The EMBO Journal vol.11 no.13 pp.4767-4778, 1992

Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits

Judith Frydman*, Elmar Nimmesgern*, Hediye Erdjument-Bromage[1], Joseph S.Wall[2], Paul Tempst[1] and Franz-Ulrich Hartl[3]

Program in Cellular Biochemistry and Biophysics and [1]Program in Molecular Biology, Rockefeller Research Laboratories, Sloan-Kettering Institute, New York, NY 10021 and [2]Brookhaven National Laboratory, Biology Department, Upton, NY 11973, USA
*The first two authors contributed equally to this work
[3]Corresponding author

Communicated by W.Neupert

T-complex polypeptide 1 (TCP-1) was analyzed as a potential chaperonin (GroEL/Hsp60) equivalent of the eukaryotic cytosol. We found TCP-1 to be part of a hetero-oligomeric 970 kDa complex containing several structurally related subunits of 52–65 kDa. These members of a new protein family are assembled into a TCP-1 ring complex (TRiC) which resembles the GroEL double ring. The main function of TRiC appears to be in chaperoning monomeric protein folding: TRiC binds unfolded polypeptides, thereby preventing their aggregation, and mediates the ATP-dependent renaturation of unfolded firefly luciferase and tubulin. At least *in vitro*, TRiC appears to function independently of a small co-chaperonin protein such as GroES. Folding of luciferase is mediated by TRiC but not by GroEL/ES. This suggests that the range of substrate proteins interacting productively with TRiC may differ from that of GroEL. We propose that TRiC mediates the folding of cytosolic proteins by a mechanism distinct from that of the chaperonins in specific aspects.

Key words: firefly luciferase/protein folding/t-complex polypeptide/tubulin

Introduction

The folding and oligomeric assembly of newly synthesized proteins *in vivo* requires the function of certain accessory proteins, so-called 'molecular chaperones' (Ellis, 1987) or 'polypeptide chain binding proteins' (Rothman, 1989). These components recognize proteins in non-native conformations, thereby preventing premature folding and aggregation, and mediate the acquisition of the native structure (Gething and Sambrook, 1992; Hartl *et al.*, 1992; Lorimer, 1992). Essential roles in these processes have been established for the constitutively expressed stress proteins of the Hsp60 family (ibid.). The Hsp60s, also termed 'chaperonins' (Hemmingsen *et al.*, 1988) (*Escherichia coli* GroEL, chloroplast Rubisco binding protein, mitochondrial Hsp60), have a characteristic oligomeric structure: two heptameric rings of ~60 kDa subunits are stacked on top of each other, forming a large double ring complex (Hendrix, 1979; Hohn *et al.*, 1979; Pushkin *et al.*, 1982). The chaperonin complex binds one or two molecules of substrate protein, probably in the conformation of an early folding intermediate, and supports its folding to the native state in an ATP-dependent reaction. A co-chaperonin protein (*E.coli* GroES, mitochondrial Hsp10), which is a heptameric ring complex of ~10 kDa subunits, is required for full function of the chaperonin double ring.

Recent evidence suggests that the Hsp60s have the potential to act in a sequential folding pathway accepting folding substrate proteins from Hsp70. The Hsp70s are able to recognize polypeptides in an extended conformation (Flynn *et al.*, 1991; Palleros *et al.*, 1991; Landry *et al.*, 1992) as they may emerge from ribosomes or at the *trans* side of membranes during translocation (Beckmann *et al.*, 1990; Kang *et al.*, 1990; Scherer *et al.*, 1990; Sanders *et al.*, 1992). DnaK, the *E.coli* homolog of Hsp70, cooperates with the stress protein DnaJ in stabilizing proteins against aggregation by maintaining a partially folded conformation, which is made available to Hsp60 for folding to the native state (Langer *et al.*, 1992a). Sequential interaction of first Hsp70 and then Hsp60 has been demonstrated for proteins that fold following import into mitochondria (Manning-Krieg *et al.*, 1991) and is also likely to occur in chloroplasts and in the bacterial cytosol.

Given these findings, it is intriguing that a structural and/or functional equivalent of Hsp60 has not yet been defined for the eukaryotic cytosol, which is known to contain Hsp70 and also DnaJ homologs (Caplan and Douglas, 1991; Luke *et al.*, 1991; Atencio and Yaffe, 1992). Recently, t-complex polypeptide-1 (TCP-1) has been proposed as a candidate cytosolic chaperonin based on a weak sequence homology to the Hsp60s (Ellis, 1990; Gupta, 1990). This possibility has been strongly supported by the analysis of TF55, an archaebacterial stress protein, which forms a double ring complex containing 8- or 9-membered rings of 55 kDa subunits and which has some chaperonin properties (Phipps *et al.*, 1991; Trent *et al.*, 1991). Sequence analysis revealed that TF55 has 40% sequence identity with mouse TCP-1, indicating a significant structural relationship (Trent *et al.*, 1991). Mouse TCP-1 is encoded within the so-called t-complex on chromosome 17 and has been implicated in the phenomenon of male-specific transmission ratio distortion (Silver *et al.*, 1979; Silver and Remis, 1987). However, this remains controversial (Lyon, 1990) and a more general function is suggested by the presence of TCP-1 not only in different mammalian cell types (Willison *et al.*, 1986; Kirchhoff and Willison, 1990; Kubota *et al.*, 1991) but also in *Drosophila* (Ursic and Ganetsky, 1988) and in yeast (Ursic and Culberston, 1991). TCP-1 is highly expressed in developing sperm cells (Willison *et al.*, 1990), which are actively engaged in microtubule assembly. The protein appears to be generally present in the cytosol of eukaryotes. Yeast TCP-1 is essential for growth and a temperature-

© Oxford University Press

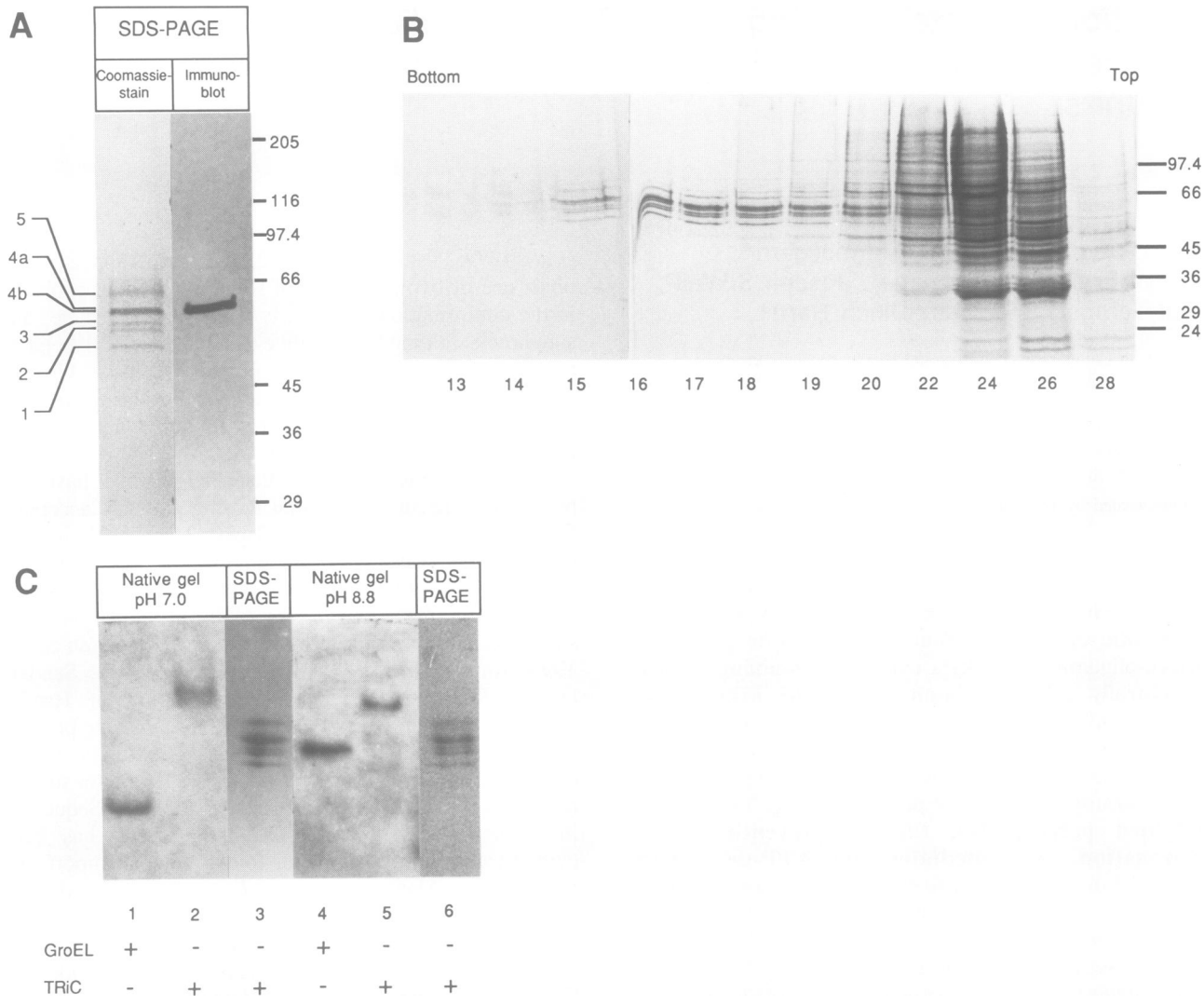

Fig. 1. Properties of purified TCP-1 ring complex (TRiC) of bovine testis. **A**, SDS−PAGE of TRiC purified by Mono-Q chromatography, sucrose density gradient centrifugation and ATP−agarose chromatography as described in Materials and methods. The position of molecular weight markers is indicated in kDa. The presence of a polypeptide that crossreacted with TCP-1 was determined by Western blotting with the monoclonal rat anti-mouse TCP-1 antibody 91A (Willison *et al.*, 1989; kindly provided by K.Willison). Individual proteins are numbered. **B**, Sucrose density gradient centrifugation of Mono-Q fraction enriched in TRiC. Fractions were analyzed by SDS−PAGE and Coomassie staining. **C**, Analysis of purified TRiC by 4−10% non-denaturing polyacrylamide gels (native PAGE) at pH 8.8 and pH 7.0 followed by Coomassie staining. The only band detectable in both systems was excised and applied to a 12.5% SDS−polyacrylamide gel as described (Trent *et al.*, 1991). Purified GroEL is included in the native gel analysis for comparison.

sensitive yeast mutant affecting Tcp-1 shows a defect in mitotic spindle formation (Ursic and Culberston, 1991).

We report here the structural and functional characterization of TCP-1 as a subunit of a hetero-oligomeric ring complex, TRiC. This confirms recent observations of Lewis *et al.* (1992) for the mouse protein. Based on partial sequence analysis we demonstrated that at least three subunits of the complex are structurally related to TCP-1. Purified TRiC has ATPase activity, associates with non-native polypeptides and mediates the ATP-dependent folding of luciferase and tubulin. A comparative analysis of TRiC and GroEL/ES suggests that TRiC differs from the chaperonin GroEL in its spectrum of substrate proteins. Under the conditions *in vitro*, TRiC function was independent of an added co-chaperonin protein. TRiC may constitute a member of a new class of chaperonin-like components that function in protein folding in the eukaryotic cytosol.

Results

TCP-1 is a subunit of a hetero-oligomeric ring complex

Based on the observation that TCP-1 is expressed to high levels in developing sperm cells (Willison *et al.*, 1990), bovine testis was chosen as the starting material for the isolation of the protein. The monoclonal antibody 91A, directed against mouse TCP-1 (Willison *et al.*, 1989), was used to monitor purification. This antibody recognized a soluble 59.9 KDa protein of bovine testis (Figure 1A) which co-migrated with mouse TCP-1 on SDS−polyacrylamide gels (not shown). Crossreacting polypeptides of the same size but lower abundance were detected in bovine liver and spleen and in rabbit reticulocytes (not shown). Sequence analysis confirmed that the crossreacting polypeptide in bovine testis is the equivalent of mouse TCP-1 (see

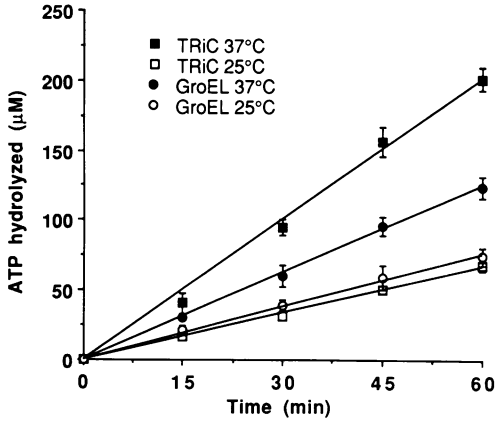

Fig. 2. ATPase activities of TRiC and GroEL. ATPase activities were determined colorimetrically using the malachite green reagent (Lanzetta *et al.*, 1978; Martin *et al.*, 1991) at 25 and 37°C as indicated. Assays contained 0.24 μM purified TRiC or GroEL (with respect to the double ring complex) and $MgCl_2$/ATP (5 mM/2 mM). Mean ± SD for three independent determinations is shown.

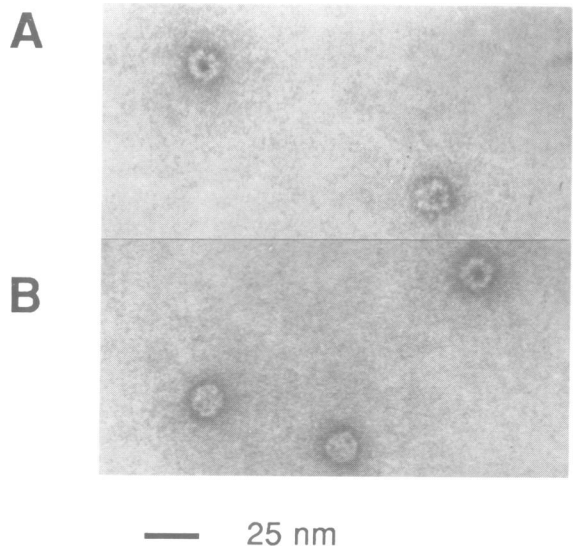

Fig. 3. Scanning transmission electron microscopy of TRiC. Samples of purified TRiC lacking ATP (**A**) or containing ATP/$MgCl_2$ (2 mM/5 mM) (**B**) were prepared for scanning transmission electron microscopy by negative staining (Wall and Hainfeld, 1986; Trent *et al.*, 1991). The right particle in (A) may represent a partial side view of the complex. M_r measurements were performed on unstained, freeze-dried specimens (Wall and Hainfeld, 1986). Particles for M_r analysis were selected on the basis of their separation from their neighbors, clean background, sharp edges and lack of obvious defects.

Figure 4). Consistent with a cytosolic, non membrane-associated localization, >90% of the protein was in a 100 000 *g* supernatant of a total homogenate. Purification was achieved by Mono-Q ion exchange chromatography followed by sucrose density gradient centrifugation (Figure 1B) and then chromatography on ATP−agarose. The polypeptide crossreacting with TCP-1 was in a high molecular weight fraction of ~20S that consistently copurified with at least five other polypeptides in the size range of 52−65 kDa.

We addressed the possibility that the copurifying proteins were contaminants of a homo-oligomeric complex consisting exclusively of TCP-1. The fraction containing TCP-1 was analyzed by native PAGE at pH 7.0 and pH 8.8. In both cases, TCP-1 was found to migrate as a distinct band more slowly than GroEL (Figure 1C) but with a mobility similar to that of its archaebacterial homolog, TF55 (not shown; see Trent *et al.*, 1991). When the bands containing TCP-1 were excised from the native gels and subsequently separated by reducing SDS−PAGE, TCP-1 was found to be accompanied by the same pattern of polypeptides detected in the high molecular weight fraction of the sucrose gradient. Furthermore, the band crossreacting with TCP-1 and the additional polypeptides bound to ATP−agarose columns and co-eluted at a concentration of 6−8 mM ATP in the presence of Mg^{2+} (not shown; see Figure 1A). Indeed, the purified TCP-1 preparation had ATPase activity comparable to that of GroEL (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990; Martin *et al.*, 1991) (Figure 2). ATP hydrolysis showed a pronounced temperature-dependence and was only observed in the presence of Mg^{2+}. The TCP-1 complex hydrolyzed ATP, GTP, CTP and UTP at similar rates (not shown).

Examination of the purified TCP-1 preparation by negative stain electron microscopy revealed ring-shaped complexes with a dark central core, sometimes partly filled, reminiscent of the previously described end-on views of TF55 particles (Figure 3) (Trent *et al.*, 1991). The number of subunits in the ring, probably eight or nine, could not be exactly determined. In contrast to TF55, the TCP-1 complex appeared to be composed of globular domains which were variable in size. With preparations lacking ATP these presumably end-on views were predominant (Figure 3A). However, in the presence of ATP, some of the complexes appeared as 2-fold symmetrical layered structures with four striations which are very similar to the side views of the TF55 and chaperonin double rings (Figure 3B). The dimensions of the TCP-1 complex were ~16×19 nm, somewhat larger than those of *E.coli* GroEL, which measures 14×16 nm (Hendrix, 1979; Hohn *et al.*, 1979; Langer *et al.*, 1992b). Analysis of unstained freeze-dried TCP-1 complex in the scanning transmission electron microscope (Wall and Hainfeld, 1986) indicated a defined M_r of 970 000 ± 54 000 (mean ± SD, n = 220).

Taken together, these results indicate that TCP-1 is part of a large, presumably double ring complex which has ATPase activity. In contrast to TF55 and the chaperonins, the TCP-1 complex is hetero-oligomeric. Although it seems likely that there is a single type of complex containing the different polypeptides at a defined stoichiometry, the existence of several closely related oligomeric species with varying subunit composition cannot be excluded. Henceforth we will use the abbreviation TRiC (for *T*CP-1 *ri*ng *c*omplex) to specify the TCP-1-containing hetero-oligomer.

TRiC is composed of structurally related subunits

At least six polypeptides of M_r 52 800−65 300 were reproducibly found in the purified high molecular weight complex (Figure 1A). On SDS−polyacrylamide gels with higher resolution, the band containing TCP-1 could be separated into two closely migrating polypeptides (P4 a and b; see also Figure 1B) of which P4a crossreacted with the mouse TCP-1 antibody 91A (not shown). As judged by Coomassie staining, P4a was about twice as abundant as each of the other subunits, which appeared to be present in close to 1:1 stoichiometry. To investigate the possibility that the

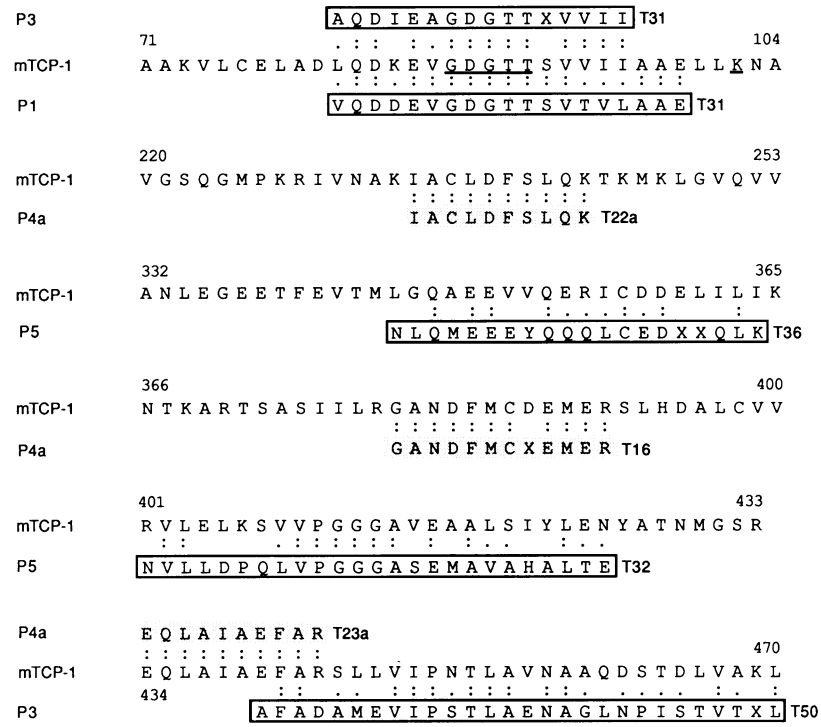

Fig. 4. Homology of the 52−65 kDa protein cluster of TRiC with mouse TCP-1. Sequences of tryptic peptides derived from P1, P3 and P5 (55.9, 57.6 and 65.3 kDa, respectively) (see Figure 1A) are boxed and are shown above or below the homologous stretch of amino acid sequences of mouse TCP-1b (GenBank M12899). Sequences of P4a (bovine TCP-1; 59.9 kDa) are shaded. ':' indicates identical amino acids, '.' indicates exchanges with a score of ≥ 0 in the PAM250 matrix which is based on the analysis of amino acid replacements among related proteins (Dayhoff *et al.*, 1978). 'x' denotes position in peptide sequence for which no assignment was made for that particular degradation cycle. Peptides are numbered according to their retention time in minutes on HPLC (see Materials and methods). A potential nucleotide binding motif of the type found in cAMP-dependent protein kinases (Bairoch and Claverie, 1988) is underlined. The initial yields for peptides P1/T31, P3/T31, P3/T50, P4a/T16, P4a/T22a, P4a/T23a, P5/T32 and P5/T36 were 4.4, 5.0, 4.9, 6.4, 0.8, 0.7, 4.5 and 1.5 pmol, respectively. All peptide sequences were searched against the complete translated GenBank (release 71) using the program 'FASTA' (Pearson and Lipman, 1988). The program 'RDF2' (Pearson and Lipman, 1988) was used to evaluate the statistical significance of the homologies found. The respective regions of the mouse TCP-1b sequence were shuffled 200 times (ktup 1, uniform shuffling). None of the shuffled sequences yielded a higher optimized score than that of the unshuffled sequence, indicating that the homologies found are statistically significant (Pearson, 1990). For each peptide the z-value [(score of test sequence − mean)/SD] was analyzed as another measure of statistical significance of homology (Pearson, 1990). Z-values ≤ 3 indicate the absence of statistically significant sequence relationship. For the perfectly matching peptides P4aT22a and P4aT23a, z-values were ~9 and 8, respectively. Z-values of peptides P1/T31, P3/T31, P3/T50, P4a/T16, P5/T32 and P5/T36 were ~9, 7, 8, 9, 5 and 4, respectively.

subunits of the complex were structurally related to TCP-1, peptides derived from the different proteins were analyzed. Purified TRiC was separated by SDS−PAGE and transferred to nitrocellulose. The amino-termini of several of the proteins were blocked, preventing amino-terminal sequencing. Therefore, the stained protein bands of P1, P3, P4a and P5, excised from the nitrocellulose blot, were subjected to tryptic digestion. Three HPLC purified peptides of 10−12 residues derived from P4a were sequenced (Figure 4). They were essentially identical to sequences in mouse TCP-1, consistent with the >96% identity shared between the TCP-1 sequences of several mammalian species (Willison *et al.*, 1986; Ahmad and Gupta, 1990; Kirchhoff and Willison, 1990; Kubota *et al.*, 1991). Since P4a has exactly the same apparent molecular weight as mouse TCP-1 and since it crossreacts with the monoclonal anti-TCP-1 antibody (Figure 1), this confirmed the identification of P4a as the bovine TCP-1 homolog. Several peptides (16−28 residues) derived from P1, P3 and P5 were also found to be highly homologous to mouse TCP-1 (Figure 4), as well as to the TCP-1 sequences of other species (not shown). The levels of sequence identity with mouse TCP-1 were in the range 40−74%. A 20 residue peptide of P1 (P1/T31), which was 74% identical to mouse TCP-1, was also related to a 16 residue peptide of P3 (P3/T31). The corresponding region of TCP-1 (residues 81−99) is also homologous to the Hsp60s (Gupta, 1990). It contains the motif GDGTT, which is completely conserved in TCP-1, P1/T31, P3/T31 and several Hsp60 homologs. In TCP-1 and human Hsp60 (Jindal *et al.*, 1989) this is followed by a lysine residue 11 and 16 amino acids downstream, respectively. Overall, this region resembles a nucleotide binding motif of cAMP-dependent protein kinases (Bairoch and Claverie, 1988). In summary, the TRiC subunits P1, P3 and P5 have a significant structural relationship to TCP-1. Apparently, these proteins represent the members of a new protein family.

TRiC binds different polypeptides in non-native conformations

The structural and functional properties of TRiC established above were consistent with its potential role as a molecular chaperone. We therefore tested whether TRiC was able to bind unfolded polypeptides upon dilution from 6 M guanidinium−Cl (GdmCl). Such complex formation was

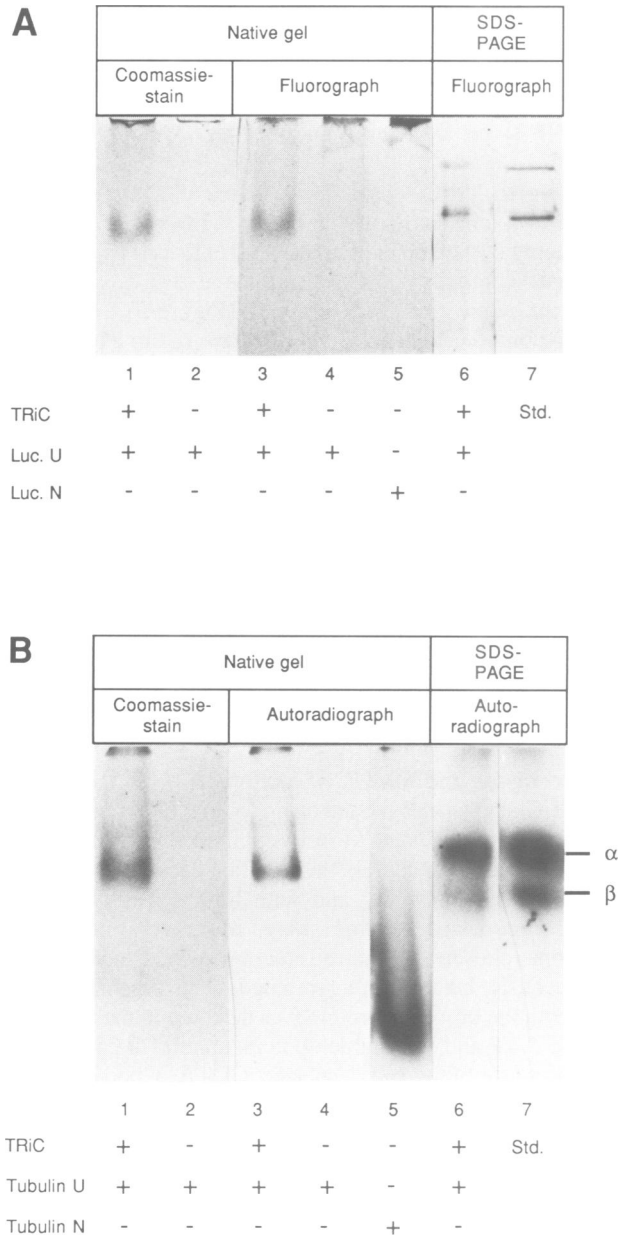

Fig. 5. Binding of denatured polypeptides to TRiC analyzed by native PAGE. **A**, Firefly luciferase was synthesized in an *E.coli* S30 extract in the presence of [^{35}S]methionine (New England Nuclear) by linked transcription/translation using the plasmid pBESTLUC (Promega) according to the instructions of the supplier. Luciferase was denatured by 4-fold dilution of S30 in buffer B (see Materials and methods) and incubation for 2 h at 25°C. **B**, Tubulin (α/β dimer) was purified from bovine brain by phosphocellulose chromatography (Karr *et al.*, 1979; kindly provided by D.Purich) and was ^{125}I-labeled as described in Materials and methods. Tubulin was denatured at 73 μM in buffer B as above. Denatured proteins (luc. U and tubulin U) were diluted 100-fold into 50 μl of buffer C at 25°C in the presence or absence of 0.2 μM purified TRiC. Protein aggregates were removed by centrifugation (see Materials and methods). In one reaction, luciferase or tubulin were added without prior denaturation (luc. N and tubulin N). Binding reactions were separated by 4–10% native PAGE at pH 7.0. The Coomassie blue stained gels were analyzed by fluorography/autoradiography and the TRiC-containing bands were re-analyzed by SDS–PAGE as above; native luciferase and tubulin were run as standards (Std.). Tubulin α and β subunits were separated on SDS–polyacrylamide gels containing urea (Lee *et al.*, 1973).

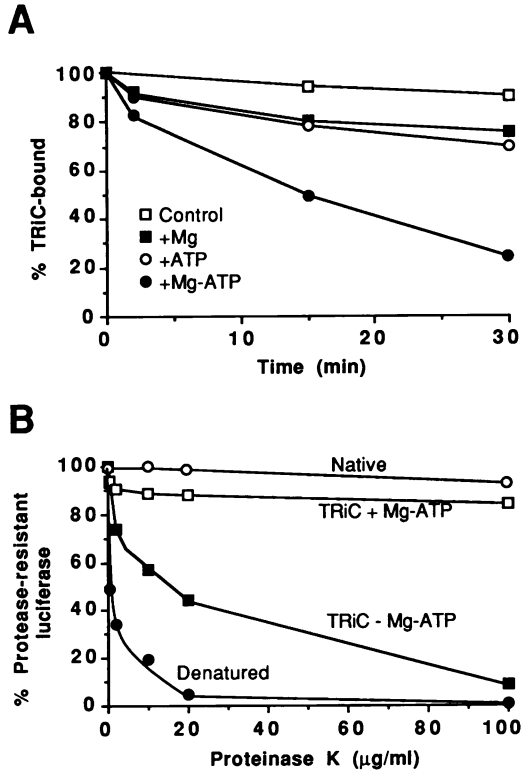

Fig. 6. ATP-dependent release from TRiC and acquisition of protease resistance of luciferase. **A**, Release of TRiC-bound luciferase upon incubation at 30°C with Mg^{2+} (■), ATP (○), Mg-ATP (●) or without further addition (□). Purified firefly luciferase was unfolded in buffer B at 20 μM (see Figure 5) and diluted 100-fold into buffer C containing 0.2 μM TRiC. Luciferase aggregates were sedimented for 15 min at 25 000 *g* at 25°C. Aliquots of the supernatant were incubated at 30°C. Where indicated, 5 mM $MgCl_2$ and 2 mM ATP were present. Reactions were stopped by cooling on ice and were separated by native PAGE (pH 7.0) at 4°C. The band that contained TRiC was excised from the native gel and re-analyzed by SDS–PAGE. Luciferase was detected by Western blotting using anti-luciferase antiserum and the luminescence based ECL system. Amounts of protein were quantified by densitometry of exposed films and are expressed as a percentage of total TRiC-bound luciferase. **B**, Intrinsic protease resistance of TRiC-bound luciferase upon incubation with (□) or without Mg-ATP (■), and protease-resistance of native (○) and denatured luciferase (●) in the absence of TRiC. TRiC-bound luciferase prepared as in (A) was incubated for 40 min at 30°C with or without Mg-ATP. Each reaction was divided into six parts and incubated for 10 min at 0°C with 0–100 μg/ml proteinase K. In the absence of TRiC, protein concentrations were adjusted by adding BSA. The protease action was stopped by adding 2 mM PMSF and TCA precipitates were analyzed by SDS–PAGE. GdmCl-denatured luciferase diluted into buffer lacking TRiC and native luciferase were analyzed as controls. Luciferase was analyzed as in (A). Amounts of protein are expressed as a percentage of the total luciferase in the reaction.

previously observed with the archaebacterial TF55 (Trent *et al.*, 1991) and with the chaperonins (see Lorimer, 1992 for review). Co-electrophoresis of bound polypeptides with the chaperone protein on native PAGE was employed as an established criterion for physical interaction (Musgrove *et al.*, 1987; Trent *et al.*, 1991). Like GroEL, TRiC indeed bound to several different denatured proteins including luciferase of the firefly *Photinus pyralis* (62 kDa), the α and β subunits of bovine brain tubulin (55 kDa) (Figure 5) and the fusion protein Su9-DHFR (26 kDa) (not shown), in

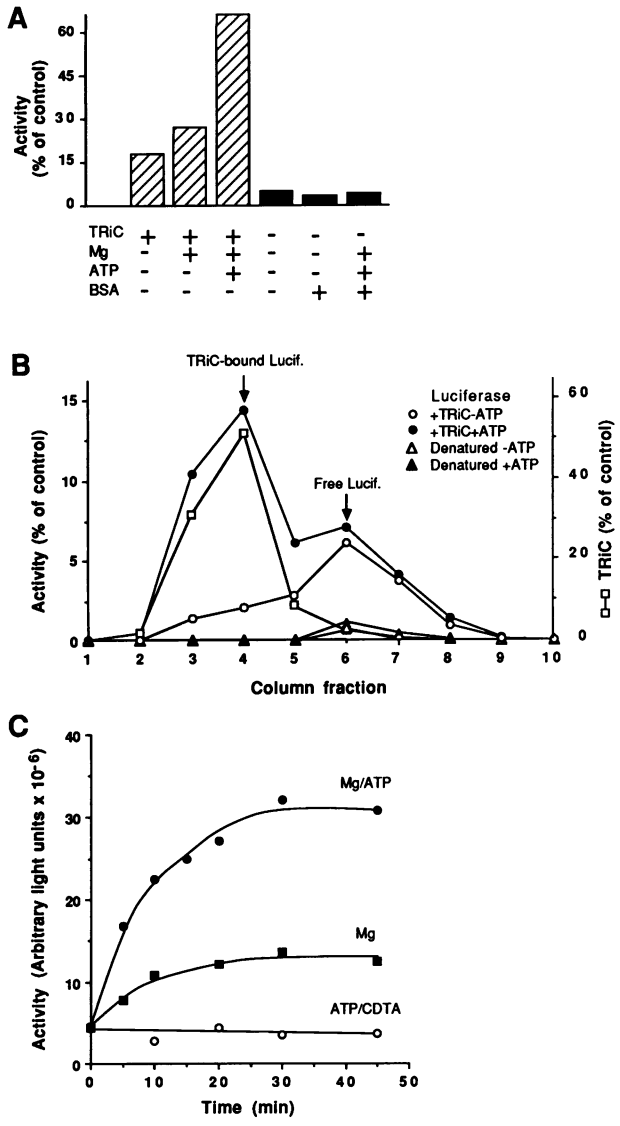

Fig. 7. TRiC-mediated reactivation of GdmCl-denatured luciferase. **A**, Denatured luciferase (20 μM) was diluted into buffer C with or without 1 μM TRiC as in Figure 6, except that addition of CDTA and sedimentation of aggregates were omitted. Reactions were incubated for 40 min at 30°C after addition of $MgCl_2$ (5 mM), ATP (2 mM) or bovine serum albumin (BSA; 3 μM) as indicated. 10 μl aliquots were diluted 5-fold into luciferase assay mix and activities were determined as described in Materials and methods. Luciferase activities are expressed as a percentage of the native enzyme control. **B**, Size exclusion chromatography of TRiC-bound luciferase. Denatured luciferase was diuted as in (A) into buffer containing TRiC or into buffer lacking chaperone. After 10 min at 25°C, the reactions were cooled to 4°C and separated on a 0.4 cm×18 cm Sephacryl S300 column (Pharmacia) equilibrated with buffer C. The void volume was discarded and 150 μl fractions were collected. Each fraction was incubated for 30 min at 30°C with or without Mg-ATP. Luciferase activities were determined as in (A). Native luciferase (applied to the column at 0.2 μM) co-fractionated with a BSA marker (indicated by an arrow). Aliquots of column fractions were analyzed by SDS−PAGE and the TRiC was quantified by densitometry of the Coomassie stained gel. **C**, Time course of TRiC-mediated reactivation. Fractions 3 and 4 of the S300 column in (B) were pooled, divided into three reactions and incubated at 30°C. When indicated, 2.5 mM of the Mg^{2+} chelator CDTA was added. CDTA carried over into the luciferase assay (see Materials and methods) had no inhibitory effect. Enzyme activities were measured after the times indicated and are given in arbitrary light units corresponding to recorded c.p.m.

which a mitochondrial precursor targeting sequence is fused to mouse dihydrofolate reductase (Pfanner *et al.*, 1987). These proteins were synthesized in cell-free translation systems in the presence of [^{35}S]methionine (luciferase, Su9-DHFR) or were used in purified form (luciferase, tubulin).

Binding to TRiC was only detected with the GdmCl-unfolded proteins and not with native luciferase or tubulin. The identity of the bound proteins was confirmed by excising the bands containing TRiC from the native gel and re-separating them by SDS−PAGE (Figure 5). Coupled transcription/translation in *E.coli* S30 gave rise to a truncated form of luciferase of 48 kDa which also bound to TRiC when unfolded (Figure 5A). In the absence of the chaperone, both full-length and truncated proteins aggregated upon dilution from denaturant. The native luciferase produced by cell-free synthesis was not detectable on the non-denaturing gels. Both α and β subunits of tubulin bound to TRiC on dilution from GdmCl (Figure 5B), whereas aggregate formation was observed when the unfolded proteins were diluted into buffer lacking the chaperone (not shown). The native α/β tubulin dimer migrated to a distinct position on the non-denaturing gel. Apparently, TRiC has the potential to bind to non-native proteins, thereby preventing aggregation, an essential feature of the functional definition 'molecular chaperone'.

TRiC mediates the folding of luciferase

Was TRiC able to mediate the chain folding of a monomeric protein to the native state? Firefly luciferase was chosen as a substrate protein to address this question because its activity can be measured rapidly and with high sensitivity by a luminescence-based enzyme assay. Moreover, under the conditions chosen, denatured luciferase did not refold spontaneously but rather aggregated. The association of TRiC with the unfolded protein was detected in the absence of added ATP and was stable upon incubation at 25−30°C in the presence of the Mg^{2+} chelator CDTA. Upon addition of Mg-ATP, luciferase was released from TRiC as detected by native PAGE (Figure 6A). After 30 min of incubation, only ~20% of the initially bound protein remained associated with the chaperone. Both ATP and Mg^{2+} were required for this reaction and the non-hydrolyzable ATP analog AMP-PNP did not support release (not shown).

ATP-dependent release was accompanied by the folding of luciferase into a protease-resistant conformation characteristic of the native protein (Figure 6B). In contrast, when chaperone-bound luciferase was incubated in the absence of Mg-ATP, the protein remained protease-sensitive, indicating that it was in a non-native conformation. Significant degradation of TRiC itself was observed at concentrations of proteinase K of >20 μg/ml. TRiC-associated luciferase (in the absence of Mg-ATP) was more resistant towards digestion than the denatured protein diluted into buffer solution lacking chaperone. This suggests that in the absence of ATP hydrolysis, the TRiC-bound protein may be stabilized in a partially folded conformation or that it may be shielded to some degree by the chaperone.

Consistent with these observations, spontaneous reactivation of unfolded luciferase upon dilution into refolding buffer reached only 2−5% of the native enzyme control after 40 min at 20−30°C (Figure 7A). The presence of bovine serum albumin did not improve the yield of spontaneous renaturation. Strikingly, refolding to ~60% of

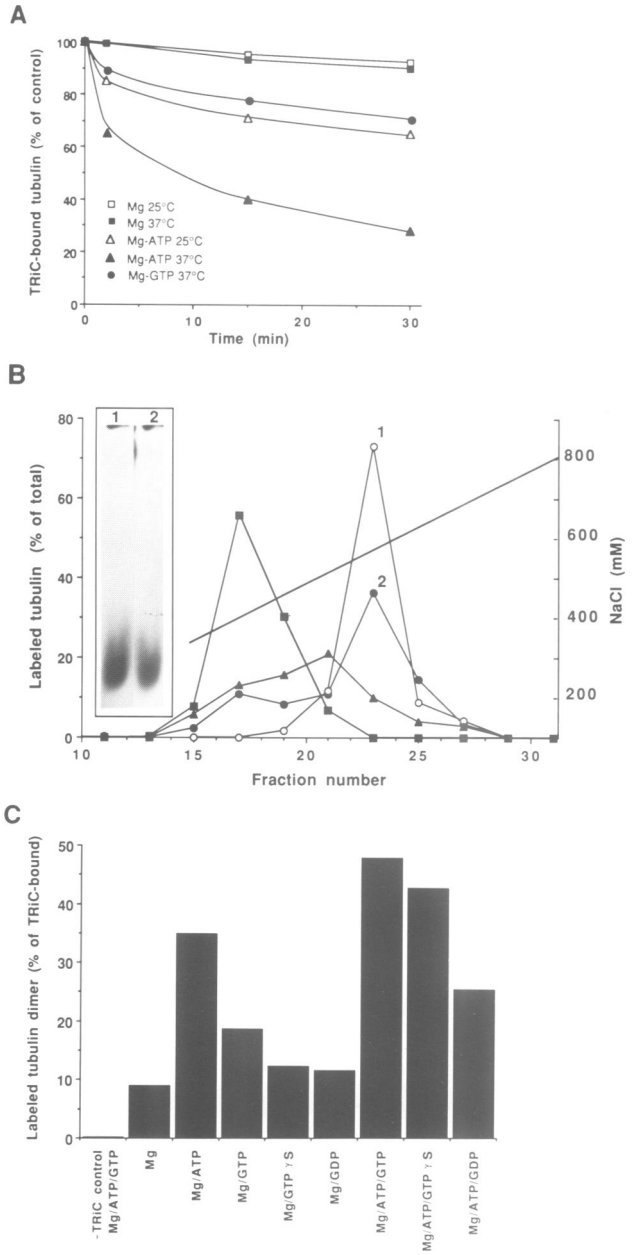

Fig. 8. TRiC-dependent assembly of α/β tubulin dimers. **A**, Release of TRiC-bound tubulin upon incubation at 25°C (□, △) or 37°C (■, ▲, ●) in the presence of Mg (□, ■), Mg-ATP(△, ▲) or Mg/GTP (●). ^{125}I-labeled tubulin was denatured in 6 M GdmCl and bound to 0.2 μM TRiC in buffer C as described in Figure 5. After centrifugation for 15 min at 25 000 g to remove aggregates, reactions were incubated at 25°C or 37°C in the presence or absence of 5 mM $MgCl_2$, 2 mM ATP or 2 mM GTP when indicated. Aliquots were removed after the times indicated, cooled on ice and were then analyzed by native PAGE (pH 7.0) at 4°C followed by autoradiography. Amounts of TRiC-bound tubulin were determined using a Phosphorimager (Molecular Dynamics) and are given as a percentage of the total TRiC-bound tubulin. **B**, Formation of tubulin α/β dimers by subunit exchange with native dimers. Analysis by ion exchange chromatography and native PAGE (insert). GdmCl-unfolded ^{125}I-labeled tubulin was diluted 100-fold into buffer C (-CDTA) containing 0.2 μM TRiC (■, ▲, ●) or 3 μM BSA (-TRiC control). Aggregates were sedimented from the TRiC-containing reaction and the supernatant was divided into three parts. Unlabeled native tubulin (0.2 mg/ml, final concentration) was added (■, -TRiC control) and all reactions were brought to 30% glycerol/buffer D. 1 mM ATP and GTP were added (▲, ●, -TRiC control). All samples were incubated for 30 min at 37°C and loaded onto a Mono-Q HR5/5 column (Pharmacia) in buffer D. ^{125}I-labeled native tubulin dimer was run as standard (○). Bound protein was eluted with a 20 ml gradient of 0–1 M NaCl in buffer D/5% glycerol. TCA-precipitates of column fractions (0.5 ml) were analyzed by SDS–PAGE and autoradiography. Amounts of tubulin were quantified as above and are expressed as a percentage of the total labeled tubulin analyzed on the column. Denatured tubulin (-TRiC control) was only recovered by a column wash with 8 M urea (not shown). **Insert** shows analysis by native PAGE (pH 7.0) of equal volumes of fraction 23 of dimer standard (Std., #1) and of dimer formed in the TRiC-dependent reaction (#2). **C**, Effects of various nucleotides at 1 mM concentration in the TRiC-dependent formation of labeled tubulin dimer were tested as in (B) in the presence of 0.2 mg/ml unlabeled native tubulin. Reactions were analyzed by native PAGE and the newly formed dimer was quantified as in (A). Amounts of dimer formed are given as a percentage of TRiC-bound tubulin.

the control activity was achieved when the denatured protein was diluted into a solution containing a 5-fold molar excess of TRiC (based on a M_r of 970 000) in the presence of Mg^{2+} and ATP. Without adding ATP (but in the absence of CDTA), 15–20% of the control activity was produced. This reactivation was enhanced in the presence of Mg^{2+}, presumably allowing the efficient hydrolysis of ATP which is tightly bound to TRiC.

That active luciferase was indeed produced from the TRiC–luciferase complex was confirmed by the following experiment. Denatured luciferase was diluted into a solution containing TRiC in the absence of ATP and, after 10 min at 30°C, the chaperone-bound protein was isolated by sizing chromatography (Figure 7B). Each fraction that eluted from an S300 gel filtration column was divided into two parts which were incubated for 20 min at 30°C in the presence or absence of Mg-ATP. ATP-dependent reactivation of luciferase was only observed in the fractions containing TRiC. The low activities detected in these fractions upon incubation in the absence of ATP were due to the presence of Mg-ATP in the luciferase enzyme assay. About 15% of the total active luciferase had apparently been released from the chaperone during the incubation prior to column fractionation. It behaved like the monomer, as would be expected at the low enzyme concentrations used (at higher concentrations, firefly luciferase is a dimer; T.Baldwin, personal communication). This reactivation was not observed when CDTA was present during the binding step (not shown). We noted, however, that the presence of Mg^{2+} during binding increased the efficiency of luciferase reactivation, perhaps by stabilizing TRiC in a functional form. As a control reaction, denatured luciferase was diluted into buffer solution lacking chaperone. Very little luciferase activity was recovered when this material was analyzed on the S300 column. Next, we incubated the luciferase–TRiC complex of the pooled column fractions 3 and 4 to determine the kinetics of TRiC-dependent refolding (Figure 7C). In the presence of Mg-ATP, reactivation occurred with a half-time of ~10 min. Less efficient but significant production of enzyme activity was again noted upon incubation with Mg^{2+} alone, consistent with the presence of TRiC-bound ATP. Addition of CDTA prevented this reactivation, except for a low level of activity which was generated during the brief incubation in the assay mix containing Mg-ATP.

These data indicate that TRiC mediates the renaturation of an unfolded protein, firefly luciferase, under conditions that do not allow significant spontaneous refolding. This

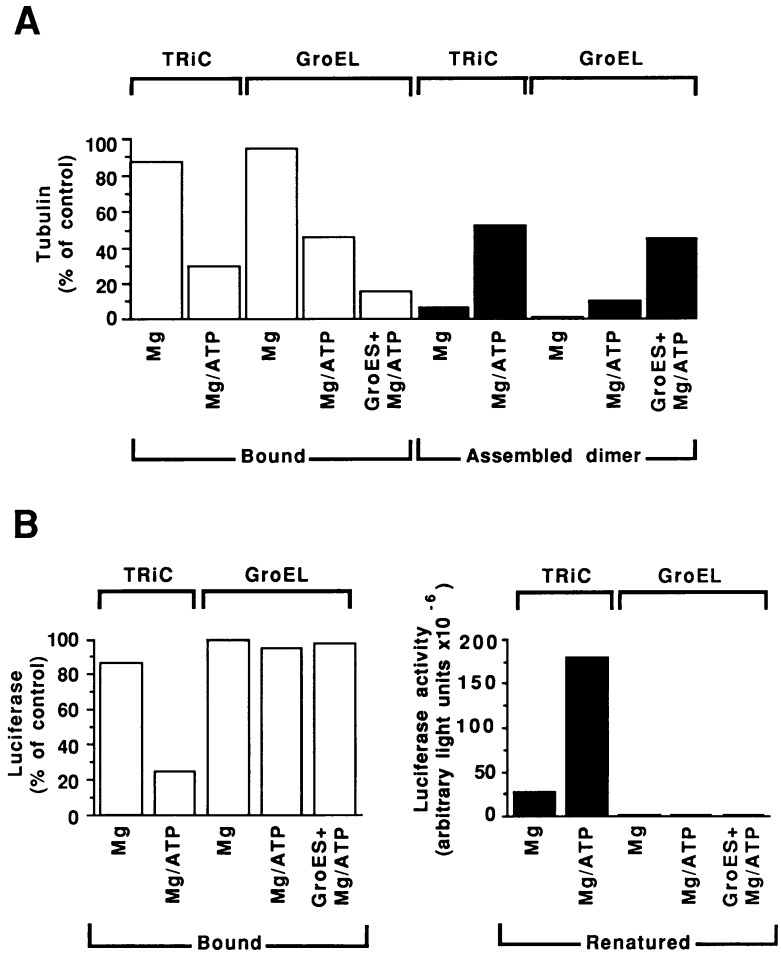

Fig. 9. Comparison of protein folding properties of TRiC and the bacterial chaperonins GroEL/ES. **A**, Tubulin release from TRiC or GroEL and formation of tubulin dimers. ^{125}I-labeled tubulin–chaperone complexes were formed and analyzed by native PAGE as in Figure 8. TRiC and GroEL were used at 0.2 μM concentrations with respect to the double ring complex. When indicated, 0.4 μM GroES heptamer was present. Reactions were incubated with Mg^{2+} or with Mg-ATP and GTP for 30 min at 37°C. Amounts of ^{125}I-labeled tubulin bound to chaperone or assembled to dimer were quantified on a Phosphorimager and expressed as a percentage of total tubulin bound to chaperone prior to incubation. GroEL bound the unfolded tubulin ~50% more efficiently than TRiC. **B**. Unfolded luciferase (20 μM) was diluted into reactions containing 0.1 μM TRiC or GroEL (see Figure 6). When indicated, 0.2 μM GroES and Mg/ATP (5 mM/2 mM) were added. Reactions were incubated for 30 min at 30°C. One-fifth of each reaction (10 μl) was assayed for luciferase activity and the remainder was analyzed by native PAGE at pH 7.0. Amounts of chaperone-bound luciferase were analyzed as in Figure 6 and are expressed as a percentage of the total protein bound to chaperone prior to incubation. TRiC and GroEL bound to unfolded luciferase with similar efficiencies.

reaction requires only the purified chaperone protein and Mg-ATP.

TRiC functions in tubulin assembly

Two lines of observation suggested a role of TCP-1 in chaperoning microtubule assembly. Firstly, TCP-1 is highly expressed in developing sperm cells, which synthesize microtubules to form the flagellum (Willison *et al.*, 1990). Secondly, a cold-sensitive Tcp-1 mutant of yeast shows a defect in mitotic spindle formation and an increased sensitivity to certain anti-microtubule drugs (Ursic and Culbertson, 1991).

Can purified TRiC mediate the folding of bovine brain tubulin into an assembly-competent form? Microtubule assembly *in vitro* has been reconstituted starting with the assembled tubulin heterodimer which consists of the two similar ~55 kDa α and β subunits (Purich and Kristofferson, 1984). Assembly from the fully unfolded monomers has not yet been achieved (Yaffe *et al.*, 1988). To produce assembly-competent, radiolabeled tubulin, purified α/β tubulin dimer was first assembled into microtubules at 37°C in the presence of glycerol and GTP (Na and Timasheff, 1982; Mitchison and Kirschner, 1985). The microtubules were then labeled with ^{125}I-labeled Bolton–Hunter reagent and were subjected to Ca^{2+}-dependent disassembly at 4°C. The resulting tubulin dimer migrated to a distinct position on native gels (Figure 5B) and was able to re-assemble into microtubules with 35–45% efficiency (not shown). Both subunits were iodinated but the specific activity of the α subunit was ~6-fold higher than that of the β subunit (Figure 5B, lane 7). Labeled tubulin dimer was unfolded by incubation in 6 M GdmCl. As shown in Figure 5B, both denatured α and β tubulin subunits bound to the chaperone with similar efficiencies (lane 6). Upon incubation with Mg-ATP, the proteins were released as judged by the disappearance of radiolabeled tubulin co-fractionating with TRiC on native PAGE (Figure 8A). Release was more efficient at 37°C than at 25°C and Mg-GTP had little effect compared with Mg-ATP. In contrast to the native dimer, the form of tubulin produced under these conditions was not detectable on the native gel. Subsequent experiments

suggested that the tubulin subunits were released from TRiC as structurally unstable monomers.

It was assumed that the efficient formation of stable tubulin dimer occurs only above a certain concentration of monomers. The dissociation constant of heterodimeric tubulin is relatively high, ~1 μM (Detrich and Williams, 1978). This is considerably higher than the concentrations of TRiC-bound tubulin (~0.2 μM) that were reached in our experiments, due to the strong tendency of tubulin to aggregate upon dilution from denaturant. We analyzed therefore whether the chaperone-associated tubulin could assemble by subunit exchange with native, unlabeled tubulin dimers, which did not themselves interact appreciably with TRiC. This was indeed the case: assembled tubulin formed with 45–70% efficiency. It co-eluted on a Mono-Q column with a standard of native dimer at a characteristic position of 570 mM NaCl (peaks 1 and 2) (Yaffe *et al.*, 1988) (Figure 8B). Both labeled α and β tubulin were found in the pool of assembled dimer (not shown). TRiC-bound tubulin eluted at a lower salt concentration (410 mM). Mono-Q chromatography also allowed us to visualize the form of tubulin that was released from TRiC in the absence of unlabeled tubulin. It fractionated in a broader distribution and with lower recovery between 420 and 550 mM salt. This material most probably represented structurally labile monomers. Tubulin monomers synthesized in a cell-free extract have very similar fractionation properties (Yaffe *et al.*, 1988). Aggregated tubulin produced by dilution from denaturant in the absence of chaperone was not recovered from the column as a distinct peak (not shown).

The nucleotide requirement of TRIC-mediated tubulin assembly was analyzed in more detail. Quantification of the radioactivity cofractionating with a tubulin dimer standard on native PAGE (Figure 8B, insert) revealed that TRiC-dependent assembly was most efficient in the presence of Mg-ATP in combination with GTP or GTP-γS. Mg-GTP alone was less efficient and the non-hydrolyzable analog did not support assembly. GTP and GTP-γS bind to tubulin and are known to promote microtubule formation (Purich and Kristofferson, 1984). GTP binding may stabilize the tubulin monomers released from TRiC.

Dimer assembly was carried out at concentrations of total tubulin of 50–200 μg/ml. This was below the critical concentration required for efficient microtubule assembly under these experimental conditions (Na and Timasheff, 1982). However, radiolabeled dimer, formed from TRiC-bound subunits, co-assembled into microtubules when the concentration of unlabeled dimer was raised to 2 mg/ml. The efficiency of microtubule assembly of the labeled dimer was then the same as with native tubulin, as judged by carrying out cycles of GTP-dependent assembly and Ca^{2+}-dependent disassembly (not shown). We conclude, that TRiC releases tubulin as folded monomers which are competent to assemble in a concentration-dependent manner. This is consistent with a basic role of TRiC in protein folding.

Functional differences between TRiC and the chaperonins

The experiments described above suggest that TRiC has chaperonin-like properties in facilitating protein folding. However, apart from its hetero-oligomeric structure, another notable difference between TRiC and the chaperonins is its apparent independence of the addition of a small co-chaperonin, such as GroES. To establish this distinction further, we compared the functional interaction of TRiC and of GroEL/ES with tubulin. Unfolded tubulin bound efficiently to GroEL upon dilution from denaturant (Figure 9A). Incubation of the tubulin–GroEL complex with Mg-ATP resulted in the release of a significant amount of the bound tubulin as judged by analysis on native PAGE. Interestingly, only a small percentage of the protein assembled to the tubulin dimer, even in the presence of unlabeled native tubulin. Instead, nonspecific, sedimentable aggregates were formed, which, unlike assembled microtubules, could not be resolved in a Ca^{2+}-dependent manner (not shown). In the presence of GroES, however, GroEL-bound tubulin was released in a conformation that had much less tendency to aggregate. Tubulin dimer was then produced in an ATP- and GTP-dependent reaction as observed with TRiC (Figure 9A). This underscores the known requirement of GroES for GroEL-mediated protein folding (Goloubinoff *et al.*, 1989; Buchner *et al.*, 1991; Martin *et al.*, 1991; Mendoza *et al.*, 1991).

We also tested the capacity of GroEL to assist in the refolding of firefly luciferase. Surprisingly, GroEL/ES-mediated folding of this protein could not be demonstrated. Unfolded luciferase bound to GroEL with normal efficiency but apparently was not released from the chaperonin, whether or not GroES was present (Figure 9B). These functional differences between TRiC and the chaperonins further support the notion that TRiC is a member of a new class of chaperonin-like folding proteins.

Discussion

Recent evidence suggests that TCP-1 has both a structural and a functional relationship with members of the GroEL/Hsp60 family (chaperonins), as well as with the archaebacterial stress protein TF55 (Ellis, 1990; Gupta, 1990; Trent *et al.*, 1991; Gao *et al.*, 1992; Lewis *et al.*, 1992; Yaffe *et al.*, 1992). We have shown here for the bovine protein that TCP-1 is a subunit of a high molecular weight ring complex, TRiC, which can be described as a chaperonin-like component but with distinct structural and functional properties. Like the chaperonins, TRiC binds unfolded polypeptides, thereby preventing their aggregation, and mediates their folding to the native state in an ATP-dependent process. In contrast to the chaperonins, TRiC is a hetero-oligomer containing structurally related subunits and can function independently of a small, dissociable co-factor such as GroES.

The chaperonins of eubacteria and mitochondria are homo-oligomeric complexes containing 14 subunits of ~60 kDa in stacked heptameric rings (Hendrix, 1979; Hohn *et al.*, 1979; Pushkin *et al.*, 1982). Archaebacterial TF55 is also a homo-oligomer, but of 8- or 9-membered rings (Phipps *et al.*, 1991; Trent *et al.*, 1991). In contrast, TRiC consists of at least six distinct subunits of 52–65 kDa, one of which is the bovine equivalent of TCP-1. The existence of different subunits in a chaperonin is not completely unprecedented, however: The chloroplast Rubisco binding protein contains two types of subunits which are 50% identical (Hemmingsen and Ellis, 1986; Martels *et al.*, 1990). Clearly, the subunit composition of TRiC is far more complex. Peptide analysis revealed that at least three of the proteins are structurally related but not identical to TCP-1. This homology is not

confined to a distinct region of the TCP-1 sequence. The level of overall sequence identity may be in the range 40–60% but complete sequence information will be required to establish this definitively. Nevertheless, the different subunits of TRiC are in all likelihood encoded by a new multigene family. This is consistent with the identification of several TCP-1 related genes in humans and mice (K.Willison, personal communication). In principle, the proteins in the TRiC preparation could each form homo-oligomeric double toroid complexes with similar properties. The following lines of evidence argue against this, however: Under several different chromatography conditions we saw no change in the stoichiometry of the TRiC subunits. More importantly, all six polypeptides co-electrophoresed as a single band on native polyacrylamide gels at different pH values. Negative-stain electron microscopy of TRiC suggested the presence of globular subunits of different shapes and sizes, and analysis by scanning transmission electron microscopy indicated a distinct molecular weight of the complex. However, we do not exclude the existence of different species of TRiC with variable subunit composition, differing perhaps in certain functional aspects. Polypeptide patterns similar to that described here for bovine TRiC have recently been observed with a preparation of TCP-1 from mouse testis (Lewis *et al.*, 1992) and from rabbit reticulocytes (Yaffe *et al.*, 1992).

Are TRiC and the chaperonins functionally equivalent? The mechanism of substrate protein binding by TRiC may be similar to that of the chaperonins (Lorimer, 1992), which appear to recognize conformational intermediates generated rapidly during folding (Martin *et al.*, 1991). As proposed for the chaperonins, the basic function of TRiC seems to be in the folding of monomeric proteins, such as luciferase, or the folding of subunits of oligomeric structures, such as α and β tubulin. As indicated by studies with GroEL (Langer *et al.*, 1992b), the central pore of the double ring may be of functional importance. However, the mechanism by which TRiC mediates protein folding appears to be distinct from that of the chaperonins in certain aspects. Chaperonin-dependent folding strictly requires the regulatory function of the co-chaperonin GroES, at least with proteins such as tubulin that do not refold efficiently *in vitro* (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990; Martin *et al.*, 1991). In the absence of GroES, GroEL releases these proteins upon ATP hydrolysis with reduced efficiency and in a conformation unproductive for further folding (Martin *et al.*, 1991; this study). This may be due to an 'uncoupling' of the chaperonin subunits so that they perform their function less efficiently. Although the existence of a component(s) regulating TRiC in a GroES-like manner is possible, the successful reconstitution of TRiC function with different substrate proteins argues against the strict requirement of an added co-chaperonin. Binding of GroES, a heptamer of 10 kDa subunits, to GroEL results in a distinct asymmetrical change of the chaperonin structure, which then appears as a 'capped' double ring on electron micrographs (Saibil *et al.*, 1991; Ishii *et al.*, 1992; Langer, *et al.*, 1992b). It seems possible that the TRiC in our preparation simply contains its release factor(s) in a stably bound form. As judged by the polypeptide composition of the complex and and by its morphology, such a component would then differ from GroES in its structure and function. Alternatively, the hetero-oligomeric subunit composition of TRiC results in functional properties that allow the productive release of a folding protein without regulation by an additional component. Presumably, its specific quarternary structure also enables TRiC to mediate the folding of proteins, such as luciferase, that do not interact productively with GroEL/ES.

Is TRiC of general importance for the folding and assembly of cytosolic proteins? There are not yet sufficient data from *in vivo* studies to answer this question. Our results indicate that TRiC is able to bind several different polypeptides in non-native conformations. Tubulin is one likely physiological substrate. As demonstrated here *in vitro*, TRiC is able to mediate the renaturation of unfolded α and β tubulin subunits into a form that is competent to assemble to the heterodimer. Assembly of tubulin by subunit exchange has previously been established with newly synthesized subunits made in a reticulocyte lysate (Yaffe *et al.*, 1988), and very recently TRiC has been demonstrated to mediate this reaction in the reticulocyte lysate system (Yaffe *et al.*, 1992). Taken together, it seems likely that TRiC has a basic function in microtubule assembly *in vivo* by mediating the folding of tubulin monomers. This reaction requires ATP hydrolysis and is most efficient in the presence of GTP. Each tubulin subunit is known to bind one molecule of GTP (Purich and Kristofferson, 1984), which may have to be introduced into the refolded monomers to stabilize them in an assembly-competent state.

During the preparation of this manuscript, a study by Gao *et al.* (1992) was published which reports the isolation of a chaperonin-like complex from rabbit reticulocyte lysate that mediates the folding of β-actin. The polypeptide pattern of the purified complex suggests that it is the rabbit equivalent of TRiC. Although evidence for the physiological requirement of TRiC in actin biogenesis is not yet available, it appears likely that at least two major cytoskeletal components, tubulin and actin, are natural substrate proteins of the new cytosolic chaperone.

Materials and methods

Purification of TRiC

Bovine testes (40 g) were homogenized in 4 vol of ice-cold buffer A (50 mM HEPES–KOH, pH 7.6, 2 mM DTT, 2 mM EDTA, 100 mM NaCl) containing 250 mM sucrose, 1 mM phenylmethane-sulfonylfluoride (PMSF), 0.5 mM 1,10-phenanthroline, 0.3 μM aprotinin, 1 μM leupeptin and 2 μM pepstatin A. The homogenate was centrifuged for 1 h at 100 000 *g*. The supernatant (2.66 g protein) was applied, in two batches, onto a Mono-Q HR16/10 column (Pharmacia) equilibrated in buffer A/10% glycerol. Bound proteins were eluted using a 350 ml 100–400 mM NaCl gradient. The presence of a polypeptide crossreacting with TCP-1 was determined by Western blotting with the monoclonal rat anti-mouse TCP-1 antibody 91A, directed against an epitope of mouse TCP-1 between residues 306 and 492 (Willison *et al.*, 1989; kindly provided by K.Willison). The pooled fractions of the major TCP-1 containing peak (150 mg protein) eluting at 285 mM NaCl, were concentrated by ultrafiltration (100 kDa filter, Millipore) and were separated on a 10–40% sucrose gradient in buffer A at 26 000 r.p.m. for 18 h at 4°C (Beckman SW28 rotor). Fractions 14–17 of the sucrose gradient (see Figure 1B) were pooled (18 mg protein) and sucrose was removed by three cycles of 10-fold dilution with buffer A/10% glycerol followed by ultrafiltration. The fraction containing TCP-1 was made 5 mM in $MgCl_2$ in buffer A and was applied to a 10 ml ATP–agarose column (C-8 linked, Sigma). After washing the column with buffer A/$MgCl_2$, bound protein was eluted with a gradient of 0–15 mM ATP in the same buffer. ATP was removed by a desalting step in buffer A/10% glycerol. Aliquots were stored at −80°C. The final yield of purified TRiC was 8–12 mg.

Peptide analysis

20 μg of purified TRiC were separated on several lanes of a 25 cm long 12.5% SDS–polyacrylamide gel, transferred to nitrocellulose and stained with Ponceau S; protein bands P1–5 were excised. *In situ* tryptic digestion was performed ('sequencing grade' trypsin, Boehringer Mannheim) and the resulting peptides were separated by narrow bore (2.1 mm) reversed phase HPLC after being S-alkylated with 4-vinylpyridine as described by Ghosh *et al.* (1990) and Tempst *et al.* (1990). Sequencing was carried out using an optimized Applied Biosystems 477A instrument (Tempst and Riviere, 1989).

Binding of denatured proteins to TRiC

Denaturation of native substrate proteins was achieved by 4-fold dilution of protein solutions in buffer B (8 M GdmCl/2 mM DTT, 30 mM Tris, pH 7.2) and incubation for 2 h at 25°C at the protein concentrations specified in the figure legends. Usually, denatured proteins were diluted 100-fold into 50 μl of buffer C (50 mM KCl, 30 mM HEPES pH 7.4, 2 mM DTT, 0.5 mM *trans*-1,2-cyclo-hexane-diaminetetraacetate [CDTA]) at 25°C in the presence of 0.2 μM purified TRiC. The final concentration of GdmCl was 60 mM. Unless indicated otherwise, insoluble aggregates were removed by centrifugation for 15 min at 25 000 *g* at 25°C and the supernatant was used for further analysis.

Luciferase reactivation assay

Purified firefly luciferase (>90% purity, Sigma) was unfolded in buffer B at 20 μM (see above) and diluted 100-fold into buffer C (lacking CDTA, unless otherwise indicated) containing 1.0 μM TRiC. After incubation at 30°C as indicated in the figure legend, 10 μl aliquots were diluted 5-fold into luciferase assay mix [20 mM Tricine pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 3.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin and 530 μM ATP] (de Wet *et al.*, 1986). Activities were determined at 20°C in a Beckman LS6000 IC scintillation counter adjusted to count photons for 3 s at 17 s intervals. Reactions were started every 20 s and recording took 2 min, starting from dilution of the sample into assay mix. Luciferase activities are expressed as a percentage of native enzyme control or are given in arbitrary light units corresponding to recorded c.p.m.

Tubulin assembly assay

Preparation of ^{125}I-labeled tubulin. Tubulin (α/β dimer) was purified from bovine brain by phosphocellulose chromatography as described by Karr *et al.* (1979) (kindly provided by D.Purich). Purified dimer (4 mg/ml, final concentration) was brought to 30% glycerol in buffer D (80 mM PIPES–KOH pH 6.8, 1 mM EGTA, 2 mM $MgCl_2$) containing 1 mM GTP, and microtubules (MT) were polymerized by incubation for 30 min at 37°C in a reaction volume of 50 μl (Na and Timasheff, 1982; Mitchison and Kirschner, 1985). MTs were added to dried ^{125}I-labeled Bolton–Hunter reagent (1 mCi) [*N*-succinimidyl-3(3,3′-diiodo-4-hydroxyphenyl) propionate, New England Nuclear] (Bolton and Hunter, 1973) and incubated for 15 min at 37°C. Glycine was added to 10 mM and MTs were sedimented through a 200 μl cushion of 50% sucrose in buffer D at 180 000 *g* for 10 min at 37 °C (TL100.1 rotor, Beckman). The pellet was washed in buffer D at 37°C and resuspended in 40 μl buffer D containing 1 mM GTP and 10 mM $CaCl_2$. Following incubation for 1 h at 0°C with gentle agitation, the solution was centrifuged at 180 000 *g* for 3 min at 4°C to remove insoluble aggregates. The supernatant was subjected to a second cycle of polymerization after adding EGTA to 15 mM followed by depolymerization as before. The specific activity of iodinated α tubulin ($>10^8$ c.p.m./mg) was ~6-fold higher than that of β tubulin, perhaps due to the fact that labeling was performed with assembled microtubules. ^{125}I-labeled tubulin was stored in aliquots at −80°C. It behaved in native PAGE, sizing chromatography and ion exchange chromatography exactly as the unlabeled tubulin dimer.

Assembly assay. GdmCl-unfolded ^{125}I-labeled tubulin (73 μM) was diluted 100-fold into buffer C containing either 3 μM BSA or 0.2 μM TRiC as specified in the figure legend. After 5 min at 25°C, unlabeled native tubulin was added (0.2 mg/ml, final concentration) and the reaction was brought to 30% glycerol/buffer D. Where indicated, 1 mM ATP and GTP were present and the reactions were incubated for 30 min at 37°C. Analysis was then performed on a Mono-Q HR5/5 column (Pharmacia) in buffer D/5% glycerol using a 20 ml gradient from 0 to 1 M NaCl, or by native PAGE (see below) followed by Coomassie blue staining and autoradiography. To visualize α and β tubulin subunits, the radioactive bands corresponding to TRiC-bound tubulin and to the released dimer were excised and subjected to 12.5% SDS–PAGE in gels containing 8 M urea (Lee *et al.*, 1973). To analyze the incorporation of newly formed radiolabelled dimer into MTs, dimer assembly was first carried out as above and then unlabeled native tubulin dimer was added to 2 mg/ml. Following incubation for 30 min at 37°C, MTs were sedimented through 50% sucrose (see above). Pellets were resuspended in buffer D containing GTP and $CaCl_2$ and were subjected to one or two additional cycles of assembly and disassembly.

Miscellaneous

Non-denaturing gel electrophoresis was performed using 4–10% polyacrylamide gradient gels (native PAGE) in either 0.37 M Tris–HCl, pH 8.8 or 80 mM MOPS–KOH, pH 7.0. Gels were run for 16 h at 120 V and stained with Coomassie blue. When indicated, protein bands were excised and subjected to SDS–PAGE using 12.5% gels as described (Trent *et al.*, 1991). The following procedures were carried out according to published methods: colorimetric determination of ATPase activity (Lanzetta *et al.*, 1978); purification of GroEL and GroES (Martin *et al.*, 1991); protein determination (Bradford, 1976); SDS–PAGE (Laemmli, 1970); electrotransfer to nitrocellulose membranes (Towbin *et al.*, 1979) and detection of bound antibodies using the ECL system (Amersham) (Vachereau, 1989).

Acknowledgements

We thank Dr K.Willison for anti-TCP-1 antibody and purified mouse TCP-1, and Dr D.Purich for purified tubulin. The help of S.Geromanos and L.Lacomis with the peptide analysis and of T.Langer with the GroEL/ES assays is gratefully acknowledged. J.F. was supported by a postdoctoral training grant from the NIH.

References

Ahmad,S. and Gupta,R.S. (1990) *Biochim. Biophys. Acta*, **1089**, 253–255.
Atencio,D.P. and Yaffe,M.P. (1992) *Mol. Cell. Biol.*, **12**, 283–291.
Bairoch,A. and Claverie,J.-M. (1988) *Nature*, **331**, 22.
Beckmann,R.P., Mizzen,L.A. and Welch,W.J. (1990) *Science*, **248**, 850–854.
Bolton,A.E. and Hunter,W.M. (1973) *Biochem. J.*, **133**, 529–538.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
Buchner,J., Schmidt,M., Fuchs,M., Jaenicke,R., Rudolph,R., Schmid,F.X. and Kiefhaber,T. (1991) *Biochemistry*, **30**, 1586–1591.
Caplan,A.J. and Douglas,M.G. (1991) *J. Cell Biol.*, **114**, 609–621.
Chanderasekhar,G.N., Tilly,K., Woolford,C., Hendrix,R. and Georgopolous,C. (1986) *J. Biol. Chem.*, **261**, 12414–12419.
Dayhoff,M.O., Schwartz,R.M. and Orcutt,B.C. (1978) In Dayhoff,M.O. (ed.), *Atlas of Protein Sequence and Structure*. National Biomedical Research Foundation, Silver Spring, MD, pp. 345–352.
Detrich,H.W. and Williams,R.C. (1978) *Biochemistry*, **17**, 3900–3907.
de Wet,J.R., Wood,K.V., Helinski,D.R. and DeLuca,M. (1986) *Methods Enzymol.*, **133**, 3–14.
Ellis,R.J. (1987) *Nature*, **328**, 378–379.
Ellis,R.J. (1990) *Science*, **250**, 954–959.
Flynn,G.C., Rohl,J., Flocco,M.T. and Rothman,J.E. (1991) *Nature*, **353**, 726–730.
Gao,Y., Thomas,O., Chow,R.L., Lee,G.-H. and Cowan,N.J. (1992) *Cell*, **69**, 1043–1050.
Gething,M.-J. and Sambrook,J. (1992) *Nature*, **355**, 33–45.
Ghosh,S., Gifford,A.M., Riviere,L.R., Tempst,P., Nolan,G.P. and Baltimore,D. (1990) *Cell*, **62**, 1019–1029.
Goloubinoff,P., Christeller,J.T., Gatenby,A.A. and Lorimer,G.H. (1989) *Nature*, **342**, 884–889.
Gupta,R.S. (1990) *Biochem. Int.*, **4**, 833–839.
Hartl,F.-U., Martin,J. and Neupert,W. (1992) *Annu. Rev. Biophys. Biomol. Struct.*, **21**, 293–322.
Hemmingsen,S.M. and Ellis,R.J. (1986) *Plant Physiol.*, **80**, 269–276.
Hemmingsen,S.M., Woolford,C., van der Vies,S.M., Tilly,K., Dennis,D.T., Georgopoulos,C.P., Hendrix,R.W. and Ellis,R.J. (1988) *Nature*, **333**, 330–334.
Hendrix,R.W. (1979) *J. Mol. Biol.*, **129**, 375–392.
Hohn,T., Hohn,B., Engel,A., Wortz,M. and Smith,P.R. (1979) *J. Mol. Biol.*, **129**, 359–373.
Ishii,N., Taguchi,H., Sumi,M. and Yoshida,M. (1992) *FEBS Lett.*, **299**, 169–174.
Jindal,S., Dudani,A.K., Singh,B., Harley,C.B. and Gupta,R.S. (1989) *Mol. Cell. Biol.*, **9**, 2279–2283.

Kang,P.-J., Ostermann,J., Shilling,J., Neupert,W., Craig,E.A. and Pfanner,N. (1990) *Nature*, **348**, 137–143.
Karr,T.L., White,H.D. and Purich,D.L. (1979) *J. Biol. Chem.*, **254**, 6107–6111.
Kirchhoff,C. and Willison,K. (1990) *Nucleic Acids Res.*, **18**, 4247.
Kubota,H., Morita,T., Nagata,T., Takemoto,Y., Nozaki,M., Gachelin,G. and Matsushiro,A. (1991) *Gene*, **105**, 269–273.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Landry,S., Jordan,R., McMacken,R. and Gierasch,L. (1992) *Nature*, **355**, 455–457.
Langer,T., Lu,C., Echols,H., Flanagan,J., Hayer,M.K. and Hartl,F.-U. (1992a) *Nature*, **356**, 683–689.
Langer,T., Pfeifer,G., Martin,J., Baumeister,W. and Hartl,F.-U. (1992b) *EMBO J.*, **11**, 4757–4765.
Lanzetta,P.A., Alvarez,L.J., Reinach,P.S. and Candia,O.A. (1978) *Anal. Biochem.*, **100**, 95–97.
Lee,J.C., Frigon,R.P. and Timasheff,S.N. (1973) *J. Biol. Chem.*, **248**, 7253–7262.
Lewis,V.A., Heynes,G.M., Zheng,D., Saibil,H. and Willison,K. (1992) *Nature*, **358**, 249–252.
Lorimer,G.H. (1992) *Curr. Opin. Struct. Biol.*, **2**, 26–34.
Luke,M.M., Sutton,A. and Arndt,K.T. (1991) *J. Cell Biol.*, **114**, 623–638.
Lyon,M.F. (1990) *Genet. Res.*, **55**, 13–19.
Manning-Krieg,U.C., Scherer,P.E. and Schatz,G. (1991) *EMBO J.*, **10**, 3273–3280.
Martel,R., Coney,L.P., Pelcher,L.E. and Hemmingsen,S.M. (1990) *Gene*, **94**, 181–187.
Martin,J., Langer,T., Boteva,R., Schramel,A., Horwich,A.L. and Hartl,F.-U. (1991) *Nature*, **352**, 36–42.
Mendoza,J.A., Rogers,E., Lorimer,G.H. and Horowitz,P.M. (1991) *J. Biol. Chem.*, **266**, 13044–13049.
Mitchison,T.J. and Kirschner,M.W. (1985) *J. Cell Biol.*, **101**, 755–765.
Musgrove,J.E., Johnson,R.A. and Ellis,R.J. (1987) *Eur. J. Biochem.*, **163**, 529–534.
Na,G.C. and Timasheff,S.N. (1982) *Methods Enzymol.*, **85**, 393–408.
Palleros,D.R., Welch,W.J. and Fink,A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5719–5723.
Pearson,W.R. (1990) *Methods Enzymol.*, **183**, 63–98.
Pearson,W.R. and Lipman,D.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2444–2448.
Pfanner,N., Tropschug,M. and Neupert,W. (1987) *Cell*, **49**, 815–823.
Phipps,B.M., Hoffmann,A., Stetter,K.O. and Baumeister,W. (1991) *EMBO J.*, **10**, 1711–1722.
Purich,D.L. and Kristofferson,D. (1984) *Adv. Protein Chemistry*, **36**, 133–212.
Pushkin,A.V., Tsuprub,V.L., Solovjeva,N.A., Shubin,V.V., Evstigneva,Z.G. and Kretovich,W.L. (1982) *Biochim. Biophys. Acta*, **704**, 379–384.
Rothman,J.E. (1989) *Cell*, **59**, 591–601.
Saibil,H., Dong,Z., Wood,S. and auf der Mauer,A. (1991) *Nature*, **353**, 25–26.
Sanders,S.L., Whitfield,K.M., Vogel,J.P., Rose,M.D. and Schekman,R.W. (1992) *Cell*, **69**, 353–365.
Scherer,P.E., Krieg,U.C., Hwang,S.T., Verstweber,D. and Schatz,G. (1990) *EMBO J.*, **9**, 4315–4322.
Silver,L.M. and Remis,D. (1987) *Genet. Res.*, **49**, 51–56.
Silver,L.M., Artzt,K. and Bennett,D. (1979) *Cell*, **17**, 275–284.
Tempst,P. and Riviere,L. (1989) *Anal. Biochem.*, **183**, 290–300.
Tempst,P., Link,A.J., Riviere,L.R., Fleming,M. and Elicone,C. (1990) *Electrophoresis*, **11**, 537–553.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **79**, 267–271.
Trent,J.D., Nimmesgern,E., Wall,J.S., Hartl,F.-U. and Horwich,A.L. (1991) *Nature*, **354**, 490–493.
Ursic,D. and Ganetsky,B. (1988) *Gene*, **68**, 267–274.
Ursic,D. and Culbertson,M.R. (1991) *Mol. Cell. Biol.*, **11**, 2629–2640.
Vachereau,A. (1989) *Anal. Biochem.*, **179**, 206–208.
Viitanen,P.V., Lubben,T.H., Reed,J., Gloubinoff,P., O'Keefe,D.P. and Lorimer,G.H. (1990) *Biochemistry*, **29**, 5665–5671.
Wall,J.S. and Hainfeld,J.F. (1986) *Annu. Rev. Biophys. Biophys. Chem.*, **15**, 355–376.
Willison,K.R., Dudley,K. and Potter,J. (1986) *Cell*, **44**, 727–738.
Willison,K., Lewis,V., Zuckerman,K.S., Cordell,J., Dean,C., Miller,K., Lyon,M.F. and Marsh,M. (1989) *Cell*, **57**, 621–632.
Willison,K.R., Hynes,G., Davies,P., Goldsborough,A. and Lewis,V.A. (1990) *Genet. Res.*, **56**, 193–201.
Yaffe,M.B., Levinson,B.S., Szasz,J. and Sternlicht,H. (1988) *Biochemistry*, **27**, 1869–1880.
Yaffe,M.B., Farr,G.W., Miklos,D., Horwich,A.L., Sternlicht,M.L. and Sternlicht,H. (1992) *Nature*, **358**, 245–248.

Received on July 29, 1992; revised on September 7, 1992