabilizes the released polypeptide in a quasi-native form.

at this time. The 180 kDa chaperone, which interacts with the N-domain and is apparently required for the release of β-tubulin from TRiC (Farr and Sternlicht, unpublished studies), is one possible candidate for such disruption.[2]

Region 380−*450*. Region 380−450 interacted weakly with TRiC. The major portion of this region (residues ∼380−425) is highly conserved but has no known function (Sullivan, 1988). Deletion of *ca*. 20 C-terminal residues from this region induced TRiC arrest (Figure 4, unpublished studies) (Fontalba et al., 1995). In actin, which shows significant parallelism to tubulin in its interaction with TRiC, removal of 10−20 C-terminal residues induced arrest (unpublished studies). These residues pack against N-terminal residues of subdomain 1 (Kabsch et al., 1990) and are distal to the TRiC-interactive core. We suspect the removal of the C-terminal residues from β-tubulin and actin allosterically perturbed the TRiC-interactive cores causing increased exposure of core residues to TRiC. Burns and Surridge proposed that release of β-tubulin and actin polypeptides from TRiC involves a competition between sequences ^{391}RKAF^{394} and ^{372}RKCF^{375}, respectively, with an homologous sequence in TCP1α for a binding site in the N-terminal portion of the polypeptides (Burns & Surridge, 1994). Their proposal for β-tubulin is consistent with our observation that mutations in sequence ^{391}RKAF^{394} caused TRiC-arrest (Driscoll et al., 1994; Dobrzynski and Sternlicht, manuscript in preparation). However, alanine-scan mutagenesis performed in this laboratory also indicated that region 390−425, and possibly a still larger region beginning at *ca*. residue 370, was sensitively linked to folding. Mutations in this region, for the most part, either induced TRiC-arrest or dimer aggregates (Dobrzynski and Sternlicht, manuscript in preparation). Other studies also argue for interactions between the C- and N-domains. Padilla et al. demonstrated an interaction between C-terminal region 423−426 and N-terminal region 107−130 of β-tubulin via cross-linking (Padilla et al., 1993). The finding that the C-terminal end of internal deletion mutant Δ85−144, was apparently less structured than that of wt (Figure 8B, Results) is consistent with the notion that this end interacts with N-terminal region *ca*. 85−144 in agreement with the Padilla et al. study. Expanding upon the Burns and Surridge hypothesis, we suggest that highly conserved region 380−425 is essential for polypeptide release from TRiC. Residues in this region are presumed either to pack against region 150−360 which inhibits binding to TRiC or to interact with intermediate residues which pack directly against the core region. Whether this region has a regulatory role in tubulin folding as suggested for sequence ^{391}RKAF^{394} still remains to be determined.

Interactive Core and Domain Structure. De la Vina et al. found three major proteolytically defined compact regions in the native protein, i.e., the N-, M-, and C-domains corresponding respectively to residues ∼1−110, 175−260, and 300−420, with the precise location of the domain boundaries uncertain by ∼15−30 residues (de la Vina et al., 1988). TRiC was shown to interact with polypeptide 252−343 (Δ2−251/Δ344−445) which overlaps portions of the M- and C-domains (Figure 9A). A portion of this polypeptide coincides with a putative interconnecting sequence that joins these domains in the native protein, which could be critical for the correct spatial placement of these domains. This sequence is rich in hydrophobic and proline residues and potentially could interact with hydrophobic residues in TRiC during the folding process [cf. Fenton et al. (1994)]. All five prolyl residues in this sequence are preceded N-terminally by a hydrophobic residue, an apparent requirement for the prolyl isomerase activity of the FKBP immunophilin family (Harrison & Stein, 1990). Interestingly, immunophilins have recently been implicated in the folding of bacterial luciferase in rabbit reticulocyte lysate (Kruse et al., 1995). Whether the interconnecting sequence actually plays an essential or pivotal role in chaperonin-mediated folding remains to be determined.

The interactive core of β-tubulin defined by the mutagenesis study encompasses portions of the M- and C-domains and an intervening sequence. This could mean that TRiC mediates domain−domain contacts during folding [cf. related study of rhodanese bound to GroEL (Hlodan et al., 1995)]. However, proteolysis studies during the quasi-native phase gave no evidence for interactions with TRiC beyond residue ∼280 which argues against such a mechanism. It is possible that a small proteolytic-resistant fragment which contained a portion of the interactive region from the C-domain was generated in our studies but was not detected. It is also possible that the chymotrypsin cleavage site in the newly synthesized polypeptide was misassigned, and a significant portion of the interactive region from the C-domain is contained in the M-fragment. Alternatively, bearing in mind that TRiC apparently interacts with nascent polypeptides (Frydman et al., 1994) (Figures 1, 5B, and 9B), mediation of domain−domain contacts by TRiC might have occurred in an earlier folding phase prior to the quasi-native phase, which was not probed by our studies.

The interdomain region may be prone to misfolding or improper interactions in the newly synthesized polypeptide. Its functional role in tubulin is unknown although genetic and mutation studies argue that this region may be involved in interprotofilament interactions (Savage et al., 1994). Chou−Fasmann and Garnier−Osguthorpe−Robson predictions suggest that this region has secondary-structure elements that flank a coil segment at position ∼268−278. Interestingly, preliminary studies in this laboratory suggest that the interactive core in actin encompasses portions of subdomains 3 and 4 and the interconnecting "hydrophobic-plug", a loop region composed of residues *ca*. 260−270 which is thought to stabilize lateral interactions in the F-actin filament (Holmes et al., 1990; Chen et al., 1995). The interdomain region of β-tubulin similarly may be important for microtubule assembly and/or tubulin heterodimer formation.[3] If this is correct, the cytosolic chaperonin may recognize structural elements or regions important for protein−protein interactions and/or self-assembly. Such elements may, for example, present difficulties during folding or may need to be protected for processing. Future studies should further clarify the molecular basis for TRiC's selectivity toward substrate and its mechanism of action.

[3] This notion that the interdomain region of β-tubulin contains a functional loop important for chaperonin selectivity is supported by recent studies of the interaction of GroEL with *E. coli* and murine DHFR (Clark et al., 1996). GroEL does not interact with *E. coli* DHFR but interacts with both native and unfolded forms of murine DHFR. With the exception of three loop regions in murine DHFR, the native structures of the two DHFRs are similar. Insertions of two of the three murine loop regions into *E. coli* DHFR generated mutants which, like murine DHFR, interacted with GroEL from both the native and unfolded states.

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REFERENCES

Angeles, M., Nieto, A. J. M., Andreu, D., & Andreu, J. M. (1990) *J. Mol. Biol. 214*, 105−120.
Beese, L., Stubbs, G., & Cohen, C. (1987) *J. Mol. Biol. 194*, 257−264.
Brown, H. R., & Erickson, H. P. (1983) *Arch. Biochem. Biophys. 220*, 46−51.
Burns, R. G., & Surridge, C. D. (1994) *FEBS Lett. 347*, 105−111.
Campo, R., Fontalba, A., Sanchez, L. M., & Zabala, J. C. (1994) *FEBS Lett. 353*, 162−166.
Chen, X., Cook, R. K., & Rubenstein, P. A. (1993) *J. Cell Biol. 123*, 1185−1195.
Chen, X., Sullivan, D. S., & Huffaker, T. C. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9111−9115.
Chou, P. Y., & Fasman, G. D. (1978) *Adv. Enzymol. 47*, 45−148.
de la Vina, S., Andreu, D., Medrano, F. J., Nieto, J. M., & Andreu, J. M. (1988) *Biochemistry 27*, 5352−5365.
Clark, A. C., Hugo, E., & Frieden, C. (1996) *Biochemistry 35*, 5893−5901.
Driscoll, J., Farr, G. W., & Sternlicht, H. (1994) *Mol. Biol. Cell 5*, 282a.
Farr, G. W., & Sternlicht, H. (1992) *J. Mol. Biol. 227*, 307−321.
Fenton, W. A., Kashi, Y., Furtak, K., & Horwich, A. L. (1994) *Nature 371*, 614−619.
Fontalba, A., Avila, J., & Zabala, J. C. (1995) *J. Mol. Biol. 246*, 628−636.
Frydman, J., & Hartl, F.-U. (1994) *Science 272*, *1497*−*1502*.
Frydman, J., Nimmesgern, E., Erdjument-Bromage, H., Wall, J. S., Tempst, P., & Hartl, F.-U. (1992) *EMBO J. 11*, 4767−4778.
Frydman, J., Nimmesgern, E., Ohtsuka, K., & Hartl, F.-U. (1994) *Nature 370*, 111−117.
Gao, Y., Thomas, J. O., Chow, R. L., Lee, G. H., & Cowan, N. J. (1992) *Cell 69*, 1043−1050.
Gao, Y., Vainberg, I. E., Chow, R. L., & Cowan, N. J. (1993) *Mol. Cell. Biol. 13*, 2478−2485.
Gao, Y., Melki, R., Walden, P. D., Lewis, S. A., Ampe, C., Rommelaere, H., Vandekerckhove, J., & Cowan, N. J. (1994) *J. Cell Biol. 125*, 989−996.
Garnier, J., Osguthorpe, D. J., & Robson, B. (1978) *J. Mol. Biol. 120*, 97−120.
Gu, W., & Cowan, N. J. (1989) *Mol. Cell. Biol. 9*, 3418−3428.
Hansen, W. J., Lingappa, V. R., & Welch, W. J. (1994) *J. Biol. Chem. 269*, 26610−26613.
Harrison, R. K., & Stein, R. L. (1990) *Biochemistry 29*, 3813−3816.
Hlodan, R. Tempst, P., & Hartl, F.-U. (1995) *Nat. Struct. Biol. 2*, 587−594.
Holmes, K. C., Popp, D., Gebhard, W., & Kabsch, W. (1990) *Nature 347*, 44−49.
Ishii, N., Taguchi, H., Sasabe, H., & Yoshida, M. (1995) *FEBS Lett. 362*, 121−125.
Jackson, G. S., Staniforth, R. A., Halsall, D. J., Atkinson, T., Holbrook, J., Clarke, A. R., & Burston, S. G. (1993) *Biochemistry 32*, 2554−2563.
Jayaram, B., & Haley, B. E. (1994) *J. Biol. Chem. 269*, 3233−3242.
Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes K. C. (1990) *Nature 347*, 37−44.
Kim, S., Willison, K. R., & Horwich, A. L. (1994) *Trends Biochem. Sci. 228*, 543−548.
Kirchner, K., & Mandelkow, E.-M. (1985) *EMBO J. 4*, 2397−2402.
Kruse, M., Brunke, M. Escher, A., Szalzy, A. A., Tropschug, M., & Zimmermann, R. (1995) *J. Biol. Chem. 270*, 2588−2594.
Kubota, H., Hynes, G., Carne, A., Ashworth, A., & Willison, K. R. (1994) *Curr. Biol. 4*, 89−99.
Levison, B. S., Wiemols, J., Szasz, J., & Sternlicht, H. (1989) *Biochemistry 28*, 8877−8884.
Lewis, V. A., Hynes, G. M., Zheng, D., Saibil, H., & Willison, K. (1992) *Nature 358*, 249−252.
Mandelkow, E.-M. Herrmann, M., & Ruhl, U. (1987) *J. Mol. Biol. 194*, 257−264.
Martin, J., Mayhew, M., Langer, T., & Hartl, F.-U. (1993) *Nature 366*, 228−242.
Mayhew, M., da Silva, A. C. R., Martin, J., Bromage, H. E., Tempst, P., & Hartl, F.-U. (1996) *Nature 379*, 420−426.
Melki, R., & Cowan, N. J. (1994) *Mol. Cell. Biol. 14*, 2895−2904.
Melki, R., Vainberg, I. E., Chow, R. L., & Cowan, N. J. (1993) *J. Cell Biol. 122*, 1301−1310.
Miklos, D., Caplan, S., Mertens, D., Hynes, G., Pitluk, Z., Kashi, Y., Harrison-Lavoie, K., Stevenson, S., Brown, C., Barrell, B., Horwich, A. L., & Willison, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2743−2747.
Paciucci, R. (1994) *Biochem. J. 301*, 105−110.
Padilla, R., Otin, C. L., Serrano, L., & Avila, J. (1993) *FEBS Lett. 325*, 173−176.
Phadtare, S., Fisher, M. T., & Yarbrough, L. R. (1994) *Biochim. Biophys. Acta 1208*, 189−192.
Reijo, R. A., Cooper, E. M., Beagle, G. J., & Huffaker, T. C. (1994) *Mol. Biol. Cell 5*, 29−43.
Saibil, H., & Wood, S. (1993) *Curr. Biol. 3*, 207−213.
Savage, C., Xue, Y., Mitani, S., Hall, S., Zakhary, R., & Calfie, M. (1994) *J. Cell Sci. 107*, 2165−2175.
Schagger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368−379.
Serrano, L., de La Torre, J., Maccioni, R. B., & Avila, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5989−5993.
Shivanna, G. D., Mejillano, M. R., Williams, T. D., & Himes, R. D. (1993) *J. Biol. Chem. 268*, 127−132.
Sternlicht, H., Farr, G. W., Sternlicht, M. L., Driscoll, J. K., Willison, K., & Yaffe, M. B. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9422−9426.
Sullivan, K. F. (1988) *Annu. Rev. Cell Biol. 4*, 687−716.
Tian, G., Vainberg, I. E., Tap, W. D., Lewis, S. A., & Cowan, N. J. (1995) *J. Biol. Chem. 270*, 1−4.
Tian, G., Huang, Y., Rommelaere, H., Vandekerckhove, J., Ampe, C., & Cowan, N. J. (1996) *Cell 86*, 287−296.
Todd, M. J., Viitanen, P. V., & Lorimer, G. H. (1994) *Science 265*, 659−666.
Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W., & Cleveland, D. W. (1981) *Nature 289*, 650−655.
Vinh, D. B.-N., & Drubin, D. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9116−9120.
Weissman, J. S., Kashi, Y., Fenton, W. A., & Horwich, A. L. (1994) *Cell 78*, 693−702.
Weissman, J. S., Rye, H. S., Fenton, W. A., Beechem, J. M., & Horwich, A. L. (1996) *Cell 84*, 481−490.
Yaffe, M. B., Farr, G. W., & Sternlicht, H. (1988) *J. Biol. Chem. 263*, 16023−16031.
Yaffe, M. B., Farr, G. W., Miklos, D., Horwich, A. L., Sternlicht, M. L., & Sternlicht, H. (1992) *Nature 358*, 245−248.

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