transbilayer transport of lipids is the function common to this ATPase subfamily.

Although PS is a charged molecule, its amphipathic character distinguishes it from the simple ions transported by most P-type ATPases. Several glutamates as well as asparagine and threonine buried within transmembrane helices 4, 5, 6, and 8 are important to cation transport, particularly by the Ca^{2+}-dependent ATPases (*17*). Tentative sequence alignment indicates that these positions in transmembrane helices 4 and 6 of the ATPase II subfamily are replaced by amino acids with bulky hydrophobic side chains, which might interact with the hydrophobic portions of the amphipathic phospholipid molecules (Fig. 4).

Down-regulation of the aminophospholipid translocase activity is an early step in apoptosis (programmed cell death) in lymphocytes (*18*), a step that contributes to the appearance of PS on the cell surface as a recognition signal for phagocytosis. Identification of a homolog in *C. elegans* may aid in elucidating whether loss of lipid asymmetry plays a role in recognition of apoptotic cells during development in this organism.

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19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Principles of Chaperone-Assisted Protein Folding: Differences Between in Vitro and in Vivo Mechanisms

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Molecular chaperones in the eukaryotic cytosol were shown to interact differently with chemically denatured proteins and their newly translated counterparts. During refolding from denaturant, actin partitioned freely between 70-kilodalton heat shock protein, the bulk cytosol, and the chaperonin TCP1–ring complex. In contrast, during cell-free translation, the chaperones were recruited to the elongating polypeptide and protected it from exposure to the bulk cytosol during folding. Posttranslational cycling between chaperone-bound and free states was observed with subunits of oligomeric proteins and with aberrant polypeptides; this cycling allowed the subunits to assemble and the aberrant polypeptides to be degraded. Thus, folding, oligomerization, and degradation are linked hierarchically to ensure the correct fate of newly synthesized polypeptides.

Although the amino acid sequence of a polypeptide chain contains the information that determines the three-dimensional structure of the functional protein (*1*), the folding of many proteins in vivo requires the assistance of a preexistent machinery of molecular chaperone proteins (*2*). These components prevent off-pathway folding reactions that lead to aggregation. Two functionally distinct chaperone families, the 70-kD heat shock proteins (Hsp70s) and the chaperonins, have been implicated in protein folding in the cytosol. The Hsp70s bind to extended hydrophobic peptide segments in an adenosine triphosphate (ATP)–dependent manner that requires the cooperation of members of the DnaJ/Hsp40 family of chaperones (*3*). The chaperonins are large cylindrical complexes consisting of two stacked rings of seven to nine subunits each. Partially folded polypeptides bind within the chaperonin central cavity and reach the native state through multiple, ATP hydrolysis–dependent cycles of binding and release. The bacterial chaperonin GroEL is homo-oligomeric, whereas the eukaryotic chaperonin TCP1–ring complex (TRiC) is composed of eight different but homologous subunits (*4–6*).

Molecular chaperones are thought to have a general role in the folding of newly synthesized polypeptides in vivo (*7–9*). However, our present understanding of chaperone mechanisms relies predominantly on the results of in vitro studies, in which a complete polypeptide, unfolded by chem-

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ical denaturant, is diluted into a solution containing purified chaperones. These studies suggested that the main function of chaperones is to unfold incorrectly folded or kinetically trapped intermediates to prepare them for another trial of folding in the bulk solution (*10, 11*). Consequently, folding would involve the kinetic partitioning of non-native intermediates between different chaperone molecules ("chaperone cycling"). In vivo, however, folding polypeptides are presented in a vectorial manner during translation, and it is unclear whether their interaction with chaperones is governed by the same principles that function when a protein is refolded from denaturant. To address this question, we analyzed the folding of model proteins upon dilution from denaturant and upon translation. In addition to firefly luciferase (~60 kD), the ~50-kD protein actin was chosen as a substrate, because its folding in the cell is known to require TRiC (*12*).

Actin and luciferase (*13*) refolded in a chaperone- and ATP-dependent process upon dilution from guanidinium-HCl into rabbit reticulocyte lysate (cytosol) (Fig. 1A) (*14*). Actin folding was monitored by measuring the specific binding of native actin to deoxyribonuclease I (DNase I) or by native polyacrylamide gel electrophoresis (PAGE) (*13*). During folding, both polypeptides formed complexes with various cytosolic chaperones, including the constitutively expressed Hsp70 (Hsc70), the Hsc70 cofactor Hsp40, and TRiC, as shown by coimmunoprecipitation with chaperone antibodies. When denatured actin (D-actin) was diluted into ATP-depleted cytosol, coimmunoprecipitation of the protein was observed with antibody to Hsc70 (anti-Hsc70) and with anti-Hsp40 but not with TRiC monoclonal antibody (mAb). After a 5-min incubation with ATP, however, actin was predominantly associated with TRiC (Fig. 1B), consistent with a partitioning of unfolded polypeptide between these chaperones. TRiC is critical for actin folding but binds with a relatively slow on-rate (*12*). The initial interaction with the Hsc70 chaperone system may stabilize actin in a folding-competent state.

To examine whether these refolding reactions involved the release of non-native protein into free solution, we used two variants of *Escherichia coli* GroEL—a double-mutant form (*10*) and an internally glutaraldehyde-crosslinked GroEL (*15*)—as "traps" for folding intermediates (T-GroEL) (Fig. 1, C and D). Both traps bind non-native polypeptide with high affinity but are unable to release it despite the presence of ATP (*10, 15*). When increasing concentrations of either type of T-GroEL were added to the reticulocyte lysate, unfolded actin and luciferase were efficiently captured and their refolding was inhibited. Notably, folding was also inhibited when the denatured polypeptides were bound to the cytosolic chaperones first, with subsequent addition of T-GroEL and ATP (Fig. 1, C and D). Thus, the refolding of D-actin and D-luciferase in the reticulocyte lysate involves the release into the bulk solution of non-native intermediates that require further interaction with chaperones to complete folding. In principle, these intermediates could be formed either upon transfer from Hsc70 to TRiC or during iterative cycles of release and rebinding from these chaperones (Fig. 1, C and D).

The mechanism of chaperone action in translation was analyzed in a synchronized translation reaction in the reticulocyte lysate (*16*). Newly synthesized actin chains were detected in a transient complex with TRiC by native PAGE (Fig. 2A). Native actin formed rapidly from the TRiC-bound material upon completion of synthesis (Fig. 2B). This observation indicated that during translation, TRiC mediated the folding of actin with much faster kinetics than it did upon dilution of the chemically denatured protein into the same extract (see Fig. 1). Incomplete actin chains prematurely released from the ribosome remained associated with TRiC. The complex with TRiC formed cotranslationally but was absent very early in translation. The majority of short chains synthesized at this point did not enter the native polyacrylamide gel but could be coimmunoprecipitated with anti-Hsc70. The chain length dependence of the observed chaperone interactions was analyzed for ribosome-bound actin polypeptides generated from truncated mRNAs (*17*). A short chain of ~15 kD (residues 1 to 133) could be coimmunoprecipitated with anti-Hsc70 but not with TRiC mAb, whereas a nascent chain of ~24 kD (residues 1 to 220) interacted with both Hsc70 and TRiC (Fig. 2C). Thus, the folding of actin is likely to involve a sequential interaction of the nascent polypeptide with Hsc70 and TRiC (*8*), consistent with the chaperone interactions observed for D-actin upon dilution into reticulocyte lysate (Fig. 1).

In principle, chaperones may interact primarily with misfolded proteins that must be unfolded to return to a productive folding track. Folding itself would then proceed spontaneously in free solution. Alternatively, the chaperone machinery may be recruited to the elongating polypeptide before any misfolding has occurred and may accompany the protein along its folding pathway. If the first hypothesis is correct, there would be several steps at which T-GroEL might be able to intercept non-native polypeptides (Fig. 3, A and B): T-GroEL

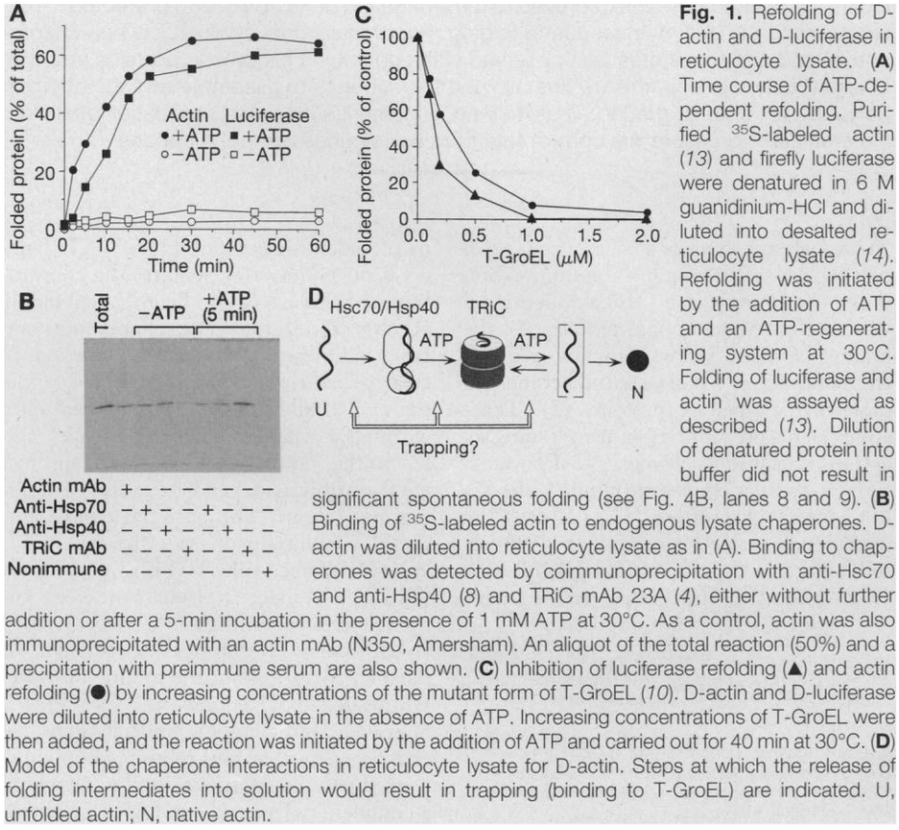

Fig. 1. Refolding of D-actin and D-luciferase in reticulocyte lysate. (**A**) Time course of ATP-dependent refolding. Purified ^{35}S-labeled actin (*13*) and firefly luciferase were denatured in 6 M guanidinium-HCl and diluted into desalted reticulocyte lysate (*14*). Refolding was initiated by the addition of ATP and an ATP-regenerating system at 30°C. Folding of luciferase and actin was assayed as described (*13*). Dilution of denatured protein into buffer did not result in significant spontaneous folding (see Fig. 4B, lanes 8 and 9). (**B**) Binding of ^{35}S-labeled actin to endogenous lysate chaperones. D-actin was diluted into reticulocyte lysate as in (A). Binding to chaperones was detected by coimmunoprecipitation with anti-Hsc70 and anti-Hsp40 (*8*) and TRiC mAb 23A (*4*), either without further addition or after a 5-min incubation in the presence of 1 mM ATP at 30°C. As a control, actin was also immunoprecipitated with an actin mAb (N350, Amersham). An aliquot of the total reaction (50%) and a precipitation with preimmune serum are also shown. (**C**) Inhibition of luciferase refolding (▲) and actin refolding (●) by increasing concentrations of the mutant form of T-GroEL (*10*). D-actin and D-luciferase were diluted into reticulocyte lysate in the absence of ATP. Increasing concentrations of T-GroEL were then added, and the reaction was initiated by the addition of ATP and carried out for 40 min at 30°C. (**D**) Model of the chaperone interactions in reticulocyte lysate for D-actin. Steps at which the release of folding intermediates into solution would result in trapping (binding to T-GroEL) are indicated. U, unfolded actin; N, native actin.

may compete with the endogenous chaperones for binding to the nascent polypeptide, may capture polypeptides as they partition between Hsc70 and TRiC, or (because complete folding requires polypeptide release from the ribosome) may interrupt folding posttranslationally. However, when actin and luciferase were translated in the presence of T-GroEL, no significant inhibitory effect on either the rate or the yield of folding was observed (Fig. 3A), even at concentrations exceeding those required to inhibit refolding of the chemically denatured proteins (*18*). Consistent with this lack of inhibition, T-GroEL did not bind to the newly translated polypeptides (shown for actin in Fig. 3, C and D). This finding indicated a fundamental difference between the actions of chaperones in the refolding of denatured and translating polypeptides. The translating polypeptide is apparently shielded from T-GroEL on its folding pathway until it reaches a conformation from which completion of folding is possible without further chaperone interactions (*18*).

This conclusion was confirmed by analysis of the chaperone-bound state of ribosome-associated and released actin chains that were synthesized in the presence of T-GroEL. As expected, full-length actin was folded and not bound to chaperones. Notably, incomplete nascent chains were bound to TRiC but not to T-GroEL (Fig. 3C, lane 1). Centrifugation through a dense sucrose solution separated the ribosome–nascent chain complexes from chaperonin-bound chains that were prematurely released from the ribosome (*19*) (Fig. 3C). These incomplete chains were enriched in the sucrose solution (Fig. 3C, lane 2). Although most of this material was associated with TRiC, a significant fraction was bound to T-GroEL. When stabilized by cycloheximide, the ribosome–nascent chain complexes barely entered the native polyacrylamide gel (Fig. 3C, lane 3). Incubation with puromycin revealed that these chains were exclusively bound to TRiC (Fig. 3C, lane 4). Thus, T-GroEL was unable to interact with the ribosome–nascent chain complex, but it could intercept those chains that were prematurely released from the ribosome and were unable to fold. Indeed, when T-GroEL and ATP were present during puromycin treatment, the otherwise TRiC-bound chains were captured by T-GroEL (Fig. 3C, lane 5). The exclusion of T-GroEL from binding to nascent chains was not attributable to a steric hindrance by the ribosome, because the somewhat larger chaperonin TRiC bound efficiently to nascent chains (Fig. 3C) (*8*). T-GroEL had a higher affinity for denatured polypeptides than did TRiC (see above); thus, the eukaryotic chaperonin is likely to be specifically recruited to the ribosome–nascent chain complex.

In contrast to proteins that are stably folded as monomers, such as actin and luciferase, the subunits of oligomeric proteins fold first and then assemble. These proteins may thus be released from chaperones as monomers that expose hydrophobic surfaces required for oligomerization. The mechanism of chaperone action in this process was analyzed with the trimeric enzyme chloramphenicol acetyltransferase (CAT) as a substrate. When T-GroEL was added during translation, the synthesis of CAT chains was unimpaired, but the formation of enzymatically active trimer was markedly inhibited (Fig. 3A) (*20*). However, as observed for actin and luciferase, T-GroEL was unable to interact with the ribosome-bound chains of CAT (*20*). Thus, full-length chains were released from the chaperone complex, probably as folded monomers that can cycle on and off the chaperonin until they have trimerized successfully. T-GroEL may have interfered with assembly by binding these monomers on their exposed hydrophobic surfaces. Similar observations were made with newly translated tubulin (*20*).

The sequestration of elongating polypeptides in a complex with chaperones suggested that these chains may be protected from the cellular degradation machinery while they are bound to the ribosome. On the other hand, polypeptides that are unable to fold must eventually be released from the nascent chain–chaperone complex to permit their degradation. We examined the behavior of two COOH-terminally truncated mutant forms of actin (actin-ΔC, comprising amino acids 1 to 220 or 1 to 368) that remained ribosome-bound after translation. Upon release from the ribosome with puromycin, these proteins did not fold (*17*) and were recovered in a complex with TRiC (shown for actin-ΔC 1–220 in Fig. 3D). An additional species, presumably representing free or Hsc70-associated actin-ΔC, migrated on native PAGE somewhat more slowly than did folded full-length actin (Fig. 3D, right panel). Although T-GroEL did not bind to translating actin chains (Fig. 3D, left panel) and did not inhibit folding of the wild-type protein (Fig. 3A), it efficiently captured both forms of newly synthesized actin-ΔC upon release from the ribosome (Fig. 3D, right panel).

Thus, aberrant polypeptides may cycle posttranslationally between chaperone-bound and free states and may thereby partition to the degradation machinery. Upon release into hemin-free lysate, actin-ΔC was indeed rapidly degraded (Fig. 3E). This degradation was ATP dependent and occurred through the proteasome pathway, which is inhibited by hemin (*21*). Ribosome-associ-

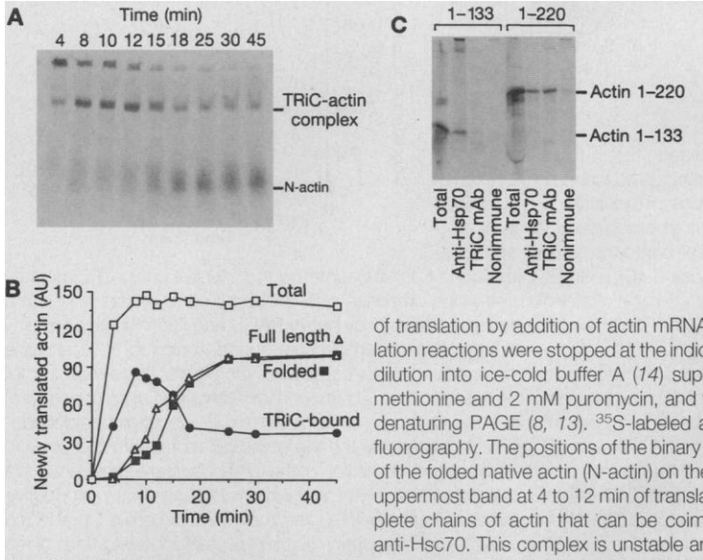

Fig. 2. Chaperone interactions of actin during translation in reticulocyte lysate. (**A**) Native PAGE analysis of a synchronized actin translation in reticulocyte lysate at 30°C (*16*). Translation was synchronized by adding 75 μM aurintricarboxylic acid (ATCA) 3 min after the initiation of translation by addition of actin mRNA. Aliquots of the translation reactions were stopped at the indicated times by threefold dilution into ice-cold buffer A (*14*) supplemented with 2 mM methionine and 2 mM puromycin, and were analyzed by nondenaturing PAGE (*8, 13*). ^{35}S-labeled actin was visualized by fluorography. The positions of the binary complex with TRiC and of the folded native actin (N-actin) on the gel are indicated. The uppermost band at 4 to 12 min of translation represents incomplete chains of actin that can be coimmunoprecipitated with anti-Hsc70. This complex is unstable and leads to the deposition of insoluble material that does not migrate into the gel. The intact complex with Hsc70 appears to migrate somewhat more slowly than does native actin (barely visible at 4 min of translation). (**B**) Folded actin is generated from the TRiC-bound material. The ^{35}S-labeled actin species separated by native PAGE in (A) were quantitated by Phosphorimager analysis (*20*). The amounts of total actin translation products and of full-length actin were quantitated from total translation reactions separated by SDS-PAGE and are given in arbitrary units (AU). (**C**) Chain length dependence of chaperone binding. ^{35}S-labeled nascent actin chains comprising amino acids 1 to 133 (15 kD) or 1 to 220 (24 kD) were synthesized in a lysate programmed with truncated mRNA (*17*). Puromycin-released chains were analyzed by coimmunoprecipitation with antibodies as indicated, followed by SDS-PAGE and fluorography. The efficiency of coimmunoprecipitation with anti-Hsc70 and TRiC mAb was 20 to 40%. Total translation reactions are shown for comparison.

ated actin-ΔC was degraded much more slowly, indicating a significant degree of protection of the nascent chain (Fig. 3E) (*22*). Full-length actin was not degraded when assayed under the same conditions (Fig. 3E). Inclusion of T-GroEL during the incubation with puromycin completely prevented the degradation of actin-ΔC (Fig. 3E), as all the polypeptide was transferred to the trap (see Fig. 3D). Apparently, the proteasome degradation machinery cannot access a polypeptide as long as it is bound within the central cavity of the chaperonin (*23*).

The final steps in the folding of actin are mediated by TRiC. To test whether the protection of the translating chain from T-GroEL is solely explained by the functional coupling of the Hsc70 and TRiC systems or whether it is also a result of the action of TRiC itself (*24*), we analyzed the folding of D-actin by purified TRiC (*25*). D-actin folded efficiently from an isolated binary complex with TRiC in an ATP-dependent manner (Fig. 4A). The addition of a 20-fold molar excess of T-GroEL to the actin-TRiC complex did not inhibit folding (Fig. 4A). Analysis of the reaction by native gel revealed a low amount of actin binding to T-GroEL, but this binding had no effect on the amount of folded actin and was probably the result of some structural lability of TRiC (Fig. 4A) (*26*). In contrast, when D-actin was diluted into a solution containing both TRiC and T-GroEL, the latter competed effectively with TRiC for binding of D-actin (see Fig. 1). Thus, T-GroEL could bind unfolded actin but was not capable of interrupting its TRiC-mediated folding. Either actin was not released into the bulk solution during folding, or it was released after TRiC caused conformational changes that rendered the protein unable to interact with T-GroEL. In any case, the trapping phenomenon observed upon dilution of D-actin into reticulocyte lysate must have resulted from the interruption by T-GroEL of the polypeptide transfer between Hsc70 and TRiC (Fig. 1). The lack of inhibition by T-GroEL during translation thus indicated the functional coupling of these chaperones in de novo folding.

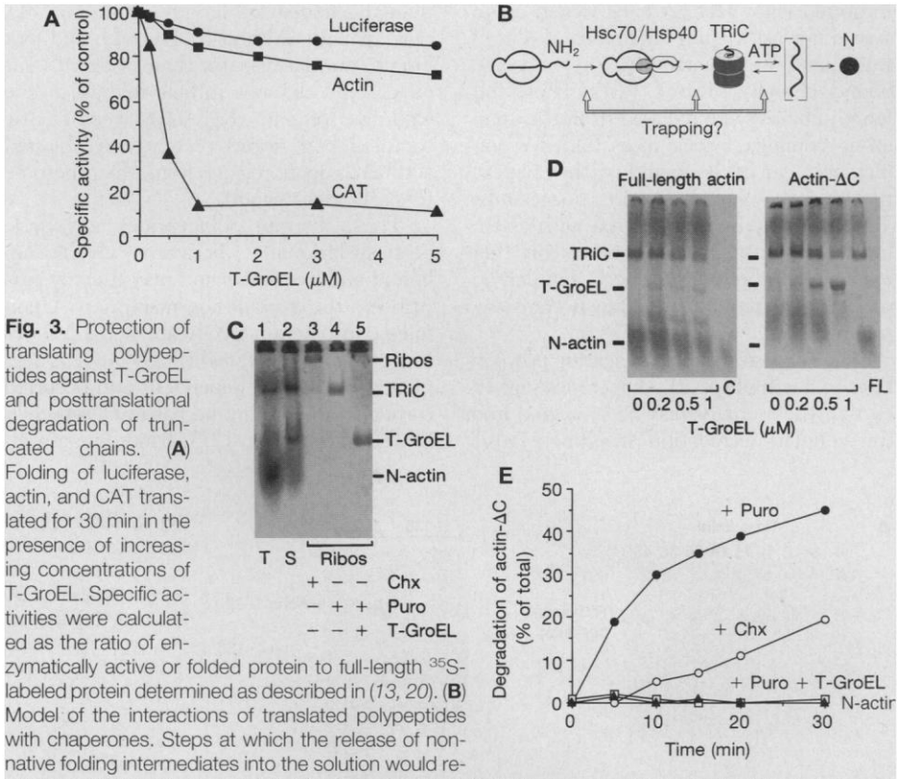

Fig. 3. Protection of translating polypeptides against T-GroEL and posttranslational degradation of truncated chains. (**A**) Folding of luciferase, actin, and CAT translated for 30 min in the presence of increasing concentrations of T-GroEL. Specific activities were calculated as the ratio of enzymatically active or folded protein to full-length ^{35}S-labeled protein determined as described in (*13, 20*). (**B**) Model of the interactions of translated polypeptides with chaperones. Steps at which the release of nonnative folding intermediates into the solution would result in trapping are indicated. (**C**) T-GroEL is excluded from ribosome-bound nascent chains. Ribosomes were isolated from actin translations performed in the presence of T-GroEL by centrifugation through a 30% sucrose solution (*19*). ^{35}S-labeled chains were analyzed by native PAGE and fluorography. Lane 1, total translation reaction (T); lane 2, prematurely released chaperonin-bound chains in 30% sucrose solution (S). Lanes 3 to 5: isolated ribosome–nascent chain complexes (ribos) stabilized with 2 mM cycloheximide (chx) (lane 3), after release of the nascent chains by incubation with 2 mM puromycin (puro) (lane 4), and after incubation with puromycin in the presence of 0.5 μM T-GroEL (in its glutaraldehyde-crosslinked form) and 1 mM ATP (lane 5). Insoluble material at the top of the gel represents mostly actin chains that have fallen off the ribosome during prolonged incubation of the extract and are either bound to Hsc70 or have self-aggregated. (**D**) T-GroEL does not interact with newly translated actin that is productive for folding but binds to nonproductive truncated chains after their release from TRiC. Either full-length actin or actin-ΔC 1–220 was translated for 30 min at 30°C in the presence of the indicated concentrations of the glutaraldehyde-crosslinked form of T-GroEL. The reactions were stopped with puromycin, and the ^{35}S-labeled newly translated polypeptides were analyzed by native PAGE and fluorography. Native ^{35}S-labeled actin purified from CHO cells (C) and a translation reaction containing full-length actin (FL) are also shown. (**E**) Degradation of actin-ΔC requires release from the ribosome. Actin-ΔC 1–220 was accumulated on the ribosome by translation at 30°C for 30 min. The complex was stabilized with cycloheximide and desalted to remove hemin, an inhibitor of the proteasome pathway (*21*). The lysate was supplemented with ATP and an energy-regenerating system [as in (*14*)] and incubated at 37°C for the times indicated, in the presence of cycloheximide, puromycin, or puromycin and 1 μM mutant T-GroEL (*10*) (Puro + T-GroEL). As a control, full-length translated actin, treated identically to the 1–220 sample, was incubated in the presence of puromycin (N-actin).

Our results provide insight into the basic principles by which molecular chaperones control the fate of newly synthesized polypeptides. Folding in the mammalian cytosol is mediated by chaperone components that are selectively recruited to the emerging nascent chain (Fig. 4C, **1**). This mechanism ensures that the elongating polypeptide is protected against aggregation and degradation. In the case of monomeric proteins such as actin and luciferase, the full-length polypeptide dissociates from the ribosome as a complex with TRiC and can reach the native state without the intermittent release of unfolded forms into the cytosol (Fig. 4C, **2**). In contrast, the subunits of oligomeric enzymes and polypeptides that are unable to fold may cycle posttranslationally between different chaperone systems and the bulk solution. This cycling allows subunits to assemble (Fig. 4C, **3**) and allows incomplete or mutated polypeptides to be degraded (Fig. 4C, **4**). The ubiquitin-proteasome system would only have access

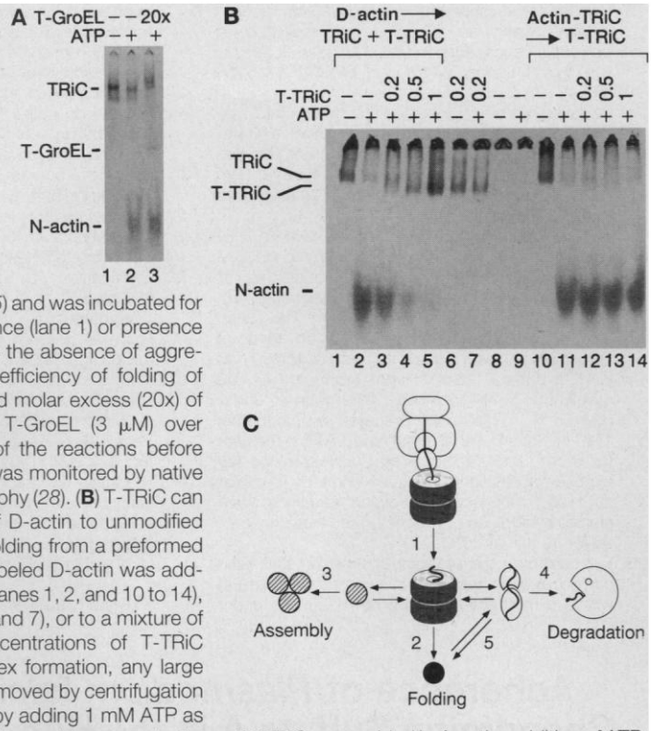

Fig. 4. Actin folding by TRiC without release of non-native intermediates into the bulk solution. (**A**) ATP-dependent folding of actin from a preformed complex with purified TRiC (*25*) is not inhibited by T-GroEL. The complex between ^{35}S-labeled D-actin and TRiC (0.15 μM) was isolated by ion exchange chromatography (*25*) and was incubated for 40 min at 30°C in the absence (lane 1) or presence (lane 2) of 1 mM ATP. Note the absence of aggregated actin and the high efficiency of folding of TRiC-bound actin. A 20-fold molar excess (20x) of glutaraldehyde-crosslinked T-GroEL (3 μM) over TRiC was added to one of the reactions before adding ATP. Actin folding was monitored by native PAGE followed by fluorography (*28*). (**B**) T-TRiC can compete for the binding of D-actin to unmodified TRiC but does not inhibit folding from a preformed TRiC-actin complex. ^{35}S-labeled D-actin was added to 0.2 μM purified TRiC (lanes 1, 2, and 10 to 14), to 0.2 μM T-TRiC (lanes 6 and 7), or to a mixture of TRiC and increasing concentrations of T-TRiC (lanes 3 to 5). After complex formation, any large aggregates formed were removed by centrifugation and refolding was initiated by adding 1 mM ATP as indicated. In lanes 12 to 14, increasing concentrations of T-TRiC were added before the addition of ATP. Omission of either form of chaperonin in the reaction resulted in the formation of actin aggregates that did not migrate into the gel (lanes 8 and 9). In this experiment, the actin-TRiC complex was not purified by ion exchange chromatography as in (A); this explains the presence of aggregated actin at the top of the gel, which does not participate in the folding reaction. (**C**) Interaction of newly synthesized polypeptides with chaperones, shown schematically for proteins that use the Hsc70 system and TRiC for folding or assembly. Reactions **1** to **4** are folding, assembly, and degradation reactions (see text for details). In **5**, proteins denatured during stress are stabilized by the Hsp70 system, possibly in association with Hsp90 or Hsp104 (*31*); kinetic partitioning may then lead to either refolding or degradation.

to a newly synthesized polypeptide after it has been given a chance to fold, consistent with a functional hierarchy of folding and degradation.

A cooperative action of Hsc70 and TRiC is probably important in vivo for the efficient folding of a subset of polypeptides with aggregation-sensitive folding pathways, including the abundant cytoskeletal proteins actin and tubulin. A functional coupling of translation and chaperone action may be achieved at three distinct stages of this pathway:

1) Hsc70 may be directed to the ribosome by an interaction with other components of the translation machinery even before the polypeptide chain emerges. For example, binding of the nascent chain–associated complex (NAC) precedes the interaction of the translating chain with Hsc70 (*29*) and may be involved in recruiting Hsc70 and Hsp40.

2) The functional cooperation with the Hsc70 system may then facilitate the selective binding of TRiC (*8*), which may recognize specific structural elements exposed by those translating polypeptides that require the chaperonin for folding.

3) TRiC can mediate folding within its central cavity without releasing the polypeptide in a non-native form (*20*, *30*). Presentation of the nascent polypeptide as a complex with Hsc70 may allow folding on TRiC to occur efficiently, perhaps in a single cycle of ATP-dependent protein release.

On the basis of our observations, caution should be exercised when extrapolating from in vitro studies to the functional properties of chaperones in de novo folding. The interactions of chemically denatured proteins with chaperones may resemble those occurring in the cell upon exposure to various forms of stress, such as high temperature (Fig. 4C, **5**). Under these conditions, the processivity of the de novo folding pathway is probably replaced by a distributional mechanism that is governed primarily by the kinetic partitioning of folding intermediates between different chaperone systems.

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13. ^{35}S-labeled actin was purified from metabolically labeled CHO cells by affinity chromatography on a DNase I–Sepharose column on the basis of the high affinity of folded actin for DNase I [K. Zechel, *Eur. J. Biochem.* **110**, 343 (1980)]. Actin folding was monitored both by native PAGE analysis on 3 to 10% polyacrylamide gels (*8*) and by binding of folded actin to DNase I–Sepharose beads with native CHO ^{35}S-labeled actin as a control. Identical results were obtained with either actin folding assay. Firefly luciferase was purchased from Sigma. Luciferase folding was assayed as described with the use of a commercially available assay system (Promega) as in (*8*).
14. Purified proteins were denatured in 6 M guanidinium-HCl, 5 mM dithiothreitol (DTT), and 50 mM Hepes-KOH (pH 7.4) for 30 min at 30°C and diluted 100-fold into reticulocyte lysate (Promega) desalted in buffer A [20 mM Hepes-KOH (pH 7.4), 100 mM KAcO, 5 mM $Mg(AcO)_2$, 0.5 mM EDTA, and 1 mM DTT]. After removing protein aggregates by centrifugation (20,000*g* for 15 min at 4°C), refolding was initiated by addition of 1 mM ATP and an ATP regenerating system [creatine kinase (50 μg/ml) and 8 mM phosphocreatine], as described [E. Nimmesgern and F. U. Hartl, *FEBS Lett.* **331**, 25 (1993)]. At different time points, the reactions were stopped on ice by the addition of 10 mM *trans*-1,2-cyclohexanediaminetetraacetate (CDTA). Both luciferase and actin aggregated when diluted into either buffer A or buffer A containing bovine serum albumin (5 mg/ml). Coimmunoprecipitation with chaperone antibodies was performed as described after stopping the reactions with apyrase (0.5 U/μl) (*8*).
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16. Translation reactions containing 50% of the final volume of nuclease-treated reticulocyte lysate (Promega) were performed as described (*8*). The volume of the reaction was adjusted by addition of buffer A. Actin mRNA was produced by in vitro transcription of pGEM–mouse β-actin linearized with Xho I [K. Tokunaga, H. Taniguchi, K. Yoda, M. Shimizu, S. Sakiyama, *Nucleic Acids Res.* **14**, 2829 (1986)], provided by J. Shephard. Luciferase mRNA was transcribed from the plasmid pGEM-luc (*8*).
17. Polypeptides translated from an mRNA lacking a stop codon remain ribosome-bound [A. H. Erickson and G. Blobel, *Methods Enzymol.* **96**, 38 (1983)]. The peptidyl-tRNA complex was stabilized with 2 mM

cycloheximide or released with 2 mM puromycin in buffer A. Ribosome-bound actin-ΔC was derived from RNAs transcribed from pGEM–mouse β-actin linearized with Apa I (amino acids 1 to 368), Xba I (amino acids 1 to 220), or Sna BI (amino acids 1 to 133). Coimmunoprecipitation with chaperones was performed as in (*8*). Truncated actin chains remained unfolded, as indicated by their sensitivity toward proteinase K and by their inability to bind to DNase I. Similar results were obtained with each truncation in the degradation assay.

18. The addition of T-GroEL at 1 and 3 μM reduced the efficiency of translation by ~30%, which explains the decrease in the amount of native actin seen in Fig. 3D. Quantification of the amount of full-length actin in these translations indicated that there was no decrease in the fraction of folded actin (see Fig. 3A). Addition of T-GroEL reduced the amount of TRiC-bound actin chains because the translation reaction contains incomplete chains that are unable to fold. In contrast to T-GroEL, addition of T-TRiC [see (*25*) and Fig. 4B] inhibited the folding of actin when added from the beginning of translation.
19. Ribosomes were isolated by sedimentation through a sucrose solution as described (*8*). A 50-μl actin translation reaction was diluted threefold in buffer B [buffer A containing 5 mM $Mg(AcO)_2$, 0.5 mM cycloheximide, 1 mM DTT, RNAsin (Promega, 1 U/μl), 1 mM phenylmethylsulfonyl fluoride, aprotinin (2 μg/ml), and leupeptin (0.5 μg/ml)], and ATP was removed by incubation with apyrase (0.5 U/μl for 5 min at 25°C). The reaction was centrifuged for 10 min at 16,000*g* to remove aggregates and placed over 350 μl of 30% sucrose in buffer B. Ribosomal pellets obtained by centrifugation for 12 min at 120,000 rpm in a TL-120.1 rotor at 4°C were washed twice in buffer B, resuspended in the initial volume of buffer C [20 mM Hepes-KOH (pH 7.4), 100 mM KAcO, 5 mM $Mg(AcO)_2$, 10% glycerol, 5% polyethylene glycol, and 1 mM DTT] and centrifuged (16,000*g*, 10 min) to remove insoluble material.
20. CAT assays were performed as described [J. P. Hendrick, T. Langer, T. A. Davis, F. U. Hartl, M. Wiedmann, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10216 (1993)]. Native purified CAT (Boehringer) was used as a control; pGEM-CAT was provided by M. Wiedmann. Specific activities were calculated as the ratio of the measured enzymatic activity to the amount of full-length ^{35}S-labeled protein as determined by SDS-PAGE and Phosphorimager analysis. Native PAGE of CAT translation reactions resolved TRiC-bound chains from assembled CAT trimers. The decrease in CAT activity upon addition of T-GroEL was accompanied by an increase in the amount of newly translated full-length CAT bound to T-GroEL. Transfer from TRiC to T-GroEL was also observed for newly translated α-tubulin. This protein can fold on TRiC into its GTP-binding conformation (*30*), but before the formation of tubulin dimers exposes hydrophobic surfaces that may allow it to cycle on and off the chaperonin. Thus, the exclusion of T-GroEL from productive nascent chains of actin and luciferase cannot be explained by an intrinsic lack of binding capacity of T-GroEL for newly translated polypeptide.
21. Hemin is essential for translation in reticulocytes but inhibits proteasome function [A. L. Haas and I. A. Rose, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6845 (1981)]; we removed it by desalting the lysates containing ribosome-associated actin-ΔC on Sephadex G-25 columns equilibrated in buffer A. Actin-ΔC was degraded through the proteasome-ubiquitin pathway, because degradation was inhibited by hemin (with the accumulation of ubiquitin conjugates) and by methylated ubiquitin [A. Hershko, D. Ganoth, J. Pehrson, R. E. Palazzo, L. H. Cohen, *J. Biol. Chem.* **266**, 16376 (1991)]. Similar results were obtained for actin-ΔC 1–220 and actin-ΔC 1–368.
22. The protection of nascent actin chains against degradation was underestimated because of the spontaneous release of nascent chains from the ribosome at 37°C (Fig. 3E), which we measured by including hemin during the incubation with cycloheximide and separating ribosome-bound and released chains by sedimentation through a dense sucrose solution (*19*).
23. Release of the chaperone as a requirement for degradation was also observed for Hsp70-bound polypeptides in mitochondria [I. Wagner, H. Arlt, L. van Dyck, T. Langer, W. Neupert, *EMBO J.* **13**, 5135 (1994)].
24. Evidence has been presented that during TRiC-mediated folding, actin undergoes cycles of ATP-dependent release and rebinding involving its discharge into the solution in a non-native state [G. Tian, I. E. Vainberg, W. D. Tap, S. A. Lewis, N. J. Cowan, *Nature* **375**, 250 (1995)].
25. TRiC was purified from bovine testes as in (*5*). ^{35}S-labeled D-actin was diluted 100-fold into 0.2 μM TRiC in buffer C (*19*). After incubation for 5 min at 30°C, aggregates were removed by centrifugation. Unbound actin was separated from TRiC-actin complexes by chromatography on a Mono-Q HR5/5 column with a gradient from 100 to 400 mM KAcO in buffer D [20 mM Hepes-KOH (pH 7.4), 100 mM KAcO, 5 mM $Mg(AcO)_2$, 10% glycerol, and 1 mM DTT]. T-TRiC was prepared by incubating TRiC in buffer D with 2 mM 8-azido-ATP in the dark for 5 min at 30°C, followed by crosslinking [by exposure to UV light (310 to 340 nm) for 10 min at 4°C] and repurification. Folding reactions were started by addition of 1 mM ATP and were incubated for 40 min at 30°C.
26. We confirmed the previous observation that actin can be released from TRiC in a non-native state (*24*) with a diluted actin-TRiC complex (25 nM) under conditions where we found bovine TRiC to be structurally labile. The lack of inhibition of actin folding by T-GroEL observed with native PAGE (Fig. 4A) was confirmed by the DNase I binding assay.
27. The crosslinked form (T-TRiC) displayed a faster mobility on native PAGE than did TRiC (Fig. 4B), either because of the added negative charge conferred by the covalently bound nucleotide or because of a conformational change triggered by nucleotide binding.
28. Identical results were obtained for T-GroEL and T-TRiC when actin folding was analyzed as a function of binding to DNase I–Sepharose beads.
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32. We thank M. Wiedmann for pGEM-CAT, J. Shephard for pGEM–mouse β-actin, and M. Mayhew, D. McColl, and B. Netzer for critical reading of the manuscript. Supported by NIH and the Howard Hughes Medical Institute.

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Adherence of *Plasmodium falciparum* to Chondroitin Sulfate A in the Human Placenta

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Women are particularly susceptible to malaria during first and second pregnancies, even though they may have developed immunity over years of residence in endemic areas. *Plasmodium falciparum*–infected red blood cells (IRBCs) were obtained from human placentas. These IRBCs bound to purified chondroitin sulfate A (CSA) but not to other extracellular matrix proteins or to other known IRBC receptors. IRBCs from nonpregnant donors did not bind to CSA. Placental IRBCs adhered to sections of fresh-frozen human placenta with an anatomic distribution similar to that of naturally infected placentas, and this adhesion was competitively inhibited by purified CSA. Thus, adhesion to CSA appears to select for a subpopulation of parasites that causes maternal malaria.

Falciparum malaria can present with various clinical manifestations, including coma, pulmonary edema, renal insufficiency, and severe anemia. The molecular basis for particular syndromes is unknown, although IRBC cytoadherence is generally considered to contribute to the development of cerebral malaria (*1*), and some authors have suggested that virulent strains (*2*) or "virulence factors" (*3*) of *P. falciparum* cause severe disease or death. Maternal malaria, a distinct clinical entity, is a major cause of pregnancy-related complications in endemic areas; it is associated with premature delivery, intrauterine growth retardation, perinatal mortality in the infant, and anemia and death in the mother (*4*, *5*).

After years of exposure to malaria, women have acquired immunity to *P. falciparum* equivalent to their male counterparts (*6*). Pregnant women, however, have a unique susceptibility to malaria infection, which diminishes as their gravid status increases (*5*). In the absence of evidence for other mechanisms, maternal malaria has been attributed to the immunosuppression of pregnancy (*7*), which is also thought to lead to increased frequency of certain viral infections such as rubella (*8*). Because susceptibility to infection is markedly lower in multigravid women than in primigravid women, immunosuppression cannot entirely explain the phenomenon (*9*).

The placenta is a preferential site for sequestration of IRBCs and can experience high parasite densities while the peripheral circulation is free of parasites (*6*, *10*). In other vascular beds, IRBCs adhere to the endothelial surface; endothelial surface molecules such as thrombospondin, CD36, intercellular adhesion molecule–1 (ICAM-1),

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