The EMBO Journal Vol.18 No.1 pp.75–84, 1999

Compartmentation of protein folding *in vivo*: sequestration of non-native polypeptide by the chaperonin–GimC system

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The functional coupling of protein synthesis and chaperone-assisted folding *in vivo* has remained largely unexplored. Here we have analysed the chaperonin-dependent folding pathway of actin in yeast. Remarkably, overexpression of a heterologous chaperonin which traps non-native polypeptides does not interfere with protein folding in the cytosol, indicating a high-level organization of folding reactions. Newly synthesized actin avoids the chaperonin trap and is effectively channelled from the ribosome to the endogenous chaperonin TRiC. Efficient actin folding on TRiC is critically dependent on the hetero-oligomeric co-chaperone GimC. By interacting with folding intermediates and with TRiC, GimC accelerates actin folding at least 5-fold and prevents the premature release of non-native protein from TRiC. We propose that TRiC and GimC form an integrated 'folding compartment' which functions in cooperation with the translation machinery. This compartment sequesters newly synthesized actin and other aggregation-sensitive polypeptides from the crowded macromolecular environment of the cytosol, thereby allowing their efficient folding.

Keywords: actin/chaperonin-assisted folding/GimC/TRiC/yeast

Introduction

A significant fraction of newly synthesized polypeptides in a given cell folds in a reaction assisted by molecular chaperones. The primary function of molecular chaperones is to promote productive folding by preventing off-pathway folding reactions which lead to protein aggregation (Ellis, 1987; Rothman, 1989; Gething and Sambrook, 1992; Hartl, 1996; Johnson and Craig, 1997). Two classes of ATP-dependent chaperones, the heat shock protein 70 (Hsp70s) and the chaperonins, have been implicated in *de novo* protein folding in the cytosol.

The Hsp70s, in cooperation with members of the Hsp40 family, bind and release hydrophobic segments of unfolded polypeptides and can interact with nascent polypeptide chains prior to their folding (Hartl, 1996; Johnson and Craig, 1997). The majority of nascent chains in yeast and mammalian cells form a complex with Hsp70 (Eggers *et al*., 1997; James *et al*., 1997; Pfund *et al*., 1998), but only a subset of newly synthesized polypeptides require a chaperonin for folding to the native state (Yaffe *et al*., 1992; Horwich *et al*., 1993; Sternlicht *et al*., 1993; Ewalt *et al*., 1997). The chaperonins are large cylindrical protein complexes consisting of two stacked rings of seven to nine subunits each (Hartl, 1996; Fenton and Horwich, 1997). Group I chaperonins, such as GroEL of *Escherichia coli*, function in conjunction with a ring-shaped cofactor, GroES, that forms the lid on a folding cage in which a wide range of aggregation-sensitive polypeptides are enclosed during folding. In contrast, such a cofactor has not been found for the distantly related group II chaperonins of archaea and eukarya. The chaperonin of the eukaryotic cytosol, known as TRiC or CCT [for t-complex polypeptide 1 (Tcp1) ring complex or chaperonin containing Tcp1, respectively], is hetero-oligomeric, containing eight different subunits per ring (Kubota *et al*., 1995; Lewis *et al*., 1996; Liou and Willison, 1997). Recent structural evidence for the archaeal group II chaperonin suggests that TRiC may also form a cage structure (Klumpp *et al*., 1997; Ditzel *et al*., 1998). Opening and closing of the cage is thought to be mediated by ATP-dependent conformational changes in the TRiC subunits, not by a GroES-like co-factor. Multiple reaction cycles of polypeptide release into the cage and rebinding may be required for folding. Only a few substrates of TRiC are known, including actin, tubulin (Kubota *et al*., 1995; Lewis *et al*., 1996) and Gα-transducin (Farr *et al*., 1997).

The mechanics of some chaperone systems are now well understood through *in vitro* studies. There is, however, much debate as to the functional integration between the various elements of this machinery at the cellular level. According to the pathway model, newly synthesized polypeptides may be channelled through a specific set of chaperone interactions which have been optimized during evolution to ensure efficient folding. For example, newly synthesized actin may first interact with Hsp70 and then be passed on to the chaperonin TRiC (Hartl, 1996; Netzer and Hartl, 1998). An alternative view holds that chaperone interactions are stochastic; proteins in non-native conformations are thought to partition freely through the cytosol, between the available chaperones and the machinery for proteolytic degradation (Buchberger *et al*., 1996; Farr *et al*., 1997; Fenton and Horwich, 1997). The term 'chaperone networks' has been coined to describe this *modus operandi* of chaperones, based on *in vitro* studies with purified components (Buchberger *et al*., 1996). For proteins such as actin and tubulin, the proposed stochastic partitioning could result if non-native states were released by the chaperonin into the cytosol in every

chaperonin reaction cycle, and were able to rebind to competing chaperones during assisted protein folding (Farr *et al*., 1997).

To provide critical *in vivo* evidence in favour of either of these models of chaperone action, we analysed the chaperonin-dependent folding pathway of actin in yeast. Our results indicate that chaperonin-assisted folding in the eukaryotic cytosol follows a sequestered pathway in which functionally coupled chaperone interactions minimize the exposure of newly synthesized, non-native proteins to the bulk cytosol. Leakage of non-native forms of actin into the cytosol is however observed when either TRiC or its novel cofactor, GimC (genes involved in microtubule biogenesis complex; Geissler *et al*., 1998), are functionally defective. Similar to GroES in the case of group I chaperonins, GimC is required for chaperonin-assisted folding to occur in a protected environment, but accomplishes its co-chaperone function by a novel mechanism.

Results

Rapid folding and transit of newly synthesized actin through chaperonin

The following criteria suggested actin as an excellent model protein for performing an analysis of chaperonin-assisted protein folding *in vivo*. Actin folding is critically dependent on the chaperonin TRiC (Gao *et al*., 1992; Sternlicht *et al*., 1993; Chen *et al*., 1994; Ursic *et al*., 1994) and can be monitored by taking advantage of the fact that only native (or native-like) actin forms a stable binary complex with deoxyribonuclease I (DNase I) (Lazarides and Lindberg, 1974; Kabsch *et al*., 1990). This assay measures the production of folded monomeric actin. Spheroplasts of *Saccharomyces cerevisiae* were labelled with [^{35}S]methionine/cysteine for 80 s at 30°C, and then chased in the presence of unlabelled methionine/cysteine and cycloheximide to inhibit protein synthesis. At different time-points spheroplasts were lysed in the presence of EDTA to stop the ATP-dependent action of TRiC, and folded actin was precipitated with DNase I–Sepharose beads. During the chase, the amount of actin which bound to DNase I increased with an apparent half-time of ~1 min (Figure 1A). Equally rapid actin folding was measured in HeLa cells (not shown). Thus, actin folding *in vivo* proceeds at least 10–20-times faster than the chaperonin-assisted re-folding of denatured actin *in vitro* (e.g. Melki and Cowan, 1994) and probably involves only a few chaperonin reaction cycles.

To compare the rate of actin folding with the rate of actin transit through TRiC, a yeast strain was constructed in which the gene encoding the essential TRiC subunit Tcp1p (Cct-1p) was replaced with a version of TCP1 encoding a C-terminal c-myc epitope. The function of TRiC is fully preserved in this strain (see Miklos *et al*.,

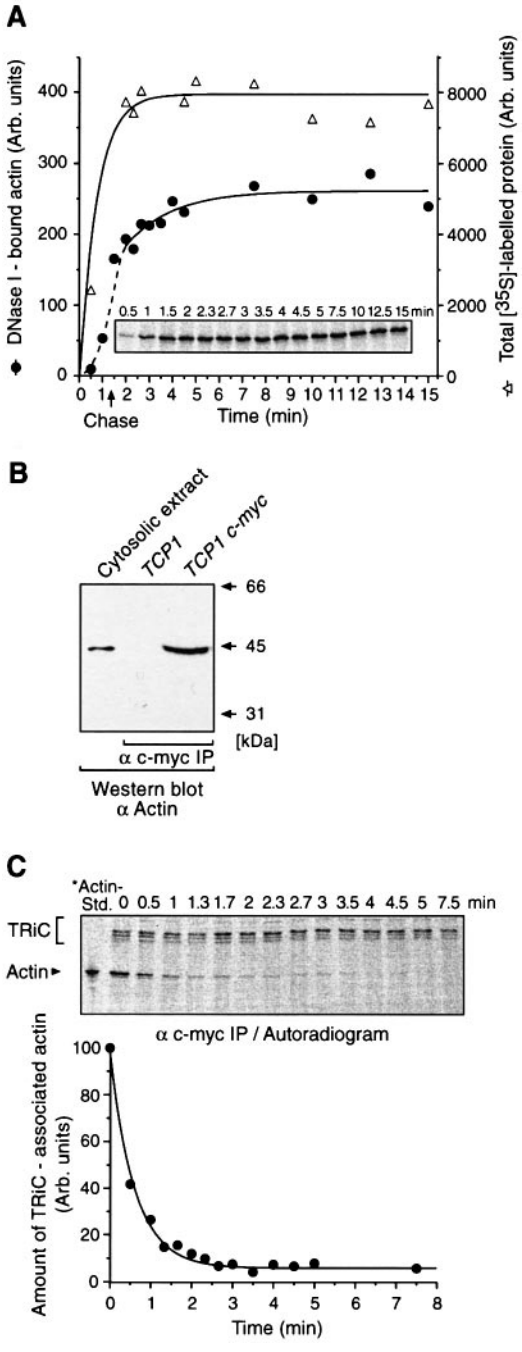

Fig. 1. Rapid kinetics of actin folding and transit through TRiC *in vivo*. (**A**) Kinetics of actin folding. Yeast spheroplasts were labelled for 80 s at 30°C followed by a chase with cycloheximide and unlabelled methionine/cysteine. At the time-points indicated, cells were lysed and actin precipitated by DNase I–Sepharose beads. Bound actin was analysed by SDS–PAGE (insert) and quantified using a phosphoimager. The incorporation of [^{35}S]methionine/cysteine into total polypeptides is shown to demonstrate the efficiency of the chase. Values before the chase (dotted line) were excluded from a single exponential fitting of the data points. (**B**) Anti-actin Western blot analysis of a cytosolic yeast extract (from TCP1 c-*myc* cells) and of anti-c-myc immunoprecipitates from extracts of TCP1 cells and TCP1 c-*myc* cells. In the latter strain actin is specifically co-immunoprecipitated with the c-myc-tagged TRiC complex. (**C**) Transit of newly synthesized actin through TRiC. Spheroplasts of TCP1 c-*myc* cells were pulse–chase labelled as in (A). During the chase TRiC was immunoprecipitated and analysed by SDS–PAGE and autoradiography (upper panel). Purified ^{35}S-labelled actin (*actin) is used as a standard. Note that the hetero-oligomeric TRiC complex migrates as a group of bands between 50–60 kDa which are only partially labelled within the short labelling time. Actin was quantified by a phosphoimager and the amount of TRiC-bound actin at the beginning of the chase was set to 100. The data points were fitted to a single exponential function. The cellular chaperonin capacity in actin folding was estimated based on a doubling time of yeast of 90 min and on the following parameters: total protein in the cytosol, 200 mg/ml; actin content, 0.1% of total protein (Karpova *et al*., 1995), i.e. 5 μM in cytosol; TRiC, 0.3 μM in cytosol (this study); rate of folding, ~0.7/min.

1994). Immunoprecipitation of TRiC with anti-c-myc antibody from unlabelled cell extracts, followed by Western blotting with anti-actin antibody, showed that significant steady-state levels of actin were specifically associated with TRiC (Figure 1B). Indeed, pulse–chase labelling experiments followed by immunoprecipitation of TRiC revealed that full-length actin was by far the major labelled polypeptide which associated with the chaperonin (Figure 1C). A number of additional TRiC-bound polypeptides were detected after longer exposure of the gels, including the tubulins that are of relatively lower abundance in *S.cerevisiae* (Barnes *et al*., 1990). The interaction between actin and TRiC occurred immediately upon synthesis. During the chase, newly synthesized actin dissociated from TRiC with a half-time of ~1 min (Figure 1C), in excellent agreement with the rate of actin folding determined above. Based on a concentration of TRiC in the cytosol of ~0.3 μM (see Materials and methods), we estimate that the chaperonin has more than twice the capacity to fold all cellular actin (see Figure 1 legend). The agreement of the rates of actin folding and transit through TRiC indicates that newly synthesized actin leaves TRiC either in its native state, or in a largely native conformation which completes folding rapidly and independently of the chaperonin.

Expression of chaperonin trap in the yeast cytosol

If chaperonin-assisted folding involves the free partitioning of unfolded species between different chaperones through the bulk cytosol, continuous expression of a mutated, heterologous chaperonin which binds, but does not release, unfolded polypeptides should interfere with the folding of actin and many other proteins in the cytosol. Such 'chaperonin traps' have been characterized for *E.coli* GroEL (Weissman *et al*., 1994). Here we used the GroEL mutant D87K, which is defective in ATP hydrolysis and binds stably to non-native polypeptides with the same high affinity as wild-type GroEL (Farr *et al*., 1997). Remarkably, we found that high-level expression of GroEL D87K (Trap-GroEL or T-GroEL) from an efficient copper-inducible promoter did not inhibit the growth of several yeast strains tested (Figure 2A). As will be shown below, trapping of newly synthesized actin by T-GroEL was only observed when the endogenous chaperonin system was defective.

T-GroEL was diffusely distributed in the cytosol, as judged by immunofluorescence analysis (not shown), and expressed to a concentration 10–30-times that of endogenous TRiC (i.e. ~3–9 μM; see Figure 2 legend). More than 80% of T-GroEL was correctly assembled to the ~800 kDa chaperonin, as determined by gel filtration and native PAGE (Figure 2B). GroEL has been shown to bind to non-native actin with higher affinity than TRiC (Melki and Cowan, 1994). Indeed, after cell lysis the assembled T-GroEL expressed in yeast was fully competent in binding denatured actin (Figure 2B and C), out-competing the much less abundant endogenous TRiC in these extracts. To determine the free actin-binding capacity of T-GroEL, radiolabelled actin was diluted from denaturant into cytosolic extracts containing either *in vivo* expressed T-GroEL or defined amounts of added purified T-GroEL (Figure 2C). Actin–T-GroEL complexes were analysed by native PAGE (Gao *et al*., 1992). The cytosol of T-GroEL-

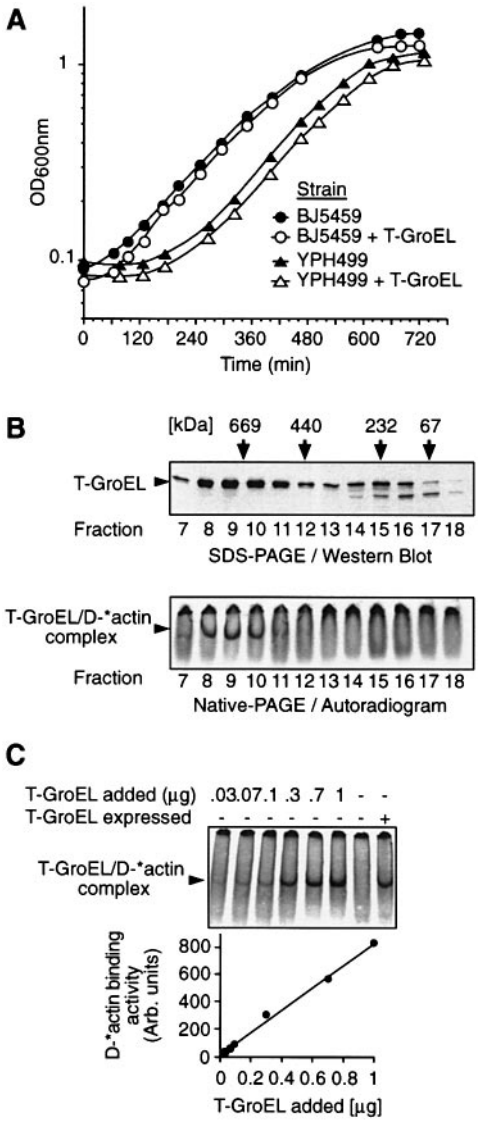

Fig. 2. Expression of functional GroEL chaperonin trap is well tolerated by yeast. (**A**) Growth rates of the strains BJ5459 and YPH499 either with or without expression of T-GroEL. Similar results were obtained with other strains used in this study (data not shown). The concentration of T-GroEL oligomer in the cytosol of these cells was estimated at 3–9 μM by quantitative immunoblotting of cytosol extracts with an affinity-purified GroEL antibody and purified T-GroEL as a standard, assuming a total protein concentration in the yeast cytosol of 200 mg/ml (not shown). (**B**) Binding of denatured actin to oligomeric T-GroEL. Upper panel, size exclusion chromatography of cytosol extract from yeast cells expressing T-GroEL followed by SDS–PAGE and Western blotting of the fractions with anti-GroEL antibody. Assembled T-GroEL oligomer fractionates at ~800 kDa. Non-assembled and partially degraded T-GroEL fractionates between 100–250 kDa. Lower panel, denatured ^{35}S-labelled actin (D-*actin) was added to each fraction from the sizing column and analysed by native PAGE and autoradiography. The T-GroEL–actin complex shows the typical migration of oligomeric GroEL on these gels (Frydman and Hartl, 1996; Farr *et al*., 1997). (**C**) Determination of the binding capacity of T-GroEL in yeast cytosol for D-*actin by native PAGE. The assay was calibrated by adding defined amounts of purified T-GroEL to an extract of yeast cells lacking endogenous T-GroEL (see Materials and methods). The amount of T-GroEL bound to D-*actin was quantified using a phosphoimager and is plotted in the lower panel. The amount of expressed, functional T-GroEL corresponds to 1% of total protein, or ~2.5 μM (with regard to the oligomer), assuming a yeast intracellular protein concentration of 200 mg/ml.

expressing yeast contained ~2.5 μM of T-GroEL capable of binding denatured actin with high affinity, in good agreement with the immunologically determined concentration of T-GroEL (see Figure 2 legend). We conclude that a major fraction of the expressed T-GroEL in the cytosol is available to bind unfolded polypeptide.

T-GroEL (~57 kDa) was detectable as a major band in cytosolic extracts of radiolabelled cells (Figure 3A, lanes 3–6) and was immunoprecipitated under conditions preserving its interaction with newly synthesized unfolded polypeptides (Figure 3A, lanes 1, 2, 7–11). However, only ~1% of total newly synthesized polypeptides in the yeast cytosol was specifically associated with T-GroEL (Figure 3A, lanes 2–10; Figure 3B, lanes 5 and 6), in striking contrast to observations in *E.coli* where GroEL, present at ~3 μM in the cytosol, is co-immunoprecipitated with ~15% of total newly synthesized polypeptides (Figure 3A, lane 11; Ewalt *et al*., 1997). Thus, most newly synthesized proteins in the yeast cytosol are excluded from binding to the chaperonin trap, suggesting that their folding does not generally involve the partitioning of unfolded states in the bulk cytosol. This sequestration of folding polypeptides explains why the expression of functional T-GroEL is well tolerated by yeast.

Actin folding occurs in a sequestered chaperonin compartment

Newly synthesized actin was not detectable among the polypeptides that bound to T-GroEL in radiolabelled yeast cells (Figure 3B, lane 5) and expression of T-GroEL had no influence on the kinetics or yield of actin folding (see Figure 6B). Neither was pre-existent actin detected by Western blotting in immunoprecipitates of T-GroEL (Figure 3B, lane 10). To assess whether the exclusion of actin from T-GroEL is mediated by the endogenous chaperonin TRiC, we analysed the partitioning of non-native actin to T-GroEL in the yeast mutant *tcp1–2*, in which TRiC function is defective in a temperature-sensitive manner (Ursic *et al*., 1994). Upon shift to the non-permissive temperature of 37°C, *tcp1–2* cells show defects in the formation of the actin and tubulin cytoskeletons and eventually lyse after about 17 h. Remarkably, in contrast to wild-type, when *tcp1–2* cells expressing T-GroEL were radiolabelled at 37°C, a significant fraction (~25%) of the newly synthesized actin (see below) associated with T-GroEL (Figure 3B, lanes 4 and 5). Actin was trapped by T-GroEL in the intact cells and not during cell lysis, because labelled actin was not co-immunoprecipitated with T-GroEL when labelled *tcp1–2* cells not expressing T-GroEL were mixed with unlabelled cells expressing T-GroEL prior to lysis (data not shown). Compared with the wild-type control, the amount of folded actin produced in the mutant cells was reduced by ~50% (Figure 3B, lanes 1 and 2). At least half of the actin which failed to fold could be captured by T-GroEL (see legend to Figure 3), thus validating the use of T-GroEL as a sensitive tool to analyse the partitioning of non-native polypeptides in the cytosol. Overexpression of T-GroEL in *tcp1–2* cells did not further decrease the efficiency of actin folding, indicating that those actin chains which folded despite the reduced function of TRiC did so without intermittent partitioning into the cytosol (not shown). Control experiments with an unrelated mutant strain,

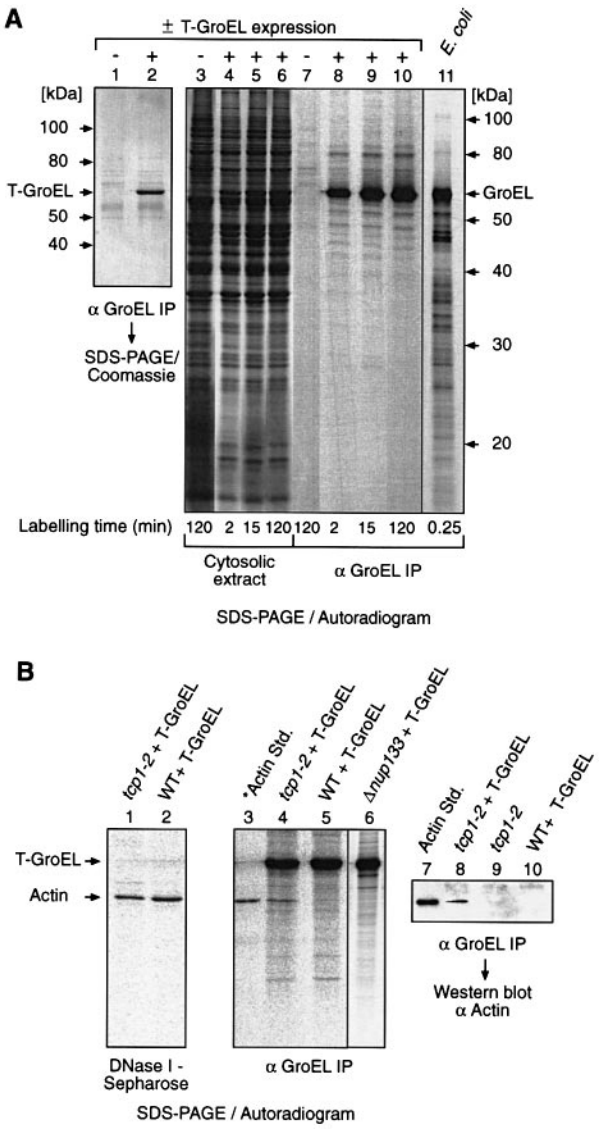

Fig. 3. Interaction of T-GroEL with newly synthesized polypeptides in wild-type and *tcp1–2* mutant yeast. (**A**) BJ5459 cells with or without T-GroEL expressed were radiolabelled for 2, 15 or 120 min, as indicated. Immunoprecipitates obtained with GroEL antibody (lanes 1, 2, 7–10) and total extracts (lanes 3–6) were analysed by SDS–PAGE and Coomassie Blue staining (lanes 1 and 2) or autoradiography (lanes 3–10), respectively. In lanes 8–10 some degradation products of non-assembled T-GroEL are seen below the band of full-length T-GroEL (see Figure 2B). Total extracts in lanes 3–6 correspond to 1/13 of the sample loaded in lanes 7–10. Lane 11 shows as a control the GroEL co-immunoprecipitate from an extract of *E.coli* cells labelled for 0.25 min (Ewalt *et al*., 1997). The fraction of total synthesized polypeptide associated with T-GroEL was determined by phosphoimager analysis taking the efficiency of GroEL immunoprecipitation into account (Ewalt *et al*., 1997). (**B**) Folding of newly synthesized actin and interaction with T-GroEL in *TCP1* wild-type (lanes 2, 5 and 10), *tcp1–2* (lanes 1, 4, 8 and 9) and Δ*nup133* mutant cells (lane 6). Cells were grown to mid-log phase at 23°C and shifted to 37°C for 6 h before radiolabelling for 15 min. Extracts containing equal amounts of protein were analysed by precipitation with DNase I beads (lanes 1 and 2) and with anti-GroEL antibody (lanes 4–6, 8–10), followed by SDS–PAGE and autoradiography (lanes 1–6) or Western blotting with anti-actin antibody (lanes 7–10). Note that lane 9 shows the anti-actin blot of an anti-GroEL precipitation from *tcp1–2* cells lacking T-GroEL to demonstrate that actin does not precipitate non-specifically. The amount of radiolabelled actin accumulated on T-GroEL in lane 4 was quantified taking the efficiency of T-GroEL immunoprecipitation and of ^{35}S-incorporation into account.

Δ*nup133*, which has a temperature-dependent growth defect due to the functional impairment of the nuclear pore protein Nup133p (Doye *et al*., 1994), showed no trapping of newly synthesized actin by T-GroEL (Figure 3B, lane 6).

It is noteworthy that the defect in TRiC resulted in the trapping by T-GroEL of only one major polypeptide species, actin. α-tubulin, another substrate of TRiC, was not detected in the complex with T-GroEL (not shown), suggesting that despite the partial defect in TRiC, this protein folds to a state that is either no longer recognized by T-GroEL or is bound by one of several cofactors required for tubulin folding and assembly (Tian *et al*., 1996). Thus, it appears that the chaperonin-assisted folding of actin and other TRiC substrates *in vivo* normally does not involve the free partitioning of non-native species in the cytosol.

Rapid actin folding on TRiC requires the co-chaperone GimC

Recent work by Geissler *et al*. (1998) described a protein complex, GimC, which is involved in the biogenesis of actin, α- and γ-tubulin in the yeast cytosol. A subsequent study showed that the mammalian homologue of GimC, termed prefoldin, can deliver unfolded actin to the chaperonin *in vitro* (Vainberg *et al*., 1998). This finding and the similarity in the phenotypes of *GIM* null cells and mutants in genes encoding subunits of TRiC raised the possibility that GimC also functions in chaperonin-assisted folding *in vivo*. To explore this possibility, we first analysed the subunit composition of GimC and the interaction of the complex with the chaperonin and its main substrates.

In addition to the previously identified subunits Gim1p–Gim5p (Geissler *et al*., 1998), purified GimC contains a sixth subunit, Gim6p (open reading frame YJL179w), as determined by the isolation of the complex via a chromosomally integrated gene fusion of *GIM2* with protein A, followed by mass spectroscopic analysis (Shevchenko *et al*., 1996) (Figure 4A). GimC does indeed interact with actin and α-tubulin *in vivo*, as shown by its co-immunoprecipitation with epitope-tagged variants of these proteins from cell extracts (Figure 4B and C). Similarly, a physical association between GimC and TRiC was demonstrated by the co-immunoprecipitation of GimC with the c-myc-tagged variant of TRiC described above (Figure 4D). The cellular amounts of TRiC and GimC are very similar, but only ~10% of GimC was co-precipitated with TRiC, suggesting that the GimC–TRiC complex is either unstable or the interaction is transient. A direct interaction between yeast GimC and bovine TRiC was confirmed with the purified proteins *in vitro* (not shown). Interestingly, deletion of the individual *GIM* genes had differential effects on the viability of three distinct conditional lethal alleles of a gene encoding one of the TRiC subunits, TCP1 (CCT1) (Table I), suggesting that the association of the two protein complexes is mediated by subunit-specific contacts.

Next, we analysed the folding of newly synthesized actin in GimC-deficient yeast strains. Strikingly, single *GIM* deletions caused a 5-fold decrease in the rate of folding, with the yield of folded actin being reduced by 40–50% (Figure 5A–C). Similar results were obtained with a Δ*gim1/2* double deletion strain and a Δ*gim1/2/3*

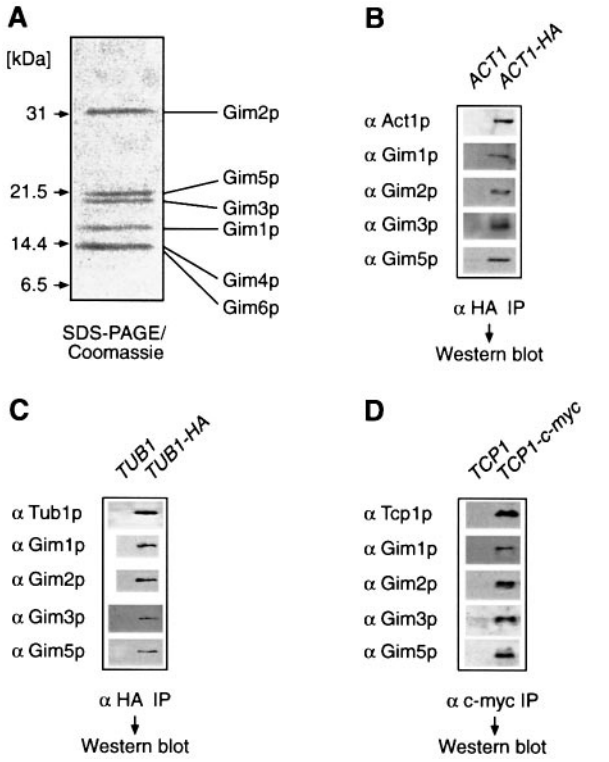

Fig. 4. GimC is a hetero-oligomer of six polypeptides and interacts with actin, tubulin and TRiC. (**A**) Isolation of GimC. Subunits of GimC were isolated from yeast cells via a protein A-tagged Gim2p subunit, separated by SDS–PAGE and Coomassie staining and identified by MALDI and nanoelectrospray mass spectrometry. Note that Gim4p and Gim6p (open reading frame YJL179w) were not resolved by the denaturing polyacrylamide gel. Mass spectrometry showed that the 13.5 kDa band consisted of a mixture of Gim4p and Gim6p (not shown). (**B** and **C**) Binding of actin (Act1p) and α-tubulin (Tub1p), respectively, to GimC *in vivo*. Anti-HA immunoprecipitates from wild-type (left lanes), *ACT1–3HA* (B) and *TUB1–3HA* (C) cells (right lane) were analysed by Western blotting with the antibodies indicated. (**D**) Binding of TRiC to GimC *in vivo*. Immunoprecipitates with anti-c-myc antibodies from extracts of TCP1 (left lane) and TCP1 c-*myc* cells (right lane) were tested for Tcp1p protein (TRiC) and GimC subunits by immunoblotting with the respective antibodies.

Table I. Allele-specific interactions of *GIM* genes and *TCP1*

	pRS414	*TCP1*	*tcp1–2*	*tcp1–3*	*tcp1–245*
Δ*gim1*	–	+	–	+/–	+/–
Δ*gim2*	–	+	+/–	+/–	+
Δ*gim3*	–	+	–	+/–	+
Δ*gim4*	–	+	+/–	–	–
Δ*gim5*	–	+	+	+/–	+/–
Δ*gim6*	–	+	+	+	+/–

Cells of *S.cerevisiae* strain SGY149 (Δ*tcp1:: HIS3MX6 pRS316-TCP1*) were deleted for *GIM1* to *GIM6*, resulting in strains KSY107 to KSY112. These strains were transformed with the indicated plasmids and tested for growth (+) on plates containing 5-fluoroorotic acid (5-FOA) at 30°C. 5-FOA allows only growth of cells which spontaneously lost the *URA3*-based pRS316-*TCP1* plasmid. KSY107–112 cells transformed with a *TRP1*-based *TCP1*-containing plasmid grew on 5-FOA, whereas KSY107–112 cells with the control plasmid pRS414 did not (–), confirming that *TCP1* is an essential gene (Ursic *et al*., 1994). Growth of KSY107–112 cells with *TRP1*-based plasmids carrying *tcp1–2*, *tcp1–3* (Ursic *et al*., 1994) or *tcp1–245* (Miklos *et al*., 1994) on 5-FOA indicates the absence of a synthetically lethal phenotype. Failure to grow reveals a synthetically lethal phenotype, while a reduction in growth is caused by a synthetically toxic defect (+/–).

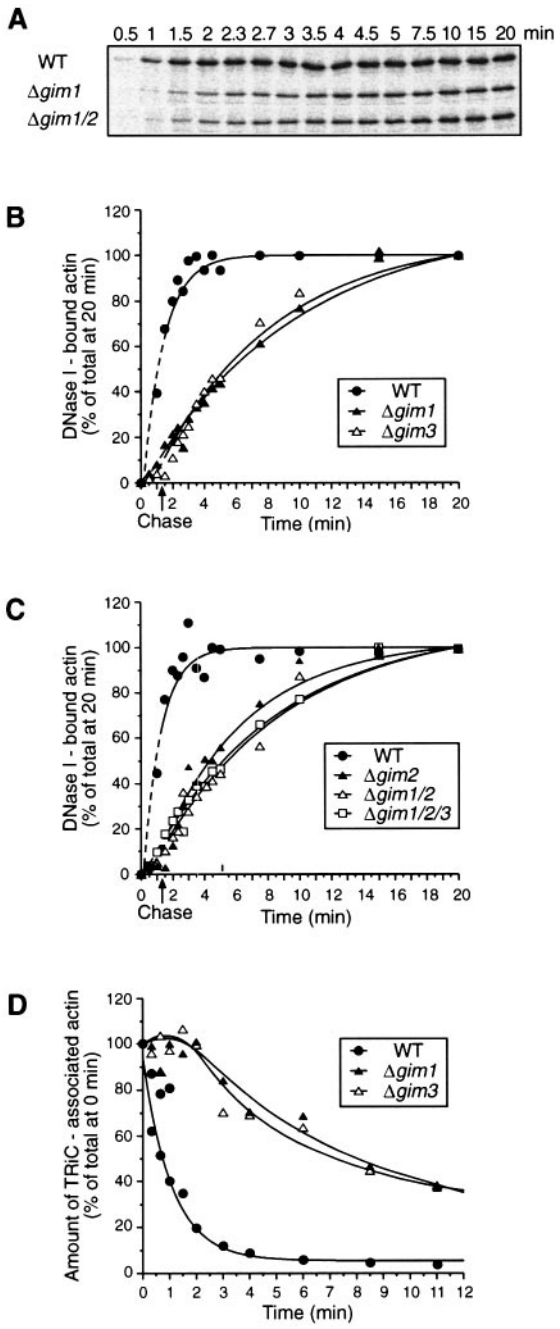

Fig. 5. Yields and rate of actin folding are reduced in GimC-deficient cells. (A–C) Actin folding in pulse–chase labelled wild-type, Δ*gim1*, Δ*gim2*, Δ*gim3* as well as Δ*gim1/2* and Δ*gim1/2/3* assayed as in Figure 1. Actin bound to DNase I–Sepharose was analysed by SDS–PAGE (**A**) and then quantified by phosphoimager analysis (**B** and **C**). The amount of actin precipitated after 20 min was set to 100. (**D**) Transit of newly synthesized actin through TRiC in wild-type, Δ*gim1* and Δ*gim3* cells, measured as in Figure 1C. Amounts of TRiC-associated actin at the beginning of the chase are set to 100. The absolute amounts of TRiC-bound actin in wild-type and Δ*gim* cells were similar. The results shown are from representative experiments. At least two independently performed experiments gave very similar results.

triple deletion (Figure 5C), suggesting that single deletions of these *GIM* genes essentially cause the complete loss of GimC function in actin folding. This is also supported by the finding that deletion of multiple *GIM* genes only slightly enhanced the growth defects observed in single *GIM* null mutants at 30°C (from 2.6 h for the single deletions to 2.8 h for a strain lacking four *GIM* genes, with wild-type cells doubling every 2.0 h).

To analyse the transit of newly synthesized actin through TRiC in GimC-deficient cells, Δ*gim1* and Δ*gim3* strains were engineered in which wild-type TRiC was replaced by the c-myc-tagged version. Pulse–chase labelling of these cells followed by immunoprecipitation of TRiC revealed that the defect in GimC resulted in 5- to 8-fold slower kinetics of actin release from TRiC (Figure 5D), corresponding to the decrease in the rate of actin folding (Figure 5B and C). The delayed clearance of radiolabelled actin from TRiC during the first two min of the chase (Figure 5D) is interpreted to reflect the rapid influx of actin into the slowly emptying TRiC pool. There was no indication that the delivery of newly synthesized actin to TRiC was impaired in GimC-deficient cells. Together, these results suggest that GimC acts primarily on the TRiC–actin binary complex to achieve rapid and efficient actin folding.

GimC function restricts the release of non-native actin during chaperonin-assisted folding

The reduced yield in actin folding in *GIM*-deficient cells suggested that actin chains are released from TRiC in a non-native state. To address this possibility, the chaperonin trap, T-GroEL, was expressed in a Δ*gim1* strain. Indeed, similar to the observations made with the TRiC-deficient mutant *tcp1–2* (Figure 3B), the majority of the newly synthesized actin that failed to fold in Δ*gim1* cells was captured by T-GroEL (Figure 6A). Interestingly, several additional newly synthesized polypeptides were also trapped by T-GroEL in Δ*gim1* cells. These unidentified polypeptides may represent other chaperonin substrates which also fold with reduced efficiency when GimC function is lacking. Notably, expression of T-GroEL did not reduce further the efficiency of actin folding in Δ*gim1* cells (Figure 6B), suggesting that non-native actin chains which have been released into the bulk cytosol are unable to return to TRiC for completion of folding. These chains may either aggregate or, more likely, be proteolytically degraded, and this is consistent with the reduced levels of α-tubulin found in *GIM* null cells (Geissler *et al*., 1998). Thus, in addition to accelerating actin folding on TRiC (Figure 5), GimC may have a 'proofreading' function in avoiding the premature release of non-native substrate from TRiC. Alternatively, TRiC may be locked with actin-folding intermediates due to the lack of GimC function, resulting in an excess of unfolded actin in the cell. This excess unfolded actin may then become a substrate for T-GroEL. Taking into account that GimC binds unfolded actin and directly interacts with TRiC, we favour the first possibility.

An alternative mechanism has been proposed for the bovine GimC homologue, prefoldin (Vainberg *et al*., 1998). These authors proposed, on the basis of *in vitro* experiments, that the most probable role of prefoldin is to bind unfolded proteins (e.g. newly synthesized or stress-denatured) and to deliver them specifically to TRiC for folding to the native state. This was concluded from the observation that in the absence of ATP the GimC-bound actin was transfered much less efficiently to GroEL or mitochondrial Hsp60 than to TRiC. To test whether such

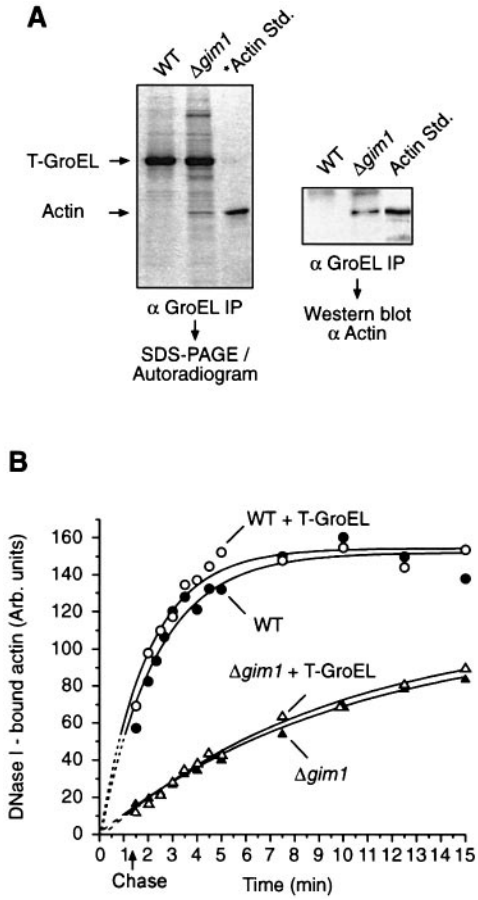

Fig. 6. Non-native actin is released into the cytosol in GimC-deficient cells. (**A**) Binding of newly synthesized actin to T-GroEL in GimC-deficient cells. Wild-type and Δ*gim1* cells expressing T-GroEL were radiolabelled for 15 min and T-GroEL immunoprecipitated, followed by SDS–PAGE/autoradiography (left panel) or Western blotting with anti-actin antibody (right panel) (see Figure 3B). (**B**) Kinetics of actin folding in pulse–chase labelled wild-type and Δ*gim1* cells in the presence or absence of cytosolically expressed T-GroEL. Amounts of folded actin in wild-type and Δ*gim1* cells are shown on the same scale.

a function of GimC could be responsible for the observed exclusion of non-native actin from the T-GroEL chaperonin trap *in vivo* (Figures 3 and 6), we incubated denatured ^{35}S-labelled actin (D-*actin) with isolated bovine GimC, followed by the addition of either bovine TRiC or an equimolar concentration of T-GroEL (Figure 7). While the GimC–actin complex alone was stable, addition of TRiC led to the ATP-independent transfer of actin from GimC to the chaperonin, as judged by native PAGE (Vainberg *et al*., 1998). However, the transfer of actin from GimC to T-GroEL was even more efficient (Figure 7). Given that *in vivo* T-GroEL was present in 10- to 30-fold excess over TRiC, our data do not support the view that in the cell GimC functions primarily by returning non-native actin chains that have been released to the cytosol to TRiC.

Discussion

Gene expression is a highly organized process subject to extensive controls and proofreading mechanisms at the level of transcription and translation. Based on the results

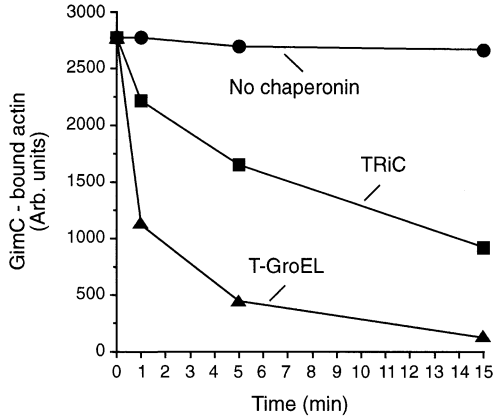

Fig. 7. GimC transfers non-native actin to TRiC or T-GroEL *in vitro*. Pre-formed bovine GimC–*actin complex was incubated at 30°C with buffer (no chaperonin), TRiC or T-GroEL as described in Materials and methods and the amount of GimC-bound *actin at the times indicated was determined by native PAGE and phosphoimager analysis.

of this study, we propose that similar principles of organization also govern the downstream process—chaperonin-assisted protein folding, taking the form of an integrated 'folding compartment'. The primary purpose of this compartmentation of folding is to minimize the exposure of aggregation-sensitive, non-native forms of polypeptides to the crowded cytosol, thereby effectively preventing off-pathway folding reactions and premature protein degradation.

Chaperone pathway of actin folding

Newly synthesized actin is maintained in a sequestered environment, inaccessible to the T-GroEL chaperonin trap, until it has reached a native or native-like conformation. The functional cooperation of components of the translation and folding machineries is critical in establishing this protected environment. First, nascent chains of actin probably interact co-translationally with the Hsp70 homologues, Ssb1 and Ssb2, like the majority of newly synthesized polypeptide chains in the yeast cytosol (James *et al*., 1997; Johnson and Craig, 1997). These chaperones are ribosome-associated and thus have immediate access to the nascent polypeptide (Pfund *et al*., 1998). In mammalian cells the ribosome-associated protein complex nascent polypeptide-associated complex (NAC) may bind to the elongating chain even prior to Hsp70 (Wiedmann *et al*., 1994). A second critical stage in the pathway of actin folding is the transfer of the newly synthesized polypeptide from the ribosome to the chaperonin TRiC; it occurs very rapidly and without the intermittent exposure of unfolded forms to the bulk cytosol. Mechanistically, the tight coupling of chain transfer could be explained by the specific recruitment of TRiC, but not T-GroEL to nascent actin before completion of translation. In support of this possibility, TRiC binds ribosome-associated polypeptides of more than ~150 amino acids in length *in vitro* (Frydman *et al*., 1994; Frydman and Hartl, 1996). Whether in living cells GimC is involved in recruiting TRiC to newly synthesized actin remains to be explored. Lastly, and most importantly, the sequestration of actin chains is maintained during their post-translational folding on TRiC, dependent on the functional cooperation between TRiC and GimC.

Our conclusion that non-native actin is sequestered from the bulk cytosol throughout its folding *in vivo* contrasts with a recent report by Farr *et al*. (1997) who observed that injection of T-GroEL into *Xenopus* oocytes inhibited the folding of newly synthesized actin. It seems possible that the level of functional GimC varies in different cell types, dependent on their metabolic activity and developmental stage. Thus, the findings of Farr *et al*. (1997) may well be explained by a relative deficiency of GimC in oocytes, which differ metabolically from typical growing and dividing eukaryotic cells (Murray, 1991).

Function of the TRiC–GimC system

TRiC most probably mediates folding by binding unfolded polypeptides and releasing them into an enclosed folding cage (Klumpp *et al*., 1997; Ditzel *et al*., 1998), similar to the mechanism of GroEL/GroES. However, according to the current model, opening and closing of the TRiC cage is mediated by the ATP-dependent movement of helical extensions of the TRiC subunits, not by the binding and release of a separate GroES-cofactor (Klumpp *et al*., 1997; Ditzel *et al*., 1998; Llorca *et al*., 1998). We found that the rate of actin folding *in vivo* is surprisingly fast, suggesting that on average actin chains interact with TRiC for fewer chaperonin cycles than thought previously (Farr *et al*., 1997). Significantly, the capacity of TRiC to fold actin rapidly in a sequestered environment depends on GimC, a hetero-oligomeric chaperone complex which is found in archaea and eukarya and has no sequence homology with GroES (Geissler *et al*., 1998; Vainberg *et al*., 1998). GimC may thus represent a general co-chaperone of group II chaperonins, necessary for efficient chaperonin-assisted folding. In contrast to GroES, GimC function is essential for cell growth only at low temperature (Geissler *et al*., 1998), or when the function of TRiC is also compromised.

Our results suggest that *in vivo* GimC acts in folding primarily in the complex with TRiC and perhaps also after the release of actin from TRiC. In GimC-deficient cells the kinetics of actin folding and transit through TRiC were slowed, suggesting that under these conditions folding involves multiple cycles of chaperonin action. This chaperonin cycling is inefficient and is accompanied by the loss of ~50% of actin chains into the cytosol in a non-native state, where they fail to fold. However, GimC does not normally prevent misfolding by returning unfolded actin chains to TRiC. We conclude this from the finding that T-GroEL does not interfere with the kinetics of actin folding *in vivo*, although *in vitro* GimC transfers unfolded actin to T-GroEL at least as efficiently as to TRiC. Instead, by virtue of its ability to bind directly to the substrate polypeptide and to TRiC, GimC may retain non-native actin on TRiC ('proofreading') and promote the formation of folding intermediates, resulting in acceleration of folding. The possibility that GimC has an additional function in modulating the ATPase activity of TRiC remains to be explored. In contrast to GimC, GroES does not interact with the substrate polypeptide directly. Thus, while both GimC and GroES cooperate with their respective chaperonin partners to achieve efficient folding in a sequestered environment, the GimC co-chaperone acts by a novel mechanism.

Compartmentation of protein folding—a general principle?

Surprisingly, the high-level expression of a functional chaperonin trap in the yeast cytosol leads to the capture of only a very small fraction of newly synthesized polypeptides, suggesting that the partitioning of non-native folding intermediates in the cytosol is generally restricted. Similar observations have been made with the expression of T-GroEL in mammalian cells (J.Frydman, personal communication). Since the endogenous chaperonin, TRiC, interacts only with a subset of newly synthesized chains (Kubota *et al*., 1995; Lewis *et al*., 1996), additional mechanisms of compartmentalizing non-native polypeptides are thus likely to exist. Most chaperonin-independent polypeptides may fold co-translationally at the level of their domains (Netzer and Hartl, 1997). Shielding by Hsp70 during elongation may explain the exclusion of these polypeptides from the chaperonin trap. The requirement for a post-translational folding compartment(s) would arise from the fact that a subset of proteins have a limited capacity to form native domain structures before completion of translation. For example, actin is a multi-domain protein having structural domains made up of interrupted amino acid sequences. The resulting complexity of its post-translational folding may explain, in part, its specific requirement for TRiC and GimC, and suggests a role for this system in the folding of other proteins with similar structural features. Another post-translational folding compartment may be defined by the Hsp90 multi-chaperone system, which also assists in the folding of a restricted set of polypeptides and presumably receives these substrates after their co-translational interaction with Hsp70 (Johnson and Craig, 1997; Nathan *et al*., 1997). It will be interesting to explore how the chaperone systems that mediate the compartmentation of folding control the transfer to the proteolytic machinery of newly synthesized chains which cannot fold productively.

Materials and methods

Yeast strains and genetic methods

Basic yeast genetic methods and media were as described previously (Guthrie and Fink, 1991). *TCP1* or *GIM* genes were deleted in strain YPH499 (Sikorski and Hieter, 1989) using PCR-amplified *kanMX4* (Wach *et al*., 1994), *HIS3MX6* (Wach *et al*., 1997), or heterologous *Kluyveromyces lactis*-base *TRP1* or *URA3* cassettes (M.Knop, K.Siegers, G.Pereira, W.Zachariae, B.Winsor, K.Nasmyth and E.Schiebel, submitted). A chromosomally integrated gene fusion to the 3′ end of *GIM2* with protein A was generated by homologous recombination of PCR-amplified cassettes in strain YPH499. Growth of wild-type YPH499 cells and mutants lacking *GIM1*, *GIM1/2*, *GIM1/2/3*, or *GIM1/2/3/4* was determined in yeast extract peptone dextrose medium at 30°C. Similar results were obtained with other single (*GIM2*, *GIM3*, *GIM5*), double, triple and quadruple *GIM* null mutants (not shown).

T-GroEL was expressed in yeast by subcloning the gene encoding GroEL D87K (T-GroEL) (Farr *et al*., 1997) into pCUP1 (2μ *TRP1*) or pSal4 (2μ *URA3*) under the control of the *CUP1* promotor/*CYC1* terminator (Mascorro-Gallardo *et al*., 1996), resulting in pCUP1–T-GroEL and pSal4–T-GroEL, respectively. pCUP1–T-GroEL was transformed into BJ5459 (*MAT*α, *ura3–52 trp1 lys2–801 leu2*Δ*1 his3*Δ*200 pep4::HIS3 prb1*Δ*1.6R can1 GAL*) (Guthrie and Fink, 1991). pSAL4–T-GroEL was transformed into the following yeast strains: DUY 558 [*MAT*α, *leu 2–3,–112 ura3–52 trp1–7 tcp1:: LEU2* (*YCpMS38; TCP1 TRP1*)]; DUY 326 [*MAT*α, *leu 2–3,–112 ura3–52 trp1–7 tcp1:: LEU2* (*YCpMS38; tcp1–2 TRP1*)] (Ursic and Culbertson, 1991); YPH499 (*MAT*α, *ura3–52 lys2–801 ade2–101 trp1*Δ*63 his3*Δ*200 leu2*Δ*1*) (Sikorski and Hieter, 1989); SGY101 (YPH499 Δ*gim1::kanMX4*)

(Geissler *et al*., 1998); KSY80-1 [YPH499 (pSG128–1; Δ*tcp1::HIS3MX6*) (pRS315 *LEU2 TCP1* c-*myc*)] (this study); KSY95-1 (KSY80-1 Δ*gim1::kanMX4*) (this study); R5453 (*MAT***a** *ade2 his3 ura3 leu2 trp1*); R5453 *nup*Δ*133* (Doye *et al*., 1994). Yeast cells were grown in synthetic complete (SC) medium at 30°C. High level, continuous expression of T-GroEL was routinely achieved by growing the cells in the presence of 100 μM $CuSO_4$.

Preparation of spheroplasts

Yeast cells were grown in 100 ml cultures to mid-log phase ($OD_{600} \approx 0.5$), harvested by centrifugation (3000 *g*, 5 min), resuspended in 5 ml of SC lacking methionine and cysteine (SC –M –C), 1.2 M sorbitol, 30 mM dithiothreitol (DTT) pH 7.5 and incubated for 10 min at room temperature. Cells were harvested as above and resuspended in the same medium without DTT containing 0.5 mg/ml Zymolyase 100T (ICN Biochemicals) and incubated for 30–60 min at 30°C until conversion of the cells to spheroplasts was >90%. The spheroplasts were harvested by centrifugation (1000 *g*, 15 min), washed twice in SC –M –C, 1.2 M sorbitol pH 5.5, resuspended in 2–5 ml of the same buffer and incubated at 30°C for radiolabelling.

Radiolabelling of yeast cells

Pulse–chase radiolabelling of spheroplasts was performed with 100 μCi/ml [^{35}S]methionine/cysteine ProMix (Amersham) followed by a chase with cycloheximide (0.36 mM) and 1 mM of unlabelled methionine/cysteine. At the time-points indicated in the figure legends, aliquots were diluted 1:1 in cold 2× lysis buffer [2× phosphate-buffered saline (PBS) pH 7.4, 10 mM EDTA, 1% Tween-20, 2× complete protease inhibitors (Boehringer Mannheim)], mixed for 10 s and immediately frozen in liquid nitrogen. At the end of the chase all reactions were thawed on ice and cell extracts cleared by centrifugation (20 000 *g*, 4°C, 10 min). Intact yeast cells were grown to mid-log phase, harvested by centrifugation, resuspended in 2–5 ml of SC –M –C and labelled as above, followed by a chase for 5 min. Cells were harvested as above, washed twice in IP-buffer [1× PBS pH 7.4, 5 mM EDTA, 5% (v/v) glycerol, 1× complete protease inhibitors] and resuspended in 0.5 ml of the same buffer. Cytosolic extracts were prepared by agitating the cells with glass-beads for 6× 30 s at 4°C. Lysates were cleared by centrifugation (20 000 *g*, 4°C, 10 min).

Yeast cytosolic extracts (35 μl of ~20 mg/ml protein) were fractionated on a Superdex 200 PC 3.2/30 column at 4°C in 50 mM Tris pH 7.2, 100 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA, and 50 μl fractions were collected.

Immunoprecipitation and Western blotting

Antibodies (affinity-purified rabbit anti-GroEL, mouse-mAB anti-c-myc (ATCC CRL1729) and anti-hemagglutinin (HA) antibodies (12CA5, Hiss Diagnostic) were cross-linked to protein A– or protein G–Sepharose 4 Fast Flow (Pharmacia), respectively, according to standard procedures. Immunoprecipitations from cell lysates of *ACT1*, *ACT1/ACT1–3HA*, *TUB1* and *TUB1 pRS315-TUB1–3HA* cells with anti-HA Sepharose beads were performed as described (Geissler *et al*., 1998). TRiC containing Tcp1p–c-myc was precipitated with anti-c-myc Sepharose beads. T-GroEL was immunoprecipitated as described (Ewalt *et al*., 1997).

Western blotting was performed using the luminescence based ECL-system (Amersham). Actin was detected with an anti-mouse actin monoclonal antibody (Boehringer Mannheim). The affinity purified anti-Gim antibodies have been described previously (Geissler *et al*., 1998). Defined amounts of purified recombinant c-myc-tagged firefly luciferase were used as standards in the quantitation of the cellular content of c-myc-tagged TRiC. A total protein concentration in the cytosol of 200 mg/ml was assumed.

Preparation of labelled denatured β-actin

The gene encoding mouse β-actin (kindly provided by J.Shephard) was subcloned into the pRSET6a vector (Schoepfer, 1993) and expressed to high levels using the *E.coli* strain BL21(DE3) pLysS in the presence of 1 mCi/ml [^{35}S]methionine/cysteine, rifampicin (0.2 mg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) (Studier *et al*., 1990). The labelled actin (D-*actin) was solubilized from isolated inclusion bodies using 8 M urea, 20 mM Tris–HCl pH 7.5, 10 mM DTT.

Kinetics of actin folding in vivo

Purified DNase I (Sigma) was crosslinked to cyanogen bromide-activated Sepharose 4B-CL (Pharmacia) as described by the manufacturer. Yeast spheroplasts were generated and pulse–chase labelled as above. At each time-point 250 μl of spheroplasts (100–200 μg of protein) were diluted with 250 μl of cold 2× lysis buffer and treated as described above. Actin in the supernatant was bound to 30 μl of DNase I-beads (1:1 suspension in 1× PBS, pH 7.4) during a 1 h incubation at 4°C (Lazarides and Lindberg, 1974). DNase I was in excess over the actin present. Beads were washed extensively as for anti-GroEL immunoprecipitations (Ewalt *et al*., 1997) prior to SDS–PAGE and phosphoimager analysis.

*Binding of D-*actin by T-GroEL in yeast lysates*

D-*actin in 8 M urea (~13 μM) was diluted 100-fold into 25 μl reactions containing 50 μg of yeast cytosolic protein (with or without T-GroEL expressed; final concentration of T-GroEL in the reaction ~50 nM), 20 mM HEPES–KOH pH 7.4, 50 mM NaCl, 2.5 mM EDTA, and 1× complete protease inhibitors), incubated on ice for 20 min, and analysed by native PAGE on 4.5% polyacrylamide gels (made and run in 80 mM MOPS–KOH pH 7.0, 0.5 mM EGTA, 1 mM $MgCl_2$). Varying amounts of purified T-GroEL was added to extracts without expressed T-GroEL. Fractions (25 μl) from the size fractionation of cytosolic extracts on a Superdex 200 column were also tested for binding to 100-fold diluted D-*actin.

Purification of yeast and bovine testis GimC

Yeast GimC was purified from a strain carrying a functional *GIM2–TEV-ProA* gene fusion. GimC containing Gim2p–Tev-ProA was bound to IgG Sepharose (Pharmacia). After extensive washing steps, the bound complex was cleaved from the IgG Sepharose using 6× His-tagged TEV protease (Gibco-BRL). TEV protease was removed by a Ni-NTA column. Bovine GimC was purified from testis essentially as described (Vainberg *et al*., 1998).

Transfer of GimC-bound actin to TRiC or T-GroEL

The bovine GimC–actin complex was formed by diluting D-*actin to a final concentration of ~0.13 μM into 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 2 mM EDTA containing GimC (0.15 μM), followed by incubation for 30 min at 30°C. TRiC, GroEL D87K trap (T-GroEL) (~0.2 μM each) or buffer were added to the GimC–actin complex. Samples were withdrawn immediately or 1, 5 or 15 min after the addition of chaperonin and frozen in liquid nitrogen. Samples were thawed and immediately separated on a 4.5% non-denaturing polyacrylamide gel.

Acknowledgements

We thank Drs M.Tector, F.Lottspeich and H.Sarioglu for performing the mass spectroscopy of T-GroEL, Dr W.Schiebel for his help in purifiying bovine GimC, F.Weber for the contribution of purified T-GroEL protein, and Dr W.Netzer for critically reading the manuscript. This work was carried out with support from the Cancer Research Campaign. K.S. and M.R.L. are holders of EMBO fellowship awards, and T.W. is a recipient of a post-doctoral fellowship from the Deutsche Forschungsgemeinschaft.

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Received October 7, 1998; revised October 26, 1998; accepted October 29, 1998