The Neurodegenerative-Disease-Related Protein Sacsin Is a Molecular Chaperone

John F. Anderson1, Efrain Siller1 and José M. Barral1,2*

1Department of Neuroscience and Cell Biology, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0620, USA
2Sealy Center for Structural Biology and Molecular Biophysics, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0620, USA

Received 23 March 2011; received in revised form 6 June 2011; accepted 10 June 2011
Available online 25 June 2011

Edited by F. Schmid

Keywords:
molecular chaperone; protein folding; neurodegeneration; ataxia; ARSACS

Various human neurodegenerative disorders are associated with processes that involve misfolding of polypeptide chains. These so-called protein misfolding disorders include Alzheimer's and Parkinson's diseases and an increasing number of inherited syndromes that affect neurons involved in motor control circuits throughout the central nervous system. The reasons behind the particular susceptibility of neurons to misfolded proteins are currently not known. The main function of a class of proteins known as molecular chaperones is to prevent protein misfolding and aggregation. Although neuronal cells contain the major known classes of molecular chaperones, central-nervous-system-specific chaperones that maintain the neuronal proteome free from misfolded proteins are not well defined. In this study, we assign a novel molecular chaperone activity to the protein sacsin responsible for autosomal recessive spastic ataxia of Charlevoix-Saguenay, a degenerative disorder of the cerebellum and spinal cord. Using purified components, we demonstrate that a region of sacsin that contains a segment with homology to the molecular chaperone Hsp90 is able to enhance the refolding efficiency of the model client protein firefly luciferase. We show that this region of sacsin is highly capable of maintaining client polypeptides in soluble folding-competent states. Furthermore, we demonstrate that sacsin can efficiently cooperate with members of the Hsp70 chaperone family to increase the yields of correctly folded client proteins. Thus, we have identified a novel chaperone directly involved in a human neurodegenerative disorder.

© 2011 Elsevier Ltd. All rights reserved.

Introduction

In order to perform their cellular functions, protein domains must acquire precise three-dimensional (native) conformations, a process known as protein folding. However, during their biogenesis and throughout their lifetime, partially folded proteins expose segments of unstructured polypeptide backbone and hydrophobic residues prone to forming nonspecific interactions in the aqueous environment of the cell. Protein misfolding occurs when these regions, normally separated in the native protein, interact during the folding process...
and form kinetically stable species. Misfolded proteins tend to self-associate into various types of aggregates driven by hydrophobic forces and interchain hydrogen bonding. It has become increasingly evident that certain proteins that fail to reach or maintain their native states undergo aggregation and are associated with a variety of human diseases known collectively as protein misfolding disorders, including Alzheimer’s, Huntington’s and Parkinson’s diseases.

In the cell, a class of proteins known as molecular chaperones has evolved to effectively counteract the tendency of proteins to aggregate, both during their synthesis and under conditions of cellular stress. In general, chaperones transiently shield the non-native segments of proteins and assist toward their proper folding, typically without contributing conformational information or becoming a part of the final biologically active structure. Several chaperone families that are nearly universally conserved throughout evolution, including the Hsp70/Hsp40 (DnaK/DnaJ in bacteria) and Hsp90 systems (absent in Archaea), as well as the cylindrical chaperonins, have been identified. These three chaperone systems utilize ATP binding and hydrolysis to regulate structural transitions that allow binding and release of polypeptide clients during their folding process.

Intriguingly, the central nervous system appears to be particularly susceptible to the effects of protein misfolding, as many of these disorders affect neurons specifically. Moreover, it has been previously shown that augmented levels of the general chaperone machinery due, for example, to recombinant overexpression or to pharmacologic induction of the stress response are capable of alleviating the neuronal toxicity associated with aggregation of various neurodegenerative-disease-related proteins. These findings suggest that neuronal toxicity arises, at least partially, from an imbalance between the load of potentially dangerous protein species and the capacity of the chaperone repertoire of affected neurons. However, very little is currently known about the chaperone machinery that operates in neurons specifically.

Molecular characterization of the sacsin protein may provide valuable insights into the role of neuron-specific chaperones in the pathogenesis of neurodegenerative disorders. Sacsin is the protein responsible for autosomal recessive spastic ataxia of Charlevoix-Saguenay, a human disorder characterized by degeneration of the cerebellum and the cervical spinal cord and appearance of inclusion-like granules in the remaining neurons. Bioinformatic analysis has revealed various domains with similarities to molecular chaperones and co-chaperones throughout the long sacsin sequence (4579 amino acids). These consist of three regions with similarity to the N-terminal domain of the molecular chaperone Hsp90, which are embedded within larger so-called sacsin repeating regions (SRRs) along the N-terminal half of the protein, and a segment with similarity to the J domain (J) domain of the co-chaperone Hsp40 close to the C-terminus. Additional domains include a ubiquitin-like (Ubl) domain at the extreme N-terminus of the protein, as well as an XPCB (xeroderma pigmentosum C binding) domain and an HEPN (higher eukaryotes and prokaryotes nucleotide binding) domain, flanking the J domain.

Several lines of evidence support the notion that sacsin participates in cellular protein quality control pathways. For example, small interfering RNA-mediated knockdown of sacsin in neuroblastoma-derived cells results in enhanced toxicity of proteins containing expanded polyglutamine tracts associated with multiple neurodegenerative disorders. Initial biochemical characterization of various sacsin domains similarly supports this hypothesis. For example, the Ubl domain of sacsin co-immunoprecipitates with a component of the 20S proteasome, and the J domain stimulates the ATPase activity of Hsp70 in vitro and is functional in an in vivo complementation assay in Escherichia coli. Thus, it appears that sacsin may collaborate with members of the Hsp70 family in the cell.

As stated above, within each SRR, there is a region of similarity to the N-terminal domain of Hsp90. This domain in Hsp90 binds and hydrolyzes ATP and displays molecular chaperone activity in vitro. We have previously shown that an N-terminal segment of sacsin containing the Ubl domain and the first SRR domain (termed RegA) (Fig. 1a) displays ATP-hydrolyzing activity, which is abrogated by a human-disease-causing mutation. However, chaperone activity for this, or any other, region of sacsin has not been documented. In this study, we report that RegA of sacsin demonstrates direct chaperone activity toward the model client protein firefly luciferase (FLuc), as it is capable of increasing its refolding yield upon dilution from denaturant. Additionally, we find that RegA is highly capable of maintaining client polypeptides in folding-competent states and can cooperate with members of the bacterial Hsp70 system to achieve high refolding yields.

**Results**

**RegA of sacsin displays molecular chaperone activity**

In addition to displaying ATPase activity, the N-terminus of Hsp90 has been demonstrated to possess chaperone activity independently of the rest of the molecule, and we have previously demonstrated that RegA of sacsin has ATPase...
activity.\textsuperscript{15} Thus, the presence of an ATPase-active region of similarity to Hsp90 in this SRR domain led us to consider whether these domains might constitute chaperone modules along the sacsin molecule. In order to directly examine the chaperone activity of sacsin, we utilized purified RegA (Fig. 1a)
and the model client FLuc. FLuc has been extensively utilized to characterize protein folding properties of multiple chaperones, including Hsp90 and Hsp70/DnaK. FLuc is a valuable model client due to its high propensity to aggregate when diluted from denaturant, its strong dependence on chaperones for efficient refolding and the availability of a sensitive and convenient assay of acquisition of the native state (bioluminescence). FLuc was denatured in guanidinium chloride (GdmCl), subsequently diluted out of denaturant into mixtures containing various purified components and allowed to refold to directly measure the chaperone capacity of RegA.

Fig. 1. RegA of sacsin displays molecular chaperone activity. (a) Cartoon depiction of the domain structure of sacsin. The protein fragment utilized in this study (RegA) is outlined in red and labeled. (b) Plot depicting the amount of FLuc (500 nM final) refolded upon 1:100 dilution from GdmCl (see Materials and Methods) in the presence of 500 nM RegA or the indicated concentrations of BSA, as determined by the activity of an equivalent concentration of native luciferase. Error bars are the mean and standard deviation of three independent experiments. (c) Plot depicting the data in (b) normalized as follows: each series of data points was fit to a hyperbolic equation, and the value of each data point was divided by the maximum value of the fit for that series. (d) Plot depicting FLuc refolding reactions performed as in (b) in the presence of RegA without ATP. RegA with ATP and buffer control are plotted for reference. (e) Plot depicting the data in (d), normalized as indicated for (c). (f) Histogram summarizing the maximum percent of FLuc recovered in the various experiments, plotted as the mean and standard deviation as determined from a fit to a hyperbolic equation. (g) Histogram summarizing the $t_{1/2}$ values of FLuc refolding reactions in the various experiments, plotted as the mean and standard deviation as determined from the fit to a hyperbolic equation.

Fig. 2. RegA of sacsin prevents FLuc from aggregating. (a) Dot-blot analysis of the amount of material present in the total soluble (super) and insoluble (pellet) fractions of FLuc refolding reactions after 30 min of incubation and subsequent separation by centrifugation and immunobloting (see Materials and Methods). (b) Dot-blot analysis as in (a), but refolding reactions were carried out in the presence of 3 mM ATP. (c) Histogram displaying the quantitation of soluble material as determined by densitometry and plotted as the mean and standard deviation of three independent experiments.
When FLuc was diluted into buffer, it recovered ∼12% of its activity (Fig. 1b and f). However, when diluted into buffer supplemented with a 10-fold molar excess of RegA, the fraction of FLuc that acquired the native state increased approximately twofold. Although modest, this increase was statistically significant, reproducible and unlikely to be the result of nonspecific binding, as it was not observed when reactions were supplemented with excess bovine serum albumin (BSA) (Fig. 1b and f). BSA is a stringent control for chaperone assays due to its propensity to bind to proteins through its abundant hydrophobic surface patches and is incapable of assisting in the refolding of FLuc even when present at a 64-fold excess by mass to RegA (∼190-fold molar excess). We next wished to determine whether the observed chaperone activity of RegA was influenced by nucleotide. We compared refolding reactions carried out in the presence or absence of ATP in the refolding buffer and found that the refolding curves were essentially superimposable, indicating that this activity of sacsin is not regulated by ATP (Fig. 1d and f).

In addition to enhancing the yield of refolded protein, molecular chaperones may also delay the velocity of a refolding reaction due to the iterative binding and release of unfolded and/or partially folded intermediates. Thus, we examined our results in terms of refolding rates in more detail. We plotted our data as the percent of the maximum luciferase refolded as a function of time in order to visualize the relative refolding rates (Fig. 1c and e). A qualitative examination of the curves revealed

Fig. 3. RegA of sacsin maintains luciferase in a folding-competent state and cooperates with additional chaperones. (a) Plot depicting the amount of FLuc refolded in an order-of-addition experiment as follows: FLuc (10 μM) was denatured and allowed to refold by 1:100 dilution from GdmCl in the presence of 600, 300 or 100 nM RegA or an equivalent concentration of BSA (w/w). At 30 min, a cocktail of K/J/E was added to a final concentration of 10, 2 and 6 μM, respectively. The data are plotted as mean and standard deviations of three independent experiments. (b) Plot depicting an equivalent experiment as that shown in (a), except that a version of RegA containing the D168Y mutation was utilized. (c) Plot of an identical experiment as in (a), except that ATP was initially excluded from the refolding reaction and was added simultaneously with the K/J/E cocktail at 30 min to the 600-nM RegA-supplemented reaction. The data points of RegA with ATP and BSA experiments from (a) are included for reference. (d) Histogram summarizing the maximum FLuc recovered (percent of a native control) for the indicated conditions (mean and standard deviation).
Sacsin RegA maintains luciferase in a highly folding-competent state that can be acted upon by the bacterial Hsp70 system

Our findings above that RegA only moderately assists the refolding of FLuc yet is highly capable of maintaining it in a soluble state led us to ask whether the main function of this region of sacsin is to maintain partially folded clients in a folding-competent state and subsequently deliver them to other regions within this large molecule (e.g., more C-terminal SRRs) and/or to additional chaperone systems. If this were the case, RegA of sacsin would need to bind folding-competent clients and release them within biologically relevant timescales, which would then be assisted by downstream chaperones. On the other hand, if clients were being kept soluble by binding irreversibly to RegA or if RegA were unable to discriminate between terminally misfolded and folding-competent clients, one would expect that subsequent chaperone systems would have a diminished capacity to assist them during their folding. In order to distinguish between these possibilities, we utilized the well-characterized Hsp70 system from E. coli, which consists of DnaK, DnaJ and GrpE [DnaK/DnaJ/GrpE (K/J/E)] and has previously been shown to be capable of refolding FLuc to very high yields (i.e., ~90%). We reasoned that, if the large fraction of FLuc being kept soluble by RegA upon dilution from denaturant was competent for refolding, subsequent addition of excess K/J/E would allow substantial recovery of activity. On the other hand, nonproductive binding would not yield any significant increase in activity upon addition of K/J/E, which would be expected for the large fraction of FLuc being maintained soluble by BSA (Fig. 2c).

FLuc refolding reactions were initiated, as described above, in the presence of a 6-fold molar excess of RegA or equivalent amounts of BSA (by weight, corresponding to an ~3-fold molar excess). After 30 min of incubation, K/J/E (10 μM/2 μM/6 μM, respectively) was added to the reactions, and light emission was measured over time (Fig. 3a). A substantial amount of reactivated luciferase was recovered in the RegA-supplemented reactions (~60%), whereas no additional active luciferase was recovered in the reactions supplemented with excess BSA (Fig. 3a and d). Thus, even though both RegA and BSA are capable of maintaining a large fraction of FLuc in a soluble state, only RegA is capable of releasing folding-competent intermediates in a biologically relevant timescale.

Typically, molecular chaperones exert their activity in vitro at supra-stoichiometric concentrations with respect to their clients, as they are not catalysts per se and must iteratively bind and release the majority of unfolded and partially folded molecules simultaneously en route to the native state.30 Thus,
chaperoning capacity is generally reduced as the concentration of chaperone approximates that of its client. In order to examine whether the function of RegA demonstrated above displayed a concentration-dependent behavior, we varied the RegA:FLuc stoichiometry from 6:1 to 3:1 and 1:1 at 100 nM constant FLuc. We observed a marked reduction in the capacity of RegA to maintain FLuc in a folding-competent state at the 3:1 stoichiometry, and its capacity disappeared at equimolar concentrations (Fig. 3a and d).

The function of the K/J/E system is dependent on ATP hydrolysis, and thus, this nucleotide cannot be omitted after addition of these chaperones. However, we tested whether presence or absence of ATP had any effect on RegA function by including it or omitting it during the 30 min of incubation prior to addition of K/J/E. The refolding curves of these experiments are superimposable (Fig. 3c), demonstrating that RegA does not require ATP for its chaperone activity in this assay.

We had previously described that the human-disease-causing mutation D168Y \(^{15}\) within the Hsp90-like segment of RegA results in complete abrogation of the ATPase activity of this region, and thus, we wished to determine whether this change had any effects on the robust ability of RegA to maintain FLuc in a folding-competent state. We set up a series of experiments as described above, except that we utilized the version of RegA that contains the D168Y substitution. We observed essentially identical behaviors between wild-type RegA and the D168Y mutant (compare Fig. 3a and b). Additionally, the D168Y mutant displayed a similarly subtle ability to assist directly in FLuc refolding to that of wild-type RegA (data not shown). These findings are not entirely surprising and are consistent with the notion that our luciferase refolding assays reveal an ATP-independent aspect of the chaperone activity of sacsin, which is likely only a partial reflection of its full function in the cell.

RegA of sacsin displays a high affinity for unfolded/partially folded FLuc

Since both the K/J/E system and RegA interact with unfolded/partially folded FLuc, we reasoned that we could use this system to qualitatively estimate the relative affinity of RegA for FLuc. We predicted that, if DnaK and RegA were both included at equivalent concentrations in the same refolding reaction, the behavior of the refolding reaction would be governed by the chaperone with the greatest affinity for FLuc (fast and efficient refolding for K/J/E versus slower and less efficient refolding for RegA). Therefore, we decreased the concentration of the components of the K/J/E system to where DnaK is equal to RegA (while maintaining the ratio of K/J/E as in all other refolding reactions). The combination of both RegA and K/J/E in a FLuc refolding reaction caused a considerable change in the rate and yield of refolded FLuc compared to K/J/E alone (Fig. 4a).

Significantly, the resulting curve instead resembled the curve for RegA alone (Fig. 1b), whereas the combinations of BSA (at equivalent concentrations to RegA) and K/J/E did not have any effects on the behavior of the refolding reaction. Taken together, these results suggest that the affinity of RegA for unfolded/partially folded FLuc is stronger than the nonspecific interaction between BSA and FLuc and of similar or greater magnitude than the affinity between FLuc and the K/J/E system. Interestingly, the fraction
of refolded FLuc in the mixture containing RegA and K/J/E did not reach the same level as that of the reaction containing K/J/E alone. We interpret this result as effective competition between RegA and K/J/E at these low chaperone concentrations due to a substantially higher affinity of RegA for FLuc. Consistent with these results, the binding of RegA to FLuc could be overcome by increasing the concentration of K/J/E in the refolding reaction: a 20-fold excess of K/J/E relative to RegA was able to efficiently and rapidly capture and assist the FLuc being maintained soluble by RegA (Fig. 3a). These results indicate that, in the cell, where the concentration of Hsp70 family members is in substantial excess to that of sacsin, these systems may efficiently cooperate during the folding of client polypeptides.

**RegA of sacsin can cooperate with DnaK independently of DnaJ**

The participation of DnaJ in the K/J/E-assisted folding cycle consists of two related activities: client delivery to DnaK (via its client-binding domain) and stimulation of the ATPase activity of DnaK (via its J domain). Since sacsin contains a functional J domain and at least one region that can interact with unfolded/partially folded FLuc, we wondered if sacsin could perhaps operate as a functional DnaJ-type co-chaperone. We decided to test whether RegA could functionally operate as the client delivery module of a DnaJ co-chaperone by performing experiments in the presence and absence of bacterial DnaJ. If the cooperation of RegA with K/J/E during FLuc refolding was strictly dependent on the presence of DnaJ in the mixtures, then it would be less likely that sacsin can function as a DnaJ co-chaperone. To test this, we began by examining the behavior of a FLuc refolding experiment in the presence of DnaK and GrpE [DnaK/GrpE (K/E)] but in the absence of DnaJ. K/E alone did not efficiently assist FLuc during its refolding (Fig. 4b). However, the presence of RegA in the K/E mixture substantially enhanced the yield of refolded FLuc (≈27% versus ≈47%) (Fig. 4b). Consistent with the results presented throughout this study, substitution of RegA by BSA in the same experimental set up did not result in any increases in activity beyond those observed for K/E or K/J/E (Fig. 4b). These results confirm that folding-competent clients may be delivered from RegA to DnaK and suggest that full-length sacsin, which contains a J domain, may be ideally situated to deliver clients to members of the Hsp70 family in the eukaryotic cytosol where it has been localized.

**Discussion**

In this study, we provide evidence that sacsin functions as a molecular chaperone. RegA of sacsin was moderately capable of directly assisting during the refolding of FLuc and was highly capable of maintaining it in a state that can be productively acted upon by bacterial Hsp70 family members. The capacity of sacsin to bind and release folding-competent client proteins within biologically relevant timescales, its ability to cooperate with additional chaperones and the presence of a functional J domain demonstrate that this unusually large protein contains both chaperone and co-chaperone modules within the same polypeptide chain.

The three SRRs along the sacsin molecule contain segments with considerable similarity to the N-terminal domain of Hsp90, yet the molecular chaperone activity described here for RegA is subtly different from that of Hsp90. Hsp90 is an abundant molecular chaperone that, in the eukaryotic cytosol, is involved in the maturation of a growing list of clients, which notably include protein kinases and steroid hormone receptors.24 The chaperone activity of Hsp90 has been documented in multiple experimental paradigms. For example, it has been shown to prevent the aggregation of various model proteins (including β-galactosidase and FLuc) and maintain them in a state competent for subsequent action by other molecular chaperones.24,25 RegA of sacsin is similar to Hsp90 in this regard. However, in contrast to the Hsp70/Hsp40 system, Hsp90 has not been demonstrated to be directly capable of increasing the refolding yield of several model clients.25 Thus, in this regard, RegA of sacsin was different from Hsp90 in that it was capable of increasing the refolding efficiency of FLuc. Thus, sacsin possesses a "protective" chaperone activity similar to that of Hsp90 *in vitro*. In addition, it displays a distinct, albeit modest, "folding" chaperone action not present in Hsp90.

It is important to note that the SRR domain present in RegA is only one of three SRRs present along the full-length sacsin molecule. Its intermediate capacity to directly assist in the refolding FLuc may reflect the fact that full activity may necessitate cooperation or synergy among the various SRR domains of sacsin. That is, successive copies of SRRs may display a considerably stronger chaperone activity as reflected by this type of assays. Of note, the vast majority of SRRs present in eukaryotes occur in multiples. Indeed, we have previously found evidence of convergent evolution leading to this characteristic arrangement of consecutive SRRs along the same polypeptide chain.15 Additionally, the presence of a functional J domain in the full-length sacsin molecule suggests that SRRs are perhaps not designed to function independently, but rather in concert with other chaperone systems in the cell, most probably with members of the Hsp70 family.

We have shown previously that RegA of sacsin possesses ATPase activity and that the human-
disease-causing mutation D168Y completely abrogates this activity. This suggests that this protein segment is structured and that the novel properties described in this study are not the result of nonspecific binding through hydrophobic regions that could be present if this segment were unstructured. Our extensive controls with BSA throughout this report support our conclusions that the observed properties of RegA cannot be attributed to irreversible nonspecific binding, but rather represent a novel chaperone activity associated with this region of sacsin.

In this study, we find that the D168Y mutation has no detectable effects on the capacity of RegA to assist in the refolding of FLuc. This is consistent with the notion that our luciferase refolding assays reveal an ATP-independent aspect of the chaperone activity of sacsin, which may only be a partial reflection of its full function in vivo. This is likely due to the fact that we are working with a fragment of the protein. The precise mechanisms by which the D168Y results in disease remain unclear. However, as with Hsp90, it is likely that disruption of nucleotide binding and hydrolysis in mutant sacsin results in defects of the careful orchestration of conformational states required in realizing its full function in the cell.

Additionally, RegA of sacsin is likely to functionally collaborate with other modules involved in protein quality control in the cell, either within its own polypeptide or with downstream members of the folding and proteolytic machinery of the cell. Future work may reveal likely sacsin interaction partners, including members of the eukaryotic Hsp70 family, as well as potential client polypeptides that necessitate sacsin activity to acquire and/or maintain their structure and promote neuronal survival.

Materials and Methods

Protein purification

Purification of wild-type RegA and D168Y RegA

RegA and an identical version containing the D168Y substitution are His6-tagged proteins encompassing residues 1–1456 of murine sacsin. Purification from recombinant baculovirus-infected insect cells was performed essentially as described previously. Briefly, cells were resuspended in lysis buffer [30 mM Tris (pH 7.4), 150 mM NaCl and 1× protease inhibitors without EDTA (Amresco), disrupted in an EmulsiFlex C3 (Avestin) and clarified by centrifugation at 30,000 g for 30 min at 4 °C. The supernatant was loaded on a DE52 column and eluted with a linear gradient of KCl in the lysis buffer. Fractions containing purified DnaK were collected, pooled and concentrated by ultrafiltration. Glycerol was added to 10% (v/v), and aliquots were flash frozen in liquid nitrogen and stored at −80 °C.

Purification of DnaK

E. coli DnaK was purified from BL21 E. coli cells overexpressing the wild-type protein, as described previously. Harvested cells were resuspended in 50 mM Mops [3-(N-morpholino)propanesulfonic acid] (pH 7.6), 20 mM KCl, 1 mM EDTA, 1 mM DTT and 1× protease inhibitors without EDTA (Amresco), subsequently disrupted in an EmulsiFlex C3 (Avestin) and clarified by centrifugation at 30,000 g for 30 min at 4 °C. The supernatant was loaded on a DE52 column and eluted with a linear gradient of KCl in the lysis buffer. Fractions containing purified DnaK were collected, pooled and concentrated by ultrafiltration. Glycerol was added to 10% (v/v), and aliquots were flash frozen in liquid nitrogen and stored at −80 °C.

Purification of DnaJ

E. coli DnaJ was purified from BL21 E. coli cells overexpressing the wild-type protein as described previously. Harvested cells were resuspended in 20 mM Tris (pH 6.8), 1 mM DTT, 0.6% Brij-58 and 1× protease inhibitors without EDTA (Amresco), disrupted in an EmulsiFlex C3 (Avestin) and clarified by centrifugation at 30,000 g for 30 min at 4 °C. The supernatant was loaded onto a HiTrap SP column (GE Healthcare) pre-equilibrated with 20 mM Tris (pH 6.8), 1 mM DTT and 0.6% Brij-58. After extensive washing with equilibration buffer, we accomplished elution with a linear gradient to 20 mM Tris (pH 6.8), 1 mM DTT and 1 M KCl. Fractions containing pure DnaJ were pooled, and glycerol was added to 10% (v/v). Aliquots were flash frozen in liquid nitrogen and stored at −80 °C.

Purification of GrpE

A His6-tagged version of E. coli GrpE was purified as described from BL21 E. coli cells. Cells were harvested and lysed in 1× phosphate-buffered saline (PBS), 0.1% Triton X-100 and 1× protease inhibitors without EDTA (Amresco), disrupted in an EmulsiFlex C3 (Avestin) and clarified by centrifugation at 30,000 g for 30 min at 4 °C. The supernatant was loaded on a HiTrap column (GE Healthcare), washed with 1× PBS containing 50 mM imidazole and subsequently eluted with 1× PBS containing 0.5 M imidazole. Fractions containing pure GrpE were collected and desalted into 1× PBS. Glycerol was added to 10% (v/v), and aliquots were flash frozen in liquid nitrogen and stored at −80 °C.

Protein concentrations were determined by absorbance at 280 nm with predicted extinction coefficients obtained by the ProtParam tool from the ExPASy Proteomics Server (Swiss Institute of Bioinformatics). Protein purity was assessed by SDS-PAGE followed by methanol/acetic acid fixation and Coomassie Brilliant Blue staining.

Refolding of luciferase

Luciferase from Photinus pyralis was obtained from Sigma. The lyophilized powder was resuspended in 0.5 M Tris succinate (pH 7.7). Luciferase (5 or 10 μM) was denatured in 6 M GdmCl and 5 mM DTT for 30 min at room temperature and then kept on ice. The standard refolding reaction was carried out in 1× luciferase buffer...
chemiluminescence (GE Healthcare) and exposure to film. (1:5,000; Pierce), followed by incubation with enhanced horseradish-peroxidase-conjugated goat anti-mouse and probed with anti-luciferase (1:2,500; Millipore) and membrane was then treated as a traditional Western blot in 5% milk in Tris-buffered saline with Tween 20. The supernatant was removed, and 1 volume of 2× Laemmli buffer was added to the remaining volume to desired total, followed by aliquoting into small volumes (typically 1 mL) and immediately freezing at −80 °C. Final concentrations were confirmed by absorbance at 260 nm. ATP aliquots were discarded after one thawing.

Dot-blot analysis

Luciferase refolding reactions were performed as described above. At 30 min, an aliquot was removed from each reaction (and kept as a reference for the total reaction), and the remainder was centrifuged for 30 min at 20,000g at 4 °C. The supernatant was removed, and 1 volume of 2× Laemmli SDS sample buffer was added to 1 volume of total and super samples. The pellet was resuspended in an equivalent volume of 1× Laemmli SDS sample buffer. The reactions were spotted onto a nitrocellulose membrane utilizing the Bio-Dot microfiltration apparatus (Bio-Rad). After extensive washing, we removed the membrane and blocked it with 5% milk in Tris-buffered saline with Tween 20. The membrane was then treated as a traditional Western blot and probed with anti-luciferase (1:2,500; Millipore) and horseradish-peroxidase-conjugated goat anti-mouse (1:5,000; Pierce), followed by incubation with enhanced chemiluminescence (GE Healthcare) and exposure to film.

Acknowledgements

We thank E. R. Anderson, H. F. Epstein, D. F. Boehning, R. O. Fox and A. F. Oberhauser for helpful discussions. This work was supported in part by the Research Grant No. 5-FY07-641 from the March of Dimes Foundation and by an award from the Pew Scholars Program in the Biomedical Sciences to J.M.B.

References

the Hsp90 molecular chaperone in vivo. EMBO J. 17, 4829–4836.