The Interaction of the Chaperonin Tailless Complex Polypeptide 1 (TCP1) Ring Complex (TRiC) with Ribosome-bound Nascent Chains Examined Using Photo-Cross-linking

Christine D. McCallum,* Hung Do,* Arthur E. Johnson,*‡§ and Judith Frydman‖

*Department of Medical Biochemistry and Genetics, ‡Department of Chemistry, and §Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843-1114; and ‖Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

Abstract. The eukaryotic chaperonin tailless complex polypeptide 1 (TCP1) ring complex (TRiC) (also called chaperonin containing TCP1 [CCT]) is a hetero-oligomeric complex that facilitates the proper folding of many cellular proteins. To better understand the manner in which TRiC interacts with newly translated polypeptides, we examined its association with nascent chains using a photo-cross-linking approach. To this end, a series of ribosome-bound nascent chains of defined lengths was prepared using truncated mRNAs. Photoactivatable probes were incorporated into these ^{35}S-labeled nascent chains during translation. Upon photolysis, TRiC was cross-linked to ribosome-bound polypeptides exposing at least 50–90 amino acids outside the ribosomal exit channel, indicating that the chaperonin associates with much shorter nascent chains than indicated by previous studies. Cross-links were observed for nascent chains of the cytosolic proteins actin, luciferase, and enolase, but not to ribosome-bound preprolactin. The pattern of cross-links became more complex as the nascent chain increased in length. These results suggest a chain length–dependent increase in the number of TRiC subunits involved in the interaction that is consistent with the idea that the substrate participates in subunit-specific contacts with the chaperonin. Both ribosome isolation by centrifugation through sucrose cushions and immunoprecipitation with anti-puromycin antibodies demonstrated that the photoadducts form on ribosome-bound polypeptides. Our results indicate that TRiC/CCT associates with the translating polypeptide shortly after it emerges from the ribosome and suggest a close association between the chaperonin and the translational apparatus.

Key words: protein folding • actin • luciferase • translation • chaperonin

Introduction

Understanding how proteins fold in the cell is one of the central problems in modern biology. In recent years it has become clear that this process is assisted by several protein families generically termed molecular chaperones. Two major chaperone systems have been implicated in cytoplasmic protein folding in eukaryotes (for reviews see Ellis, 1994; Hartl, 1996; Bukau and Horwich, 1998): the 70-kD heat shock protein cognate (Hsc70)[1] and the cytoplasmic class II chaperonin tailless complex polypeptide 1 (TCP1)-ring complex (TRiC; also called chaperonin containing TCP1 [CCT]). Although both chaperone systems have ATPase activity and bind and release unfolded polypeptides in a nucleotide-dependent manner, they are structurally and mechanistically different and appear to recognize different determinants in their substrate proteins.

The Hsc70 class of molecular chaperones has been the focus of extensive studies (Hartl, 1996; Bukau and Horwich, 1998). In contrast, little is known about the mechanism and binding determinants of the chaperonins found in eukaryotic cells (reviewed in Willison and Horwich, 1996; Gutsche et al., 1999). The chaperonin TRiC/CCT is a

Address correspondence to Judith Frydman, Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020. Tel.: (650) 725-7833. Fax: (650) 723-8475. E-mail: jfrydman@leland.stanford.edu

Dr. McCallum's present address is Merck & Co. Inc., Rahway, NJ 07065.

Dr. Do's present address is Targeted Therapy, Inc., Oklahoma City, OK 73104.

[1]*Abbreviations used in this paper:* ϵANB, N^{ϵ}-(5-azido-2-nitrobenzoyl); CCT, chaperonin containing TCP1; GIMc, genes involved in microtubule biogenesis complex; Hsc70, 70-kD heat shock protein cognate; pPL, preprolactin; SRP, signal recognition particle; TCP1, tailless complex polypeptide 1; TRiC, TCP1-ring complex.

ring-shaped complex that consists of eight different, yet homologous, subunits ranging between 50 and 60 kD (Frydman et al., 1992; Gao et al., 1992; Lewis et al., 1992). The substrate binds in the central cavity and is folded by the chaperonin in an ATP-dependent manner. Unlike Hsc70, TRiC does not interact with short extended peptides or proteins (Frydman et al., 1994; Frydman and Hartl, 1996; Rommelaere et al., 1999). The mode of interaction between TRiC and its substrates remains to be determined. TRiC was originally proposed to be a chaperone specialized for the folding of actin and tubulin (Lewis et al., 1996), but recent experiments suggest a broader substrate spectrum in vivo (Farr et al., 1997; Won et al., 1998; Srikakulam and Winkelmann, 1999; Thulasiraman et al., 1999).

Whereas the mechanism of chaperone-mediated folding was classically studied using full-length denatured protein substrates, in the cell, proteins enter the cytosol vectorially during translation. The vectorial nature of the translation process constrains the folding of the nascent chain, as the NH_2 terminus enters the cytosol first and the initial folding attempts may be localized at the NH_2-terminal end of the polypeptide (Frydman et al., 1994; Netzer and Hartl, 1997; Nicola et al., 1999). However, the cooperative nature of the interactions that stabilize folded structures makes it necessary that a complete folding domain (~50–300 amino acids) is available for productive folding into a native tertiary structure (Jaenicke, 1991). Recent studies showing that the process of translation influences the folding pathway (Frydman et al., 1999) underscore the importance of elucidating the mechanism of protein folding in the context of translation. Clearly, this will require a better understanding of how chaperones interact with polypeptides as they emerge from the ribosome. A number of studies indicate that the molecular chaperones Hsc70 and TRiC bind to ribosome-associated polypeptides during translation (Frydman et al., 1994; Hansen et al., 1994; Dobrzynski et al., 1996; Frydman and Hartl, 1996; James et al., 1997; Pfund et al., 1998; Yan et al., 1998). However, it has been proposed recently that TRiC interacts posttranslationally with newly made polypeptides, whereas the nascent chains interact cotranslationally with a novel chaperone complex named genes involved in microtubule biogenesis complex (GIMc) prefoldin (Hansen et al., 1999). Many of these studies have relied primarily on techniques such as immunoprecipitation and nondenaturing PAGE, where chaperone-bound ligands (i.e., nascent chains) are separated at some point from free ligands. In such experiments, complexes are detected only if their dissociation rates are slow compared with the rate of separation of free and bound ligands. These techniques therefore favor the detection of high-affinity interactions.

To clarify the controversial interaction of TRiC with nascent chains, we have used another approach, photo-cross-linking, that can detect highly dynamic and transient nascent chain–chaperone interactions. As described herein, this approach reveals that TRiC interacts with ribosome-bound nascent chains. The interaction begins at a much earlier stage than we had previously detected using other techniques. Furthermore, these short nascent chains cross-link to TRiC even before they can form stable high-affinity complexes with the chaperonin, suggesting that TRiC is positioned in close proximity to the site on the ribosome from which the nascent chain emerges. Our results provide further support for the notion that the chaperone machinery is functionally coupled to translation and may even interact directly with the ribosome.

Materials and Methods

Materials

[^{35}S]Methionine was purchased from NEN Life Science Products. Puromycin, apyrase, cycloheximide, and protein A–Sepharose were purchased from Sigma-Aldrich. *N*-5-azido-2-nitrobenzoyloxysuccinimide was purchased from Pierce Chemical Co. Rabbit reticulocyte lysate was prepared as described (Merrick, 1983). Anti–TCP-1α mAb (23C) was obtained from StressGen Biotechnologies (Lewis et al., 1992). Dog pancreas signal recognition particle (SRP) was purified as described (Walter and Blobel, 1983). All other reagents were of the highest quality available.

Preparation of mRNA

pGEM-mouse β-actin was linearized in the coding region by digestion with BglII, SnaBI, Asp718, ScaI, or ApaLI, and pGEM-luciferase was linearized by digestion with HnfI, BslI, BbvI, AflIII, EcoRI, or BspEI restriction endonucleases (New England Biolabs, Inc.). Truncated mRNAs coding for nascent actin polypeptides were generated by RNA transcription of these linearized plasmids in vitro, using SP6 RNA polymerase as before (Krieg et al., 1989; Frydman and Hartl, 1996). The nascent chains thus generated for actin contained 84, 133, 220, 301, 337, or 371 total amino acids and contained 5, 7, 10, 13, 17, or 18 lysine residues, respectively. The nascent luciferase polypeptides contained 77, 92, 125, 164, 197, or 232 amino acids and 6, 6, 6, 13, 14, or 15 lysines, respectively. pBSK-enolase (p-eno46; Holland et al., 1981) was linearized by digestion with DdeI, EcoRI, or BglI to yield polypeptides of 137, 251, or 375 amino acids (and 12, 22, and 34 lysines, respectively) by in vitro RNA transcription with T3 polymerase. A preprolactin (pPL) nascent chain of 86 amino acids (four lysines) was generated as described previously (Krieg et al., 1989).

Translations, Photolysis, and Analysis

Yeast $tRNA^{Lys}$ was purified and aminoacylated as described elsewhere (Crowley et al., 1993) and then modified as before (Krieg et al., 1986) to yield photoreactive N^{ϵ}-(5-azido-2-nitrobenzoyl)-Lys-$tRNA^{Lys}$ (ϵANB-Lys-$tRNA^{Lys}$) (750–800 pmol Lys/A_{260} unit of tRNA; 75–80% ϵ-labeled). Nuclease-treated rabbit reticulocyte lysate was pretreated to remove endogenous lysine using a Sephadex G-25 spin column at 4°C (typically 1 ml lysate was placed on a 5-ml column of Sephadex G-25 prespun in water and centrifuged at 1,750 *g* for 1 min). Translations contained 50% (vol/vol) nuclease-treated and desalted lysate, 80 mM KOAc, pH 7.5, 1 mM $Mg(OAc)_2$, 50 μM hemin hydrochloride, 2 μCi/μL [^{35}S]methionine, 0.6 μM ϵANB-Lys-tRNA, and an energy generating system containing all amino acids except lysine and methionine as described elsewhere (Crowley et al., 1993). After translation in the dark for 40 min at 26°C, the ATP present in the lysate was depleted by addition of apyrase to a final concentration of 0.1 U/μL and incubation for 5 min at 26°C. When indicated, DTT (20 mM final) was added to the lysate to inactivate the cross-linker, either before the translation reaction or after photolysis. The presence or absence of 2 mM cycloheximide during photolysis did not affect the outcome of the experiments. In some experiments, puromycin was added to a final concentration of 2 mM and incubated for 20 min at 26°C, either before or immediately after photolysis. The effect of ATP on the cross-linking reaction was assessed by either omitting the apyrase treatment before photolysis (which left the endogenous ATP regenerating system functional) or by supplementing with additional ATP (1 mM) and $Mg(OAc)_2$ (2 mM) before photolysis. Both conditions yielded similar results. In either case apyrase was added after photolysis.

After photolysis for 10 min at 0°C as before (Do et al., 1996), unlabeled methionine (2 mM) and DTT were added to the reactions. Samples (4 μl for direct analysis, 50 μl for immunoprecipitation) were directly analyzed by SDS-PAGE or immunoprecipitated by rocking overnight at 4°C with 2 μl anti-TCP1α antibody (1 mg/ml) in 680 μl of buffer A (20 mM Hepes, pH 7.5, 100 mM KOAc, 5 mM $Mg[OAc]_2$, 5% [vol/vol] glycerol) and 15 μl of BSA-saturated protein A–Sepharose beads. Immunoprecipitated material was separated by SDS-PAGE, and radioactivity in dried gels was detected using a Bio-Rad GS-250 PhosphorImager.

To separate ribosome–nascent chain complexes from the translation mixture, 25 μl of translation mixture was layered over 100 μl of sucrose cushion (0.5 M sucrose, 25 mM Hepes, pH 7.5, 80 mM KOAc, 1 mM $Mg[OAc]_2$) and centrifuged in a TL100 rotor at 100,000 rpm for 4 min at 4°C. The ribosomal pellets were washed with 25 mM Hepes, pH 7.5, 80 mM KOAc, 1 mM $Mg(OAc)_2$, and resuspended in SDS sample buffer or in buffer A for immunoprecipitation.

To confirm that the photo-cross-links originated from nascent chains attached to ribosomes, samples were incubated with 2 mM puromycin after photolysis, as above. Excess puromycin was removed by gel filtration over Sephadex G-25, then samples were immunoprecipitated with 2 μl anti-puromycin antiserum (a generous gift of Dr. Peter Walter, University of California San Francisco, San Francisco, CA) in buffer A with protein A–Sepharose as above.

Nondenaturing gel electrophoresis (16 h, 4°C, 120 V) was performed using 4–10% polyacrylamide gels (native PAGE) in 80 mM MOPS-KOH, pH 7.0, 1 mM $MgCl_2$ as described (Frydman et al., 1994).

Results

Nascent Chain Length Dependence of the Actin–TRiC Interaction

An analysis of the interactions between nascent chains and molecular chaperones is complicated by two factors: the heterogeneous and changing nature of the elongating nascent chain substrates, and the dynamic and transient nature of their interaction with chaperones. These experimental constraints can, however, be overcome.

A homogeneous population of nascent chains can be achieved by exploiting the fact that translation products of truncated mRNAs lacking a stop codon remain ribosome-bound as peptidyl-tRNAs (e.g., Krieg et al., 1989). Translation in the presence of excess truncated mRNA will limit ribosomal initiation to one event per mRNA, thereby resulting in a population of ribosome-bound nascent chains whose length is dictated by the length of the truncated mRNA. This approach yields samples that are homogeneous in terms of the length of the nascent chain, and hence are at a particular state of nascent chain folding and processing. Importantly, these stable translation intermediates are effective tools for the dissection of chaperone interactions with the elongating polypeptide, particularly considering that the kinetics of translation in eukaryotic cells are already much slower (on the order of minutes) than the rate of binding of chaperones to substrate polypeptides, which appears to be diffusion-limited (Corrales and Fersht, 1995; Fekkes et al., 1995). Consequently, the time of association between chaperones and ribosome-bound polypeptides, and hence the possibility of detecting these complexes, is primarily dictated by their dissociation rates both in vitro and in vivo, where the crowded conditions prevalent in the cytosol increase the association constants by several orders of magnitude (Ellis, 1997; van Den Berg et al., 1999).

Actin mRNAs truncated at different positions within the coding region of the message were translated to generate a set of ribosome-bound nascent chains of defined length (Fig. 1). These translation reactions produced polypeptides of the expected molecular weight (Fig. 1 a), and were thus used to examine how the length of the nascent chains affects their interaction with the chaperonin.

Initially we used nondenaturing gel electrophoresis to analyze the complexes between these polypeptides and endogenous components of the rabbit reticulocyte lysate. After translation in the presence of [^{35}S]methionine, the nascent chain complexes were released from the ribosomes by incubation with the antibiotic puromycin and analyzed by electrophoresis and fluorography (Fig. 1 b). Our analysis revealed two major complexes with endogenous components. The major band (Fig. 1 b, lanes 3–6, Slow) corresponded to a complex between the ^{35}S-labeled nascent chains and the chaperonin TRiC (Frydman et al., 1994). A less intense, faster moving band was also observed in the same samples (Fig. 1 b). Although comparable levels of radioactivity were subjected to native gel analysis for all chain lengths (Fig. 1 a), the intensity of the binary complexes was much weaker for short nascent chains. Both complexes were clearly visible with nascent chains of 220

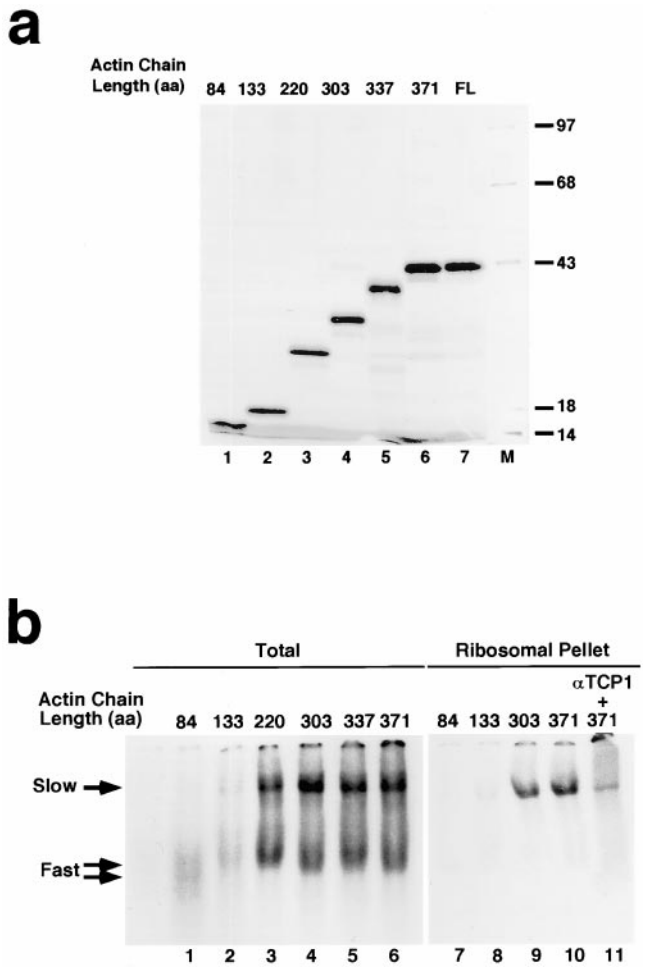


Figure 1. Actin nascent chain length dependence of interaction with TRiC. (a) Actin nascent chains of defined length. In vitro translation products of truncated actin mRNAs containing 84, 133, 220, 303, 337, 371, and 375 amino acids (lanes 1–7, respectively) were separated by SDS-PAGE in a 10–15% polyacrylamide gel. M, molecular mass markers. (b) Actin–TRiC interactions detected by native gels. Complexes of the same in vitro translation intermediates as in A were separated by nondenaturing gel electrophoresis immediately (lanes 1–6) or after purification of ribosomal complexes by sucrose density centrifugation (lanes 7–10). Incubation with anti–TCP-1 antibody (3 μl for 20 min, 4°C) prevented the migration of the TRiC complex into the gel (lane 11).

amino acids or longer, yet were barely detectable with nascent chains of 84 or 133 amino acids. Interestingly, some of the 84mer migrated as a complex with a different and faster mobility than either complex observed with longer chains (Fig. 1 b, lane 1, Fast). Thus, this analysis indicated a clear chain length dependence in the formation of both the TRiC complex and the faster moving complex. Presumably, complexes containing short chains, if formed, are too labile for this type of analysis. The same chain length dependence was observed when the interaction between actin nascent chains and TRiC was assessed by immunoprecipitation (see Fig. 3 a). Importantly, this result indicates that nascent chains shorter than ~200 amino acids do not form a stable complex with TRiC, consistent with previous observations (Frydman et al., 1994; Dobrzynski et al., 1996; Frydman and Hartl, 1996).

We next determined whether the complexes observed in Fig. 1 b, lanes 3–6 arise from ribosome-bound nascent chains. To this end, ribosome–nascent chain complexes were first isolated by centrifugation through a sucrose cushion and then analyzed by nondenaturing gel electrophoresis after puromycin treatment (Fig. 1 b, lanes 7–10). The ribosome isolation step caused a marked reduction in the levels of faster migrating complex, but not of the TRiC-containing complex, indicating that ribosome-bound chains are predominantly in a complex with TRiC. The presence of TRiC in this complex was further confirmed, as preincubation with anti-TCP1 antibodies caused a reduction in its migration into the running gel (Fig. 1 b, lane 11). The identities of the faster migrating complexes, which might include prefoldin, and their modes of interaction with nascent actin remain to be investigated (see Discussion).

Photoreactive Nascent Actin and Luciferase Chains Are Cross-linked to the Cytoplasmic Chaperonin TRiC

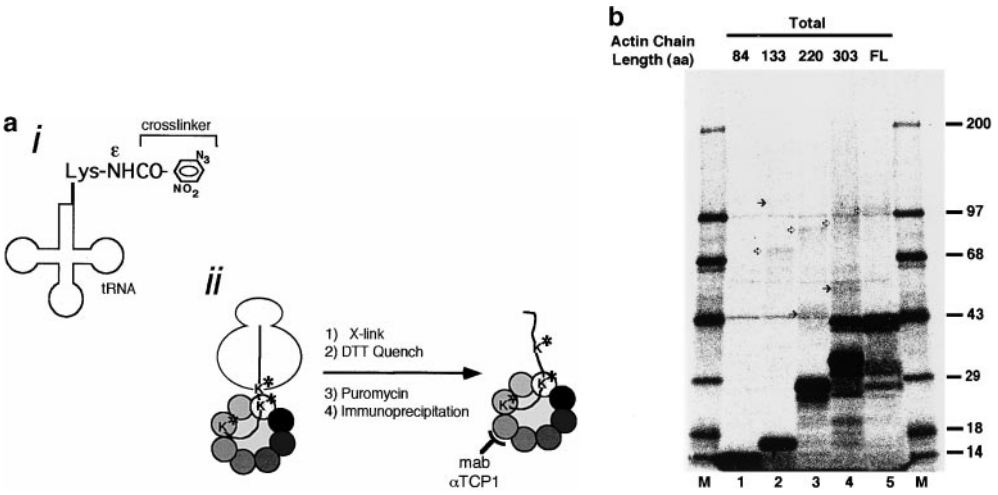
The second constraint noted above, i.e., the dynamic nature of nascent chain–chaperone interactions, can be circumvented by incorporating photoactivatable cross-linkers into a homogeneous population of nascent chains. When ribosome-bound nascent chains containing photoreactive probes are photolyzed, chaperones bound to the nascent chain may become covalently attached to the nascent chain if located close to a photoreactive probe at the time of its activation. This approach makes possible the biochemical analysis of the interactions of nascent chains by stabilizing short-range interactions between ribosome-bound polypeptides and associated proteins. Here we have employed this approach to examine the interactions of actin nascent chains with the cytoplasmic chaperonin TRiC.

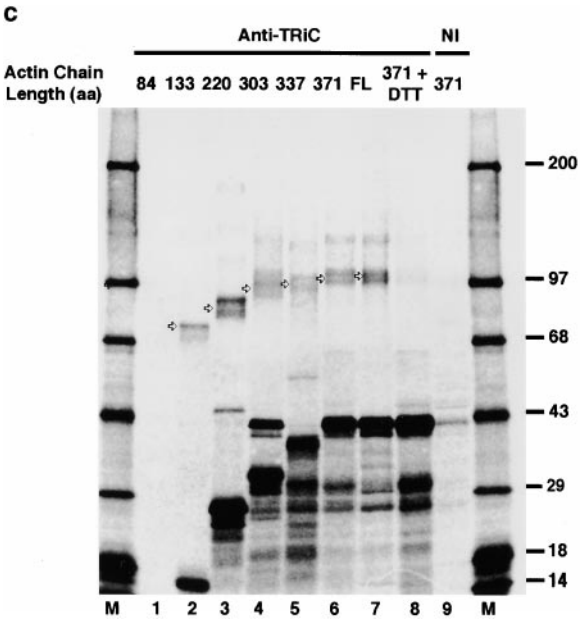
To incorporate a photoactivatable azido moiety into newly translated actin chains, ϵANB-Lys-tRNA (Krieg et al., 1986) with a photoreactive aryl azide covalently attached to the ϵ-amino group of the lysine (Fig. 2 a) was added to the in vitro translation reactions. Since the ϵANB-Lys-tRNA must compete with endogenous Lys-tRNA, only a fraction (~25%; Krieg et al., 1989) of the regularly spaced lysine residues in each actin nascent chain is replaced with a photoactivatable probe. Translation intermediates containing nascent actin chains of a specific length were prepared by translation of a particular truncated actin mRNA lacking a stop codon in reticulocyte lysate containing [^{35}S]methionine and/or ϵANB-[^{14}C]Lys-tRNA. Incorporation of the photoreactive probe in the nascent chains was confirmed by measuring the ^{14}C content of nascent chains after translation in the absence of [^{35}S]methionine (data not shown).

Photolysis of translation intermediates containing [^{35}S] actin nascent chains produced new radioactive species whose molecular weight increased along with that of the nascent chain (Fig. 2 b, open and closed symbols); this would be expected for the formation of photo-cross-links between the different nascent actin proteins and a specific protein(s). Interestingly, the major cross-linked products in this SDS-PAGE analysis of the total reaction had molecular weights consistent with photoadducts between the nascent chains and individual subunits of the TRiC complex (Fig. 2 b, open arrow). To characterize the photoadducts, samples were subjected to nondenaturing immunoprecipitation using a mAb specific for the TCP1 subunit of the hetero-oligomeric complex that immunoprecipitates the entire TRiC complex (Fig. 2 a, panel ii; Lewis et al., 1992). As shown in Fig. 2 c, the actin chains were indeed cross-linked to individual TRiC subunits. Thus, photoreactive probes in the ribosome-bound polypeptide were close enough to the chaperonin to generate a covalent bond between nascent actin and TRiC subunits after photolysis. The cross-links were not observed if the cross-linker was chemically inactivated by addition of 20 mM DTT before photolysis (Fig. 2 c, compare lanes 7 and 8).

Interestingly, cross-links were observed with actin chains as short as 133 amino acids (Fig. 2 c, lane 2). This suggests that TRiC can interact with nascent chains at an earlier stage than previously observed by coimmunoprecipitation and native gel analysis (Frydman and Hartl, 1996; see also Fig. 1 b, lanes 2 and 8, and Fig. 4, lane 2). However, a nascent chain of 84 amino acids, which only exposes ~50 amino acids to the cytosol (Malkin and Rich, 1967; Blobel and Sabatini, 1970), was not cross-linked to TRiC (Fig. 2 c, lane 1).

Firefly luciferase also interacts with TRiC during translation (Frydman et al., 1994). Furthermore, native gels and immunoprecipitation analysis showed that this interaction was also chain length–dependent. TRiC associated with luciferase nascent chains that were 197 amino acids in length, but not with chains of 77 amino acids (Frydman et al., 1994). We therefore examined the association of luciferase nascent chains with TRiC using the photo-cross-linking approach. As shown in Fig. 2 d, luciferase nascent chains were efficiently cross-linked to TRiC subunits. As observed for actin, photoadducts containing TRiC were also detected with short nascent chains, including the 77mer. Interestingly, the pattern of cross-links became more complex as the nascent chain increased in length (Fig. 2 d, panel ii). This was also the case for actin, since the 133mer actin nascent chain appeared to predominantly cross-link to one subunit of the TRiC complex, whereas longer nascent chains appeared to make contact with several subunits (Fig. 2 c). TRiC–luciferase cross-links were observed with all longer nascent chains, but the pattern of cross-links associated with TRiC became very complex for chains longer than 232 amino acid (data not shown). This could be due to a combination of factors, including the fact





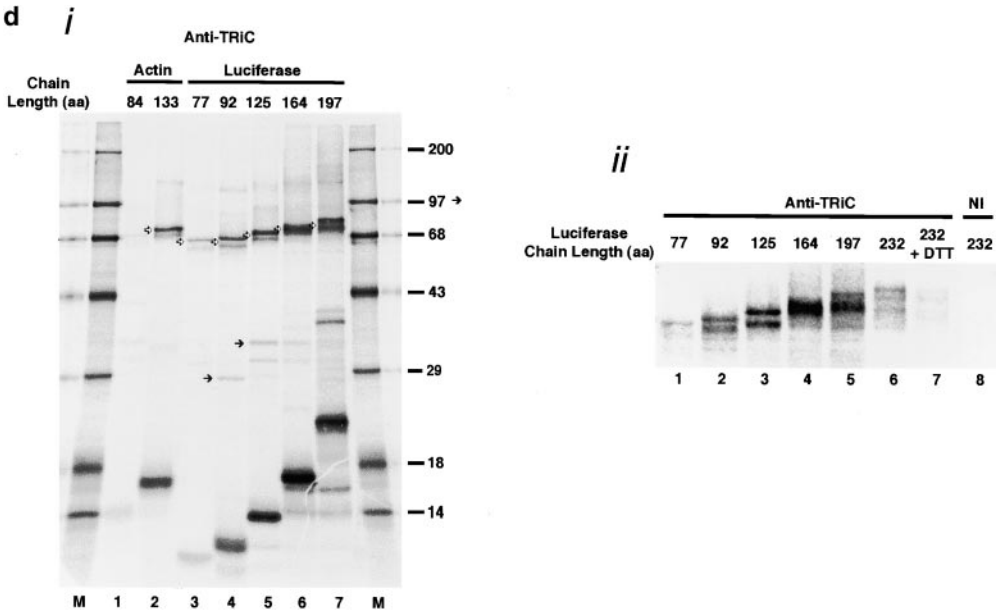


Figure 2. Photoreactive nascent chains are cross-linked to the chaperonin TRiC. (a) Schematic description of the experimental approach. (a, panel i) Structure of the ϵANB-Lys-tRNA included in the in vitro translation. (a, panel ii) Nascent actin chains are prepared by translating truncated actin mRNAs lacking a stop codon in a rabbit reticulocyte lysate translation system in the presence of [^{35}S]methionine and ϵANB-Lys-tRNA. Photolysis was performed on ice and residual cross-linker was then inactivated by addition of 20 mM DTT. In some experiments, puromycin was added to release the nascent chain from the tRNA either before or after photolysis. Photoadducts were then characterized by immunoprecipitation. (b) Total cross-links of actin nascent chains. The actin nascent chain photoadducts (generated as in a) were separated by SDS-PAGE in a 10–15% polyacrylamide gel. The major cross-linked products had molecular masses that corresponded to photoadducts between the nascent chains and a group of polypeptides of 51–57 kD (open arrows), suggesting an association with TRiC. Cross-links to other endogenous components are indicated by closed arrows. (c) Actin nascent chains cross-link to the chaperonin TRiC. Samples from b were subjected to nondenaturing immunoprecipitation using a mAb specific for the TCP-1 subunit of the hetero-oligomeric complex. Anti-TRiC (lanes 1–8) or nonimmune serum (NI; lane 9) under nondenaturing conditions. The immunoprecipitates were analyzed by SDS-PAGE. Addition of 20 mM DTT to the translation reaction before photolysis inhibited cross-linking (lane 8). Open arrows identify TRiC photoadducts (also in subsequent figures). (d) Luciferase nascent chains cross-link to TRiC. Actin and luciferase nascent chain photoadducts were immunoprecipitated as in c, and analyzed by SDS-PAGE (d, panel i). As the nascent chain increases in length, the pattern of cross-links becomes more complex (d, panel ii, lanes 1–6). NI, nonimmune serum used in immunoprecipitation. Open arrows identify photoadducts containing TRiC.

that longer chains may simultaneously cross-link to two proteins, such as TRiC plus another chaperone or two TRiC subunits. A number of photoadducts with other endogenous components were also immunoprecipitated with the TRiC-specific antibody (Fig. 2, c and d, closed arrows), suggesting that the nascent chains are in a complex with TRiC and other endogenous factors.

The change in cross-linking pattern observed for nascent chains of increasing length is intriguing. Previous experiments analyzing photoadducts generated by nascent chains bearing a single photoprobe adjacent to different sites in a target protein have not shown significant variation in photoadduct mobilities in SDS-PAGE (e.g., High et al., 1993; Mothes et al., 1994; Do et al., 1996), though one such change has been observed recently by Plath et al. (1998). It therefore seems unlikely that the photoadducts with different mobilities in Fig. 2, c and d arise from changes in the intramolecular location of nascent chain cross-links to the same TRiC subunit. The most probable explanation for the various photoadducts is that the nascent chain is cross-linking to different subunits in the complex. If so, this result may bear on the question of how TRiC recognizes its substrates. If all the subunits in TRiC possess substrate-binding sites of the same or similar specificity, the photoreactive probes would have an equal chance to react with all the subunits. Since the increase in nascent chain length is accompanied by an increase in the number of cross-linker–bearing lysines, and consequently in the probability of productive cross-linking events, the cross-links observed for longer chains should display an increase in intensity rather than the observed change in pattern. Our results are thus not consistent with a model where all TRiC subunits have equivalent substrate specificities. Instead, they suggest that emerging regions in the elongating polypeptide engage in subunit-specific interactions with different components of the ring complex.

The Specificity of TRiC Interactions with Substrates

Our finding that short actin and luciferase chains unexpectedly cross-linked to TRiC raised the possibility that TRiC has a broader range of interacting substrates than previously recognized using standard techniques. This led us to examine the pattern of cross-links of enolase, a 40-kD β-barrel protein that does not interact stably with TRiC (Fig. 3 a). After translation in reticulocyte lysate, neither enolase nor its nascent chains were associated with TRiC as determined by coimmunoprecipitation (Fig. 3 a) and native gel electrophoresis (data not shown). Surprisingly, these enolase nascent chains of 137, 251, and 375 amino acids were cross-linked to TRiC with great efficiency (Fig. 3 b). Moreover, the photoadducts with TRiC were the major products in the total cross-linking reaction (Fig. 3 b, lanes 1–3). Thus, although the interaction of TRiC with enolase is too weak to be detected by coimmunoprecipitation, the chaperone contacts the nascent chain during translation.

Although the photo-cross-linking data therefore reveal that the specificity of TRiC is broader than previously thought, not all polypeptides interact with TRiC cotranslationally. We next examined whether TRiC could cross-link to ribosome-bound nascent chains of the secretory protein pPL (Fig. 3 c). Previous studies indicated that an 86–amino acid pPL nascent chain translated in yeast extracts was cross-linked to the Hsc70 homologue SSB (Pfund et al., 1998). However, after translation in reticulocyte lysate, this ribosome-bound pPL chain cross-linked very inefficiently to TRiC subunits (Fig. 3 c, lane 3) but very efficiently to endogenous SRP54 (Fig. 3 c, lanes 1 and 2), as reported previously (Krieg et al., 1986). Moreover, the weak cross-links to TRiC were further diminished by addition of purified SRP (to 64 nM final concentration) to the lysate (Fig. 3 c, lane 4). Since the concentration of TRiC in the lysate is ~0.4 μM (Frydman et al., 1994), this result suggests that SRP favorably competes with TRiC for binding to pPL, effectively blocking its interaction with TRiC. However, we did observe cross-links to TRiC after release of the pPL nascent chain from the ribosome (data not shown). Thus, TRiC is in principle capable of interacting with the pPL 86mer, but such an interaction most likely does not occur in vivo because SRP binding will first target the ribosome to the ER membrane and translocation into the ER will proceed cotranslationally.

The TRiC–Nascent Chain Interaction Occurs Cotranslationally

We next determined whether the cross-links between the chaperone and the actin nascent chains indeed occurred while the polypeptides were ribosome-bound. This question was addressed by two independent criteria. First, ribosome–nascent chain–TRiC complexes containing the actin 133mer were purified after photolysis by centrifugation through a dense sucrose cushion. As shown in Fig. 4 a, lane 1, the ribosomal pellet contained most of the TRiC–nascent chain photoadducts. In contrast, if the nascent chains were released from the ribosome by incubation with puromycin after photolysis and before sedimentation, the TRiC cross-links were no longer associated with the ribosomes and were instead found in the supernatant of the ultracentrifugation (Fig. 4 a, lane 4). Thus, the TRiC–nascent chain photoadducts were released from the ribosomes in a puromycin-dependent manner, indicating that TRiC was cross-linked to ribosome-bound peptidyl-tRNAs.

The cotranslational nature of the cross-links between nascent chains and TRiC was tested directly by taking advantage of the chemistry of puromycin-mediated release from the ribosome (Fig. 4 b, upper panel). Puromycin mimics an aminoacyl-tRNA and reacts covalently with a peptidyl-tRNA in the ribosomal P site, a reaction that transfers the growing nascent chain from the tRNA to the puromycin. The chain thus released carries a COOH-terminal puromycin tag. To examine whether the nascent chains associate with TRiC while ribosome-bound, translation intermediates were therefore photolyzed as above, treated with DTT to eliminate unreacted photoprobes, and then incubated with puromycin. Any nascent chains that had reacted with puromycin were then immunoprecipitated with an anti-puromycin antibody. Since the puromycin reaction must be catalyzed by the peptidyltransferase center of the ribosome, only nascent chains bound functionally to ribosomes at the time puromycin was added will become covalently attached to puromycin.

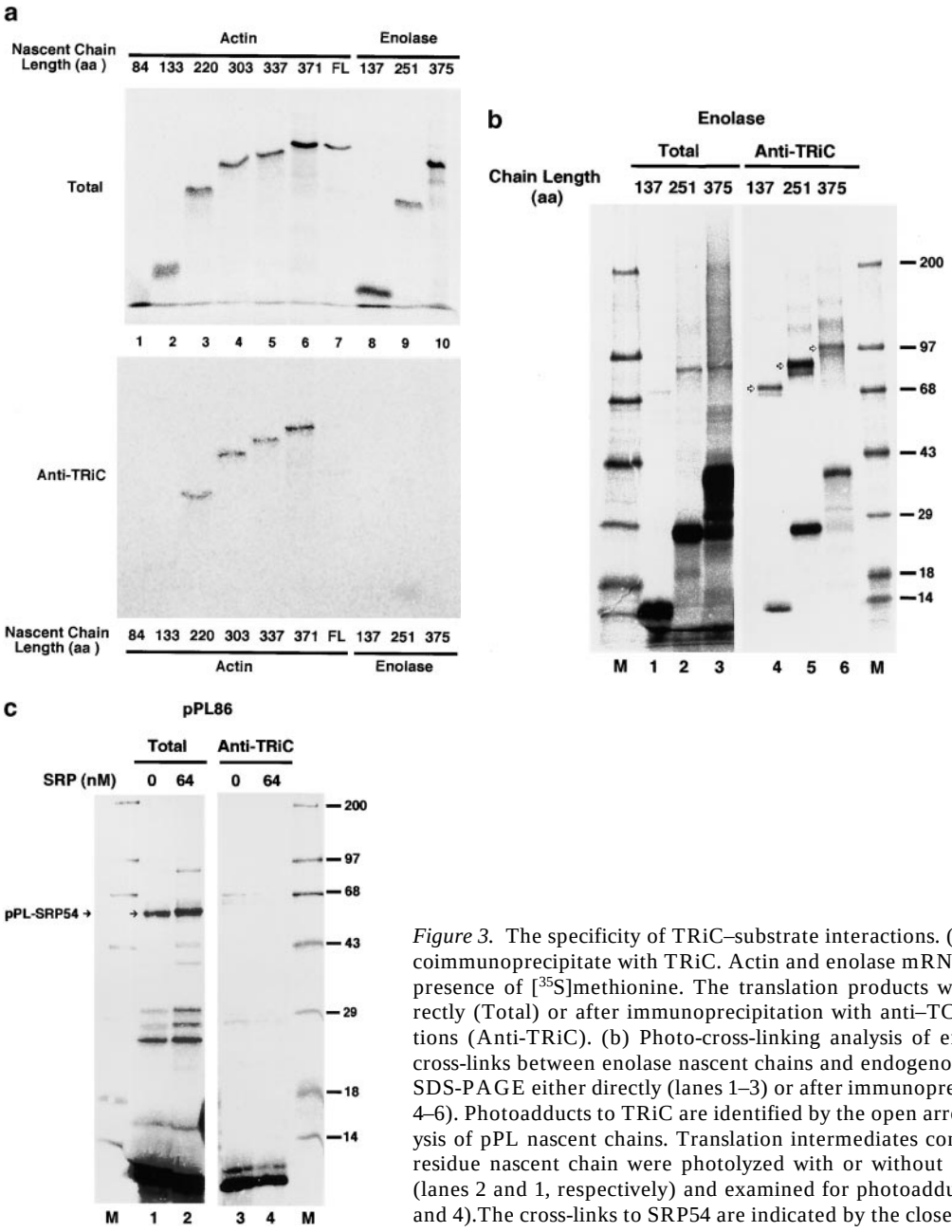


Figure 3. The specificity of TRiC–substrate interactions. (a) Enolase nascent chains do not coimmunoprecipitate with TRiC. Actin and enolase mRNAs were translated in vitro in the presence of [^{35}S]methionine. The translation products were analyzed by SDS-PAGE directly (Total) or after immunoprecipitation with anti–TCP-1 under nondenaturing conditions (Anti-TRiC). (b) Photo-cross-linking analysis of enolase nascent chains. The total cross-links between enolase nascent chains and endogenous components were analyzed by SDS-PAGE either directly (lanes 1–3) or after immunoprecipitation with anti-TCP-1 (lanes 4–6). Photoadducts to TRiC are identified by the open arrows. (c) Photo-cross-linking analysis of pPL nascent chains. Translation intermediates containing a photoreactive pPL 86-residue nascent chain were photolyzed with or without addition of 64 nM purified SRP (lanes 2 and 1, respectively) and examined for photoadduct formation with TRiC (lanes 3 and 4).The cross-links to SRP54 are indicated by the closed arrow.

Importantly, all photoprobes were inactivated, either by photolysis or by DTT, before the puromycin treatment. Consequently, the puromycin-specific antibody only immunoprecipitates photoadducts that were generated while the nascent chains were bound to ribosomes. As shown in Fig. 4 b, SDS-PAGE of these immunoprecipitations confirmed that for both actin and luciferase, the nascent chains had cross-linked to TRiC before the addition of puromycin (Fig. 4 b). Notably, the cross-links to TRiC were the predominant bands observed when using puromycin-specific antibodies. Thus, TRiC interacts with nascent chains that are associated with functional, translating ribosomes.

ATP Dependence of Cross-links to TRiC

The above photo-cross-linking data reveal that TRiC is positioned in close proximity to actin and other nascent chains, even if the ribosome-bound polypeptides are too short to form complexes with TRiC that survive immunoprecipitation. To gain further insight into the TRiC–nascent chain interaction, we examined their sensitivity to ATP. Incubation with ATP reduces the affinity of TRiC for its substrates, and thus results in their release from the chaperonin (Frydman et al., 1992). We thus compared the effect of performing the photolysis reaction in the presence or absence of ATP (Fig. 5 a). Removal of ATP from the lysate by incubation with apyrase should stabilize the

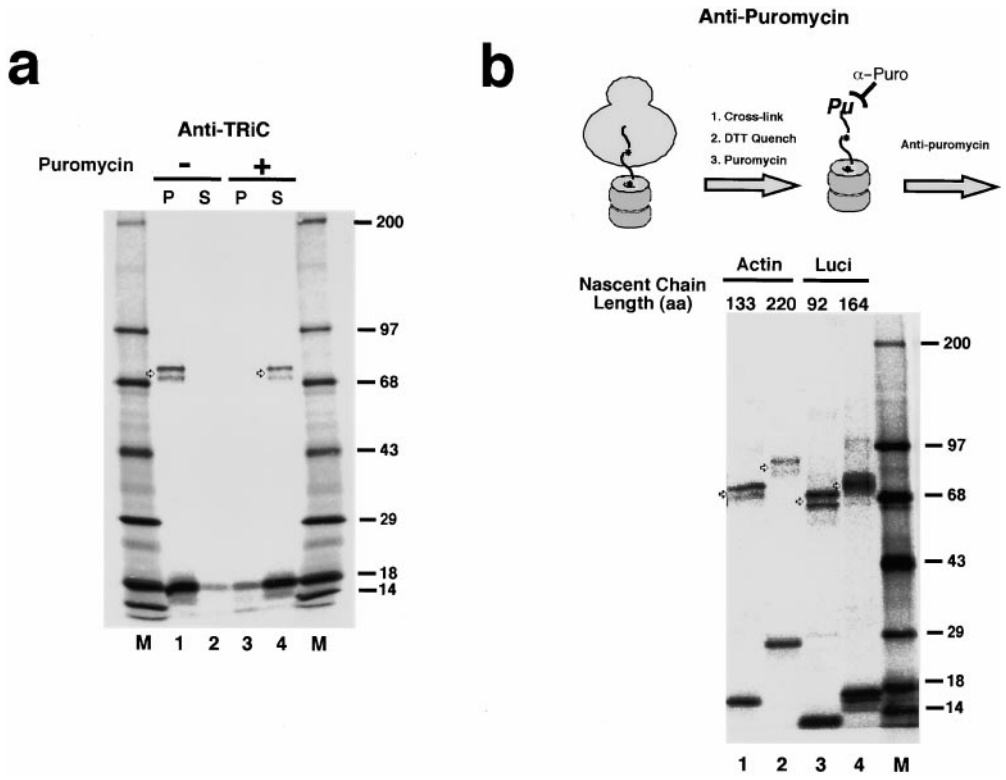


Figure 4. The TRiC–nascent chain interaction occurs cotranslationally. (a) The TRiC–actin cross-links associate with ribosomes in a puromycin-sensitive manner. After photolysis, ribosome–nascent chain complexes containing the actin 133mer were purified by sucrose density centrifugation and the pellets (P) and supernatants (S) were immunoprecipitated with anti–TCP-1 and separated by SDS-PAGE. The ribosomal pellet (P, lane 1) contained most of the TRiC–nascent chain photoadducts. In a parallel sample, the nascent chains were released from the ribosomes by treatment with puromycin before the centrifugation step (lanes 3 and 4). In this case, the TRiC cross-links were no longer found in the ribosomal pellet (lane 3), but were instead found in the supernatant (S, lane 4). (b) Nascent chains photo-cross-linked to TRiC can still react with puromycin and hence are bound to the ribosomal P site. Translation intermediates of actin and luciferase were photolyzed and then treated with puromycin to release the nascent chains from the tRNA. Excess puromycin was removed by Sephadex G-25 gel filtration, and the released nascent chain-puromycin photoadducts were immunoprecipitated with anti-puromycin antibody and separated by SDS-PAGE. The open arrows identify photoadducts to TRiC subunits.

interactions with the chaperonin. In contrast, the presence of ATP during photolysis should promote dissociation and hence diminish the amount of cross-linked product. As expected, incubation with ATP greatly reduced the extent of photo-cross-linking between TRiC and the longer nascent chains of luciferase and actin (Fig. 5 a, lanes 3, 4, 7, and 8). Surprisingly, ATP did not reduce the cross-links between TRiC and short nascent chains. Instead, these cross-links were enhanced by the presence of ATP (Fig. 5 a, lanes 1, 2, 5, and 6).

The differential effect of ATP on the extent of TRiC cross-linking to short and long nascent chains is remarkable, and suggests that the mode of nascent chain–TRiC interaction changes as the chains elongate. In particular, the unexpected ATP-dependent increase in photo-cross-linking raises the possibility that a short nascent chain is not binding to the substrate-binding site of a TRiC subunit, but is instead located in close proximity to a specific TRiC subunit. To distinguish between these possibilities, we examined the sensitivity of the cross-links of both short and long nascent chains to puromycin treatment (Fig. 5 b). We reasoned that if the short nascent chains were bound to TRiC as substrates, their interaction with the chaperonin (and the cross-links) would persist after the nascent chains were released from the tRNA and ribosomes by the action of puromycin. In contrast, if the nascent chains were not bound to a TRiC substrate-binding site, then their release from the ribosome before photolysis would eliminate any cross-links due to proximity to a surface on TRiC that is not a substrate-binding site. As shown in Fig. 5 b, puromycin treatment does not reduce TRiC cross-linking to either short (Fig. 5 b, lane 2) or long (Fig. 5 b, lane 4) nascent chains, thereby indicating that in both cases the nascent chain is bound to the chaperonin as a substrate.

The molecular basis for the ATP-dependent increase in cross-linking to short nascent chains remains unclear, but this result emphasizes the fact that the ATP dependence of TRiC function has yet to be characterized in detail. It is clear from the results presented here that TRiC binds differently to short and long nascent chains in the presence of ATP. There are several mechanisms that could account for this observation. For instance, cross-linking to TRiC may first require the ATP-dependent release of the nascent chain from an upstream cofactor, such as Hsc70. Alternatively, an ATP-mediated conformational change in TRiC may help position the chaperone in the vicinity of the ribosomal exit site, and thus facilitate binding to short nascent chains. It is also possible that individual subunits of TRiC interact differently with substrate and ATP. Future experiments addressing these possibilities may clarify the interplay between molecular chaperones and the translational machinery.

Discussion

This study demonstrates that TRiC interacts with nascent polypeptides as they emerge from the ribosome. Further-

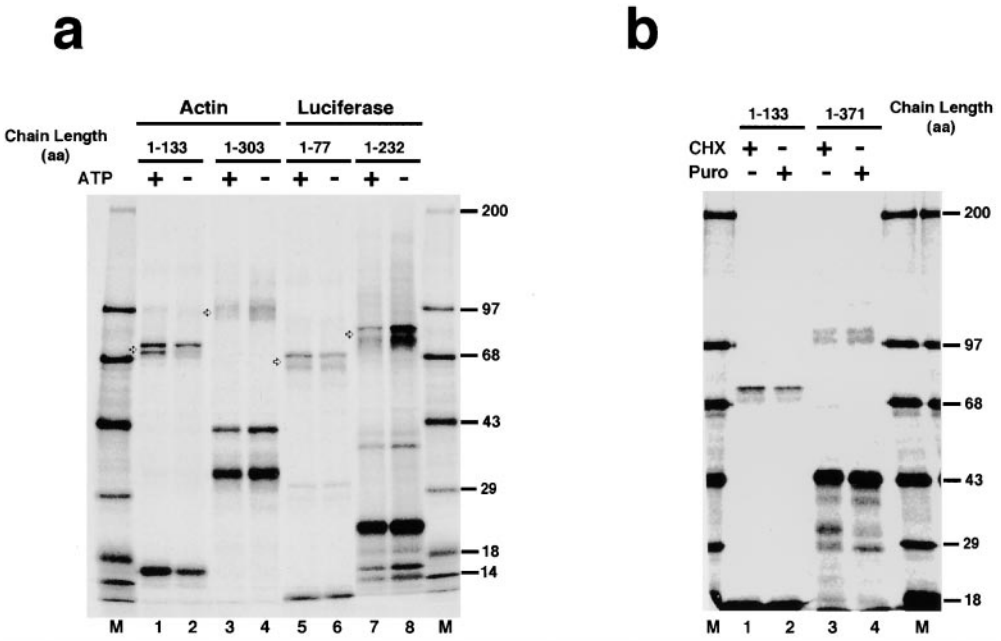


Figure 5. ATP and puromycin dependence of cross-links to TRiC. (a) ATP dependence of cross-links to TRiC. The effect of ATP on the interaction of TRiC with both short and long nascent chains was examined by incubation with ATP before and during cross-linking. In vitro translation products were incubated with 1 mM ATP + 2 mM $MgCl_2$ (lanes 1, 3, 5, and 7) or with 0.1 U/ml apyrase (lanes 2, 4, 6, and 8) for 15 min at 26°C, followed by photolysis and immunoprecipitation with anti–TCP-1, as described in Materials and Methods. (b) Puromycin dependence of cross-links to TRiC. The effect of releasing the nascent chain from the ribosome on the interaction of TRiC with both short and long nascent chains was examined by incubation of actin nascent chain–ribosome complexes for 20 min at 26°C with either 2 mM cycloheximide (CHX, ribosome-bound controls; lanes 1 and 3) or 2 mM puromycin (Puro, to release the nascent chain from its tRNA; lanes 2 and 4) before photolysis. Samples were analyzed as above.

more, TRiC association with nascent chains occurs much earlier than indicated by previous studies. The approach used here also suggests that the specificity of TRiC interactions with nascent polypeptides is broader that previously thought, and that individual TRiC subunits specifically recognize different motifs within the substrate polypeptide.

The Photo-Cross-linking Approach

The association of translating polypeptides with molecular chaperones plays a critical role in the folding process. However, the transient and dynamic nature of these associations presents a problem for the molecular analysis of this process. Here, we have generated translation intermediates consisting of nascent chains of defined lengths carrying photoreactive probes evenly located at multiple sites along the entire length of the polypeptide. Photolysis generates covalent links between the nascent chain and associated protein(s), thereby stabilizing these labile interactions for further analysis.

This approach allows the identification of endogenous proteins that bind to the nascent chain as it is being synthesized. Equally important, the interaction between the nascent chain and a particular protein will be stabilized and detected even if the affinity of the interaction is insufficient to maintain the protein–protein complex during conventional analysis, as might be the case for very short nascent chains. In addition, in the photo-cross-linking approach, the nascent chain is not released from the ribosome until after the assay (i.e., photolysis) has been completed, whereas complexes are analyzed by coimmunoprecipitation and native gel electrophoresis only after the nascent chain has been released from the ribosome. Each of these advantages was borne out when the photo-cross-linking approach was applied to actin translation intermediates.

Association of TRiC with Ribosome-bound Nascent Chains

The major cross-linked products observed for ribosome-bound actin nascent chains corresponded to photoadducts with TRiC. Two experimental approaches demonstrated that TRiC cross-linked to ribosome-bound nascent chains. First, the TRiC photoadducts sedimented with the ribosomal fraction after ultracentrifugation through a sucrose cushion. This association was not observed if the nascent chains were released from the ribosome by puromycin treatment before the ultracentrifugation step. Furthermore, to distinguish between photoadducts formed by ribosome-bound actin and by actin that had been released from the ribosome, we added puromycin to samples after photolysis had been completed. Only nascent chains functionally bound to ribosomes could react with puromycin. Consequently, immunoprecipitation with anti-puromycin antibodies selected only those photoadducts whose nascent chains are elongation-competent at the time of cross-linking. As seen in Fig. 4 b, this approach conclusively demonstrates that the photoadducts with TRiC were generated on ribosome-bound nascent chains. These results are consistent with a number of studies indicating that TRiC interacts cotranslationally with nascent chains (Frydman et al., 1994; Dobrzynski et al., 1996; Frydman and Hartl, 1996), and do not support the alternative proposal that the interaction of TRiC with its substrates is strictly posttranslational (Hansen et al., 1999).

The cotranslational nature of TRiC/CCT binding to na-

scent chains is also supported by experiments indicating that this chaperonin associates with ribosomal fractions. Comigration of the chaperonin with ribosomes upon size fractionation of cell extracts has been observed in vitro in reticulocyte lysate (Frydman et al., 1994), and in vivo in P19 embryonic carcinoma cells, where a significant fraction (5–20%) of the cellular TRiC appeared to be ribosome-associated (Roobol and Carden, 1999). Furthermore, the TRiC–ribosome interaction was confirmed by coimmunoprecipitation of ribosomes with the chaperonin (Roobol and Carden, 1999).

An analysis of the chain length dependence of cross-link formation yielded an unexpected result. TRiC association with short nascent polypeptides has not been detected previously using other techniques. Yet cross-links to TRiC were detected for actin nascent chains as short as 133 amino acids, which expose only ~90–100 amino acids outside the peptide channel. Similarly, we detected cross-links to luciferase nascent chains as short as 77 amino acids. These results indicate that chaperonins can interact with nascent chains very soon after they emerge from the ribosome. It is therefore conceivable that TRiC is already located in close proximity to the nascent chain, perhaps as a result of a specific recruitment mechanism.

Consistent with such a possibility, the study of protein targeting into organelles has produced several examples where chaperone components are physically recruited to the translocation machinery to bind to the incoming polypeptide (Brodsky and Schekman, 1993; Kessler and Blobel, 1996; Voos et al., 1996). Likewise, experiments in *Saccharomyces cerevisiae* have shown that the Hsp70 protein SSB binds to translating ribosomes through specific interactions with the translational machinery (James et al., 1997; Pfund et al., 1998). At present, there is no evidence for a direct physical interaction between TRiC and components of the basic translational machinery. However, TRiC binding to newly translated polypeptides could be facilitated by other components of the folding machinery, such as the Hsp–Hsc70 system (Frydman et al., 1994), the nascent chain–associated complex (NAC, Wang et al., 1995), and/or the recently described GIMc or prefoldin complex (Geissler et al., 1998; Vainberg et al., 1998; Hansen et al., 1999). Interestingly, incubation with ATP enhanced the cross-links between TRiC and the short nascent chains (Fig. 5), indicating that TRiC recruitment may be ATP-mediated, as expected if binding of nascent chains to the chaperonin is promoted by Hsc70. This is consistent with previous experiments showing that the interaction of TRiC with luciferase nascent chains requires the action of Hsc70 (Frydman et al., 1994). It has been suggested recently that prefoldin binds nascent chains and delivers them to TRiC (Vainberg et al., 1998; Hansen et al., 1999). However, both biochemical and genetic analyses indicate that prefoldin is not required for substrate binding to TRiC (Rommelaere et al., 1999; Siegers et al., 1999).

The specific recruitment of chaperones to bind to translating polypeptides would provide a mechanistic explanation for the observed coupling between translation and folding observed in intact eukaryotic cells, which probably contributes to the formation of a protected folding environment for nascent chains (Siegers et al., 1999; Thulasiraman et al., 1999). The mechanism for TRiC recruitment to bind nascent chains requires further investigation. We believe that the cross-linking approach described here for TRiC will permit the identification of both upstream and downstream cofactors of TRiC. Although the focus of the experiments presented here was to characterize the interaction of nascent chains with TRiC, they also revealed cross-links between nascent chains and as-yet unidentified components. Based on their molecular weight, some of the cross-links observed for longer chains might correspond to subunits of the GIMc complex (see Fig. 2 a and Fig. 3). In addition, we also observed cross-links with Hsc70 in the case of short luciferase nascent chains (77mer and 94mer; data not shown). Since the chaperone interactions detected using this approach are critically dependent on the proximity of the photoreactive probes to the chaperone binding site, elucidating the role of Hsc70 and GIMc prefoldin in the folding of newly translated proteins may require the introduction of the photoreactive group at different positions within the nascent chain.

Determinants for Substrate Binding to TRiC

Unlike its bacterial homologue, GroEL, the eukaryotic chaperonin TRiC is composed of different subunits. Most of the subunit heterogeneity resides in the putative substrate-binding site (Kim et al., 1994). However, the physiological significance of this diversity has been unclear, because little is known about what determines polypeptide binding to TRiC.

The analysis of the chain length dependence of cross-links between TRiC and both actin and luciferase nascent chains indicated that short chains appeared to contact predominantly one TRiC subunit, whereas longer chains were cross-linked to several subunits. Interestingly, enolase nascent chains are efficiently cross-linked to TRiC, but the cross-links appear to be predominantly to one TRiC subunit despite the higher proportion of lysines in the enolase nascent chains. Notably, there is a striking correlation between the extent to which the nascent chain is cross-linked to multiple TRiC subunits and the stability of the TRiC–nascent chain complexes to immunoprecipitation, supporting the idea that the frequency and number of different photoadducts indeed reflects subunit-specific interactions with different binding sites within the nascent chains. Our data are consistent with a model where stable interactions between a folding polypeptide and TRiC arise from a polyvalent set of weak interactions between defined substrate motifs and individual chaperonin subunits. Interestingly, this interpretation agrees with two recent studies on the interaction of actin with TRiC. First, deletion analysis of actin suggested that stable chaperonin binding requires at least three discrete regions in the polypeptide (Rommelaere et al., 1999). In addition, a structural analysis of the chaperonin–actin complex using immuno-EM supports the idea that the polypeptide interacts with specific subunits in the chaperonin (Llorca et al., 1999).

The possibility that each TRiC subunit contributes to the recognition of specific motifs may help explain how chaperonin substrates are selected in vivo. Identification of the chaperonin subunits that are cross-linked to specific nascent chains will provide important insights into the principles that govern substrate binding to TRiC.

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