Chaperone discovery

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Molecular chaperones assist de novo protein folding and facilitate the refolding of stress-denatured proteins. The molecular chaperone concept was coined nearly 35 years ago, and since then, tremendous strides have been made in understanding how these factors support protein folding. Here, we focus on how various chaperone proteins were first identified to play roles in protein folding. Examples are used to illustrate traditional routes of chaperone discovery and point out their advantages and limitations. Recent advances, including the development of folding biosensors and promising methods for the stabilization of proteins in vivo, provide new routes for chaperone discovery.

Keywords:
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Introduction

Chaperones are conserved molecular machines that ensure the maintenance of a functional proteome under normal and stress conditions [1]. They act in multiple cellular processes, including de novo protein folding, preventing the aggregation of misfolded proteins, assembly of oligomeric protein complexes, refolding of denatured proteins, intracellular protein transport, and assisting in proteolysis. In some cases, chaperone co-overexpression facilitates the overexpression of heterologous proteins [2, 3]. Although the working mechanism of major categories of chaperones has been relatively well established [4], their specificity for client proteins is not yet well enough defined to enable one to accurately predict the impact of a particular chaperone on a specific client. The specific nature of chaperone-client interactions means that chaperone co-expression is not a panacea that solves all the folding problems encountered in heterologous protein production [5]. Customizing the folding environment of individual target proteins is one reasonable solution, but even here it is difficult to predict which chaperone will interact with any given client protein, despite the growing knowledge of protein-chaperone interaction networks. In addition, there is no indication that the current list of chaperones and folding factors is complete. In this review, we focus on the chaperone discovery process, starting with historical examples to illustrate how chaperones have commonly been discovered. We then move on to recent and innovative techniques to monitor protein folding and stabilize proteins in vivo, describing their successful application in the discovery of novel chaperones and in the elucidation of novel functions of known chaperones.

Several chaperones have already been discovered prior to the popularization of the molecular chaperone concept. These proteins were identified either as binding partners of other proteins or via mutations that interrupt a biological process, such as bacteriophage assembly. The transcription of many chaperones is up-regulated by stress stimuli, such as heat shock [6]. This induction confers thermo-tolerance and thus contributes to an organism's stress resistance, an observation that had been made even before the functional role of heat shock proteins (Hsps) was clearly understood. In the mid 1980s, evidence accumulated that Hsps were involved in the folding or assembly of other proteins, leading to the development of the chaperone concept. To this day, the Hsps remain the most intensely studied molecular chaperones [7]. Chaperones guide the protein folding process and minimize the possibility of forming kinetically trapped species, thereby preventing premature aggregation of their client proteins. Proteins are often only marginally thermodynamically stable, resulting in a significant population of unfolded and partially folded species even under normal conditions. Stress can easily shift the conformational equilibrium towards more partially folded intermediates or unfolded forms. This increased requirement for chaperones under stress conditions goes a long way to explaining the heat inducibility of the Hsps.

Despite the fact that heat shock response studies led to the discovery of many chaperones, it is inefficient to rely on

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Abbreviations:
GFP, green fluorescent protein; **Hsp,** heat shock protein.

similar approaches to identify new chaperone candidates. Almost any change in growth conditions can be considered a "stress", and the vast majority of the myriad proteins whose synthesis rates change in response to changes in growth conditions are not directly involved in protein folding. In addition, many proteins require assistance in folding even under normal conditions; as a result, many chaperones are normally abundant and some are not further induced upon stress [8].

With the development of in vitro chaperone activity assays, biochemical verification of potential chaperones became possible, and as a result, additional proteins were added to the list of chaperones. Genomic sequencing efforts also resulted in the identification of a vast number of genes homologous to known chaperones. One wonders, however, if there are chaperones outside these homologous families that are not induced by common stress conditions and have therefore remained undiscovered. Are the current in vitro chaperone assays sensitive and specific enough to enable their discovery, or are new approaches needed? New strategies that link in vivo protein folding to easily detectable phenotypes may be one of the keys to discovering and maybe even designing new chaperones.

Discovery of Hsp70 and Hsp60: The heat shock response is associated with protein folding

The ubiquitous heat shock proteins Hsp60, Hsp70, and Hsp90 represent some of the best-studied families of molecular chaperones. It has been known since the 1960s that mild heat treatment of an organism induces different families of proteins with these molecular weights, and in the mid 1980s, many of these proteins, as well as the response itself, were shown to be conserved in species ranging from bacteria to humans [9, 10]. Although initially studied, primarily from a regulatory standpoint [11], scientists became fascinated by what these proteins might be doing. Early on, it became clear that the heat shock response acts to protect organisms from a subsequent more severe heat stress, indicating that the induced Hsps contribute to the protection [12]. Evidence suggested that some Hsps have the ability to bind to other proteins. For example, DnaK, the bacterial homolog of eukaryotic Hsp70 [9], was shown to interact with lambda replication proteins [13]. Mammalian Hsp70 accumulates in the nucleus during heat shock and was proposed to help repair heat damaged pre-ribosomes [14]. Hsp70 can be dissociated from heat-shocked nucleoli by the addition of ATP [15]. Based on these observations, Pelham [16] proposed a model in which Hsp70 binds to heat denatured proteins through hydrophobic interactions and then uses the energy provided by ATP hydrolysis to release the substrate proteins, which allows them to refold in solution. This was perhaps the first time that the heat shock response was related to protein refolding and repair.

The bacterial Hsp60 homolog, GroEL, was first identified by its requirement for lambda phage morphogenesis [17]. Defects in the *groE* locus were later shown to cause aggregation of the phage lambda head protein and phage T4 capsid protein [18]. Ellis found that the unassembled large subunits of Rubisco, a plant chloroplast protein, were co-purified with an abundant soluble protein that later was found to be homologous to GroEL [19, 20]. The concept of molecular chaperones was proposed around this time [21]. Two years later, the chaperone system consisting of GroEL, GroES, and Mg-ATP was reconstituted in vitro for the assembly of Rubisco subunits [22]. In 1989, Hartl and Horwich collaboratively isolated and characterized a mutant that affected the proper folding of imported mitochondrial proteins but did not disrupt the importing process [23]. This mutation was mapped to mitochondrial *hsp60;* later, the chaperone function of mitochondrial Hsp60 was revealed on the model substrate dihydrofolate reductase (DHFR) [24]. Thus, in the late 80s, both Hsp60 and Hsp70 were shown to be involved in protein folding. Over time, it became clear that all the major classes of Hsps play important roles in protein folding [6].

Traditional routes for chaperone discovery: Successes and limitations

The development of in vitro assays for chaperone activity allowed researchers to investigate whether proteins other than Hsps could assist the folding of model substrates. For example, a thermal aggregation assay with citrate synthase and a luciferase refolding assay have been used for this purpose. However, these assays are relatively insensitive and some are non-specific in nature. Any protein or compound that can affect the folding of model substrates can show some activity in anti-aggregation assays, including proteins that most would consider relatively inert, such as bovine serum albumin (BSA) [25]. Refolding assays are usually more diagnostic for chaperone activity, but in the absence of complete chaperone machinery, including all necessary cofactors, they may give misleading negative results. These limitations and the overall lack of sensitivity of chaperone assays have prevented their use in direct chaperone isolation via purification, which is how enzymes are often purified from crude lysates. On the other hand, once a chaperone has been definitively identified, homologous proteins become prime candidates for also having chaperone activity. Work on chaperone biology has thus been split into well-defined fields based on homology to chaperones such as GroEL, Hsp70, or Hsp90. Working within these families, tremendous advances have been made in understanding how chaperones work (for reviews, see [26–29]).

In addition to homology to known chaperones, historically chaperones have also been identified based on their ability to bind other proteins, to be induced by stress, or to cause generalized protein destabilization when the genes that encode them are mutated (Fig. 1). A few examples are included below to illustrate how these principles have guided chaperone identification in *Escherichia coli* and yeast. They also serve to point out that although many chaperones meet a number of these criteria, the manner in which they were first identified was often somewhat random.

Several yeast chaperones were discovered by homology to Hsp70

The yeast *Saccharomyces cerevisiae* contains at least 14 proteins homologous to Hsp70 [30, 31]. While several of these Hsp70 homologs are found in the cytosol, others are located in

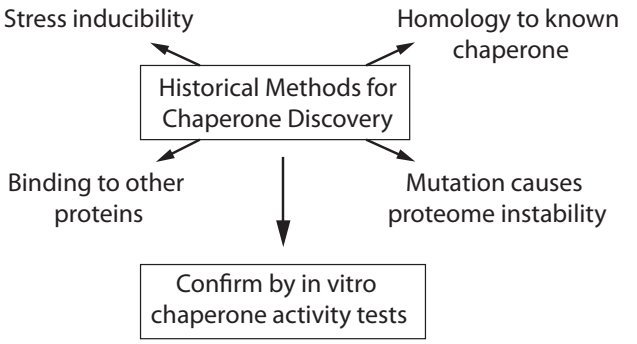

Figure 1. Historical processes of chaperone discovery. Many chaperones were discovered by these approaches. The combination of different criteria increases the sensitivity and specificity of chaperone discovery.

the endoplasmic reticulum (ER) and mitochondria. Among these, some are stress inducible and others are constitutive [32], nevertheless, all play important roles in protein folding and exhibit broad ranges of substrate specificity. Initially, the driving force for their characterization was their homology to known eukaryotic Hsp70 proteins, although occasionally some were identified in genome-wide screens for genes involved in cellular processes [33].

Many *E. coli* chaperones were identified as protein binding partners

In *E. coli*, the cytoplasmic protein trigger factor is the chaperone that many nascent polypeptides first encounter [34]. It was discovered in a biochemical screen for cytosolic components of the secretion machinery as a protein that stabilizes the precursor of outer membrane protein A (OmpA) and makes it translocation-competent [35]. Trigger factor was found to form a stable 1:1 complex with chemically denatured OmpA [36]. It was also found to associate with a nascent polypeptide chain in an in vitro translation system [37] and in a cross-linking experiment [38].

Although the periplasmic chaperone Skp was shown to have broad substrate specificity [39], it was initially identified as a binding partner to outer membrane proteins in a pull-down assay using the outer membrane porin, OmpF [40]. Another periplasmic chaperone LolA was identified as a carrier protein that facilitates the release of an outer membrane lipoprotein, Lpp, from the inner membrane [41].

IbpA and IbpB are *E. coli* homologs of the eukaryotic small Hsps [42]. They were identified as chaperones not just by this homology but also by their ability to associate tightly with inclusion bodies formed either during heterologous protein production [43] or from endogeneous *E. coli* proteins that aggregated due to heat shock [44].

Hsp33 and Hsp31 were identified by their upregulation in response to stress

As their names suggest, the cytoplasmic chaperones Hsp33 and Hsp31 were originally identified by their stress inducibility. The activation of Hsp33 is also redox-regulated on the post-transcriptional level, which distinguishes it from other temperature-regulated cytosolic chaperones [45]. Hsp31's chaperone activity is also heat-regulated on a post-translational level [46]. Its mode of action relies on temperature-driven conformational changes to expose hydrophobic regions to capture substrates. The chaperone activities of both of these chaperones were established through in vitro activity assays [45, 47].

SurA, HdeA, and other chaperones were identified by mutational phenotype

Mutational studies have been used to implicate the periplasmic chaperone SurA in the biogenesis of outer membrane proteins. The gene *surA* was originally identified as essential for survival in stationary phase [48]. Strains devoid of *surA* are slightly mucoid in rich medium and are sensitive to bacitracin, vancomycin, and bile salts, suggesting that SurA plays a role in maintaining the integrity of the outer membrane [49]. A global screening for genes that suppress one of the extracytoplasmic stress responses, the σ^E stress response, identified *surA* and two other chaperone genes, *fkpA* and *skp* [50].

Similarly, *hdeA* was identified as a chaperone gene that is important for survival at low pH [51]. Low pH causes protein denaturation. However, the HdeA dimer dissociates into disordered monomers under acidic pH and protects acid denatured proteins from aggregation [52, 53]. The *hdeA* gene is among those induced under acidic conditions [54].

Combining different strategies can increase the specificity of chaperone discovery

Although the criteria described above were historically successful in identifying chaperones, none are particularly specific. For example, in the case of protein binding, the majority of proteins interact with other proteins within a cell, so just binding to another protein by itself clearly does not qualify a protein as a chaperone. Mutational phenotypes can be hard to interpret and can often be obscured by the mutations affecting metabolism, growth, cell division, etc. Even proteins that share chaperones' relatively non-specific ability to bind other proteins may prove not to be chaperones after detailed analysis. Proteases often bind proteins relatively non-specifically, and the line between a protein being a chaperone and a protease is sometimes blurry. This is illustrated by DegP, which was reported to switch from a chaperone to a protease depending on the temperature [55], accompanied by higher order assemblies of the trimeric subunit [56].

Stress inducibility is perhaps a better initial criterion but is also not perfect. Heat shock, for instance, induces roughly 50–200 genes in various model organisms [57], and the most strongly induced proteins are often involved in protein folding. Nevertheless, many other categories of genes are heat-induced, including components of the proteolytic system and those maintaining cellular structure, regulation, and metabolism, as well as DNA/RNA binding and modification enzymes [57]. Thus, the designation Hsp does not necessarily indicate a

chaperone function. For example, yeast Hsp12 stabilizes membranes by binding to them and thus decreases membrane fluidity [58]. Bacterial Hsp15 binds RNA [59, 60] and is involved in ribosome 50S recycling [61].

To increase the specificity of chaperone discovery, one can combine multiple approaches and focus on the targets found to be in common among the different techniques used. For example, Zhao et al. [62] combined four different approaches to identify the interaction network of the Hsp90 chaperone. Genome-wide two-hybrid screening and large-scale affinity purification were used to identify 198 proteins that physically interact with Hsp90. In addition, two array-based genetic screens were used to identify 451 mutations that resulted in synthetic lethality in the absence of functional Hsp90, thereby indicating potential genetic interactions. Comparison of the physical and genetic interaction maps narrowed down the potential substrate and co-chaperone candidate genes from hundreds to 22, three of which encode established Hsp90 cofactors. Note that the four approaches also identified in total 133 uncharacterized open reading frames (ORFs). The authors reported the characterization of two novel cofactors of Hsp90 from these uncharacterized ORFs. The functions of these novel cofactors were initially inferred by homology analysis and then subsequently confirmed by showing that the corresponding knock out strains exhibited impaired ability to fold two well-established Hsp90 substrates in vivo. We consider it possible that additional chaperones or co-chaperones exist within this list of uncharacterized ORFs.

New tools for chaperone discovery

Despite the historical success of traditional chaperone discovery approaches, all require a certain degree of effort. In particular, if multiple approaches are needed to reach the desired degree of specificity, the effort required could become prohibitive. New tools to monitor the folding and stability of proteins in vivo allow for a direct assessment of the effect of chaperone action, thus providing new routes for chaperone discovery. In this review, we focus on methods for chaperone discovery that have high-throughput capacity, are easy to perform, can readily be adapted for a variety of target proteins, and require little information on the structure and function of the target protein. Although colony-based solubility screening methods such as colony filtration blot [63] and ''soluble tag availability'' [64, 65] can work in an automatic and relatively high-throughput fashion to determine the in vivo folding of test proteins, these techniques are generally costly and require multiple steps [66], making them more feasible for secondary validation than for primary chaperone discovery efforts. We will therefore focus instead on high-throughput protein folding biosensor approaches and describe how they can potentially be used for chaperone discovery.

Protein reporter-based approaches to monitor protein folding in vivo

Fusing a target protein to a reporter protein whose function is dependent on the folding status of the fusion partner has proved to be a successful technique for revealing the in vivo stability and folding efficiency of the target protein, particularly if the reporter has no effect on the folding properties of the target. In extreme cases, a poorly folding target protein can render the fusion partner insoluble or unable to assemble a functional protein. Reporter proteins are often chosen because they lead to a color or fluorescence readout or are easily assayed enzymatically or genetically. Reporters that confer a growth advantage in the presence of antibiotics, thereby allowing the rapid identification or survival of clones that express well-folded target proteins, are particularly useful. These include green fluorescent protein (GFP) [67], β-galactosidase α-peptide (lacZα) [68], chloramphenicol acetyltransferase (CAT) [69], β-lactamase [70], kanamycin nucleotidyl transferase [71], *E. coli* DHFR [72], and murine DHFR (mDHFR) [73].

Initial constructs involved fusing the reporter proteins to the ends of targets [72, 74, 75]. More recently, split reporter systems were developed to avoid several issues with the initial constructs, including interference from bulky reporters and release of functional portions of the target by proteolysis, which can lead to complementation and false-positive results [76, 77]. Reporter proteins can be split using two different approaches. In the first approach, a small independently folding fragment of the reporter is fused to the target protein and the rest of the reporter is expressed separately. In the second approach, a target protein is inserted directly into the reporter, dividing it into two portions. In both strategies, the two portions of the reporter must be able to reassemble into a functional protein that expresses a screenable or selectable phenotype.

For example, in one widely used reporter system (GFP), a small fragment with a modified sequence (GFP β-strand 11) is fused to the target protein and the complementary GFP detector (β-strand 1–10) is expressed separately [78] (Fig. 2A). A fluorescence signal results when the whole fusion is soluble and the GFP fragment is accessible to the complementary detector [78]. Misfolding of the target protein prevents the correct reconstitution of the two GFP fragments, enabling the split GFP system to be used as an in vivo folding biosensor. Circular permutation studies have revealed additional sites in GFP that allow splitting without inactivating the protein. The resulting series of GFP reporters have different degrees of stringency that are capable of accommodating target proteins with different folding propensities [79]. In a similar approach, simultaneous fusion of GFP to the C-terminus and blue fluorescent protein (BFP) to the N-terminus of a target protein allows folding of the target to be monitored by fluorescence resonance energy transfer (FRET). Efficient FRET from BFP to GFP occurs only when the target protein is well folded and the two fluorescent proteins are brought into close proximity [80].

Our laboratory has engineered two folding biosensor sandwich fusions using the selectable reporters β-lactamase and DsbA (Fig. 2B). β-lactamase encodes resistance to ampicillin and other commonly used β-lactam antibiotics, and DsbA, a periplasmic disulfide oxidoreductase, confers resistance to the toxic metal cadmium [70, 81]. Target proteins are inserted into permissive sites in β-lactamase or DsbA. If the target protein is well folded, the reporter will be reconstituted as a functional enzyme. In contrast, a misfolded target is prone to cleavage by proteases; this separates the reporter into two halves, which

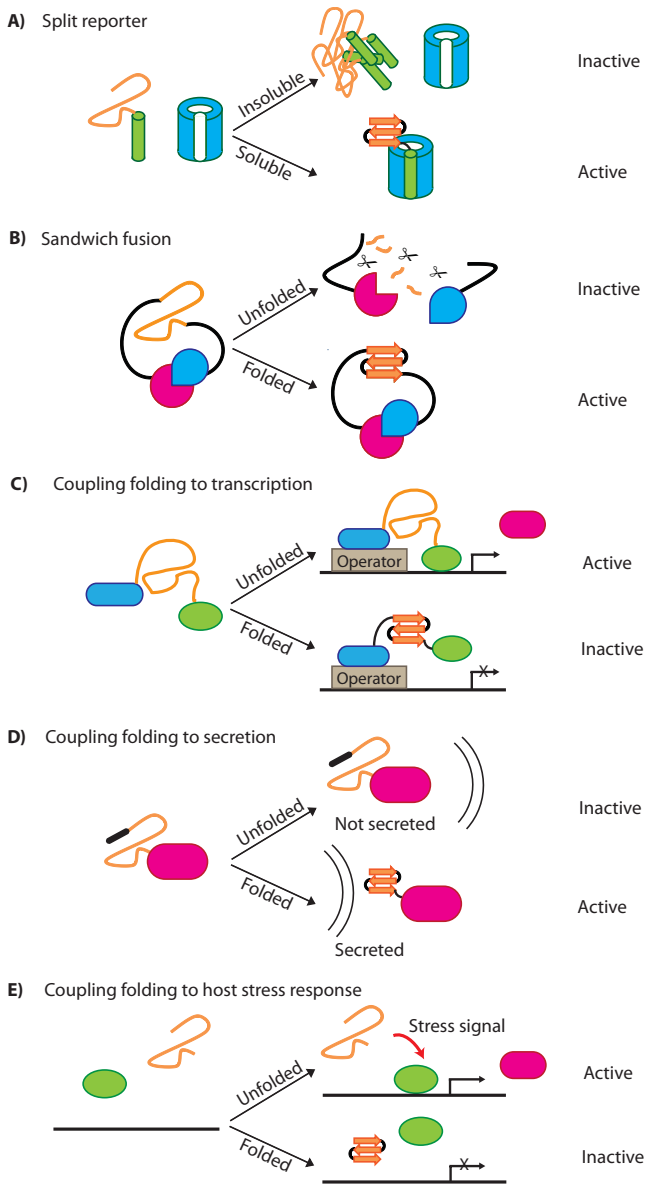

Figure 2. Protein reporter-based approaches to monitor protein folding in vivo. **A:** Split reporter as illustrated by the GFP reporter. The target protein (orange) is fused to a β strand of GFP (green), and a GFP detector missing this strand (blue) is expressed separately. **B:** Sandwich fusion as illustrated by the β-lactamase reporter. The target protein is inserted between the N-and C-termini (blue and magenta) of β-lactamase. In both A and B, only a well-folded target protein allows for the reconstitution of an active reporter. Misfolding of the target protein results in aggregation (as shown in A) or proteolysis (as shown in B) of the fusion protein, leading to a failure in the reconstitution of an intact reporter. **C:** Coupling to transcription to monitor folding. The target protein is fused to a DNA binding domain (blue) and a transcriptional activation domain (green), respectively on each end. A well-folded target protein generates a conformational constraint between the two domains, preventing their simultaneously binding to the operator and promoter regions of the reporter gene to initiate its transcription. A poorly folded target protein is more flexible, which allows for the transcription of the reporter gene and the production of the reporter protein (magenta). **D:** Use of the protein secretion quality control system to monitor folding. The target protein is fused to a secretion signal (black) on the N-terminus and a reporter on the C-terminus (magenta). A misfolded target protein is degraded by the quality control system, leaving the reporter in the wrong cellular compartment where it is inactive. Only a well-folded target protein will allow the tripartite fusion protein to reach the right compartment where the reporter is active. **E:** Use of the host stress response to monitor folding. Here, the target protein and the reporter protein are not directly connected. Misfolding of the target protein is detected by the cellular stress monitoring system, which transduces a signal to a transcriptional activator (green) to initiate the transcription of the reporter (magenta). A well-folded target protein does not activate the stress response.

are then also susceptible to proteolytic cleavage. As a result, bacteria containing fusions with well-folded target proteins are resistant to higher levels of antimicrobials than those containing fusions with poorly folded target proteins.

These various reporter systems have been used to improve the stability or solubility of target proteins via mutagenesis of the targets [82, 83], to screen for compounds that inhibit aggregation of the Alzheimer's peptide [84], and to engineer novel protease variants with desired properties [85]. Importantly, in the context of this review, and as discussed below, these systems can also be used to identify novel chaperones.

Already developed end-fusion folding reporter systems are particularly promising for conversion into sandwich folding reporter fusions. Sites that are tolerant to the insertion of large sequences are likely to have substantial overlap with sites that are tolerant to circular permutation. This is because circular permutation entails inserting a gap at a certain position within the protein while fusing its N and C termini. This process is functionally very similar to inserting a protein within a reporter, which creates a gap and, like circular permutation, has the effect of bringing the N- and C-terminal portions of the reporter into proximity. Thus, methods to discover sites capable of circular permutation can potentially be used to find sites that are suitable for construction of sandwich fusions [81].

In principle, many selectable or screenable markers that monitor protein-protein interactions could also be used to generate sandwich folding biosensors. A system analogous to the well known yeast two-hybrid system [86] was recently adapted to screen for more stable proteins. The target protein is flanked by a DNA-binding domain and a transcriptional activation domain [87] (Fig. 2C). A less stable target protein is apparently more flexible than a well-folded one, enabling the transcriptional activation domain to bind to the promoter region to initiate transcription of the reporter (β-lactamase). Therefore, more stable protein variants are obtained by looking for *slower* growing colonies on antibiotic plates [87, 88]. Note that this endpoint is in contrast to that used by many other reporter systems, in which target protein stability or solubility is positively correlated with the phenotypes of the reporter proteins.

Coupling reporter systems to in vivo protein quality control

The protein sorting and translocation quality control systems in prokaryotic and eukaryotic organisms normally make distinct fate determinations for folded and unfolded proteins; this

control feature can be combined with reporter systems to make selections or screens more powerful (Fig. 2D). In *E. coli*, the twin-arginine translocation (TAT) system only transports well-folded proteins into the periplasm. By fusing the TAT-specific signal sequence to a dipartite fusion construct of a target protein and the β-lactamase reporter, only well-folded soluble fusion proteins can be translocated into the periplasm, where the β-lactamase reporter confers cells with antibiotic resistance [89, 90]. In yeast, systems have been developed based on the observation that the secretory pathway prevents the release of incompletely folded polypeptides; thus, only folded proteins will be directed to the cell surface [91]. Recently, an elegant selection strategy was developed that involved the fusion of a selectable reporter, invertase, to a target protein that had been previously fused to an N-terminal secretion signal peptide [92]. Invertase catalyzes the hydrolysis of sucrose into glucose and fructose, which can then be taken up by the cell and used for growth. The selection is based on the observation that yeast quality control mechanisms will only enable well-folded invertase fusion proteins to release invertase into the medium, thus allowing cells expressing these well-folded fusions to grow on plates in which sucrose is the sole carbon source. This system was successfully applied to uncover folding-proficient variants of TNF-α [92].

Folding reporters induced by the host stress response

Transcriptional fusion reporters that signal the stress response of the cell upon misfolding and aggregation of a test protein have also provided an approach to assess folding in vivo. The test protein is not modified; instead, stress-responsive promoters are cloned in front of a reporter gene (Fig. 2E). Expression of the reporter is induced when the misfolded test protein accumulates. The induction level of the reporter protein is dependent on the folding state and amount of the test protein. Poorly folded test proteins evoke greater signals than well-folded test proteins. Such systems have been developed in both yeast and *E. coli*. Jonikas et al. [93] described a strategy for using the endogenous unfolded protein response (UPR) in the yeast ER as a sensor to quantify the level of accumulation of misfolded proteins. UPR induced upon stress stimuli treatment, mutation, or target gene expression is sensed by the ER transmembrane protein Ire1p, which activates the transcriptional factor Hac1p. Hac1p up-regulates a distinct set of genes, including an exogenous GFP fused to one of the Hac1p-responsive promoters. Thus, GFP fluorescence can be used as a quantitative readout for UPR, which reflects the level of misfolded proteins. Similarly, the heat shock factor σ^{32}-dependent promoters of *ibpAB* and *fxsA* have been fused in tandem with the luciferase reporter gene to allow the monitoring of protein folding status in the cytoplasm of *E. coli* [94]. Systems with various stress responsive promoters fused to other reporter proteins, such as GFP and lacZ, have also been reported [95, 96].

Applications to chaperone identification

Almost all the reporter-based techniques mentioned above can potentially be used to optimize the folding environment of specific target proteins and thereby facilitate the identification of novel folding factors including chaperones. Combining the selection or screen with a genome-wide mutagenesis approach or with the expression of plasmid libraries scanning the whole proteome may increase the sensitivity and decrease the false positive rate. Furthermore, the combination of plasmid libraries encoding a randomly mutagenized chaperone gene may work to identify chaperones with improved activity on a known substrate or with novel specificity on new substrates.

In a recent study, GFP fused to a Hac1p-responsive promoter was successfully employed together with a genome-wide gene deletion survey to reveal several hundred yeast genes with roles in ER protein folding [93] and to screen for chemical chaperones [97]. In another approach, researchers generated transgenic Drosophila cell lines that express mutant huntingtin (Htt) fragments tagged with GFP to perform a genome-wide RNA interference screen for regulators of Htt aggregation [98]. Knockdowns of 126 genes caused enhanced or suppressed aggregation of Htt. Of these knockdowns, 7% encode known chaperones or their regulators. The remaining 93% of the genes are, in our opinion, good candidates to test for proteins with chaperone function, particularly those 18% that encode proteins with no previously assigned function. This screen identified a Hsp110 homolog as being the most potent suppressor of aggregation [98]. A similar RNAi screen conducted in *Caenorhabditis elegans* with a yellow fluorescent protein-tagged misfolding-prone SOD1 mutant also identified Hsp110 as a potent suppressor for aggregation [99].

These fluorescence-based screens require large numbers of cells to be individually quantified using expensive cell sorting devices. In contrast, antibiotic resistance-based selections efficiently obtain only those clones with the desired phenotype using inexpensive genetic selections. Recent work in our laboratory resulted in the successful identification of a previously uncharacterized chaperone by using a genetic selection that directly couples in vivo protein folding to antibiotic resistance via the β-lactamase and DsbA folding biosensors described above [81]. A very unstable protein was inserted into β-lactamase and DsbA, respectively, to generate two different bipartite sandwich fusions. Genome-wide mutagenesis followed by selection for strains that were simultaneously resistant to penicillin and cadmium led to the identification of variants overproducing the chaperone Spy, a periplasmic protein of previously unknown function. Others had previously shown Spy to be induced by ethanol, butanol, and tannins [81], all of which are known to precipitate proteins. Thus, in retrospect, Spy could have been identified as a chaperone by its stress inducibility. However, the large number of proteins induced by these various stresses likely inhibited researchers from taking the laborious approach of purifying each of them and testing them in vitro for chaperone activity.

These new strategies also make it possible to optimize the efficiency or robustness of known chaperones. For instance, screening for enhanced GFP fluorescence in cells expressing a plasmid library of GroEL/S variants identified mutations to the chaperonin system that improved its ability to fold GFP [100]. Interestingly, these mutations also seemed to shift the substrate specificity of the GroEL/S variants compared to the wild type [100]. In contrast, selection for variants of the DnaK

chaperone to enhance the folding of a truncated CAT obtained 12 mutants, 4 of which also showed improved ability to fold a second protein, chemically-denatured luciferase [101]. We propose that similar strategies may be used to de novo design chaperones from template proteins that have some protein-binding activity in vitro, such as BSA, or proteins known to bind peptides, such as the periplasmic oligopeptide binding protein OppA [102]. Using directed evolution approaches, their chaperone activities may be enhanced and their substrate spectrum may be enlarged.

Conclusions

The list of known chaperones may not be complete. Traditional chaperone discovery approaches have led to the identification of the vast majority of chaperone families. However, upon entering a new era of designing bacteria or other expression organisms for customized folding of recombinant proteins, we need more direct and sensitive ways to vigorously explore the folding environment. The ability to optimize folding in vivo using a variety of in vivo folding biosensors provides new routes for chaperone discovery and for improving our understanding of how protein stability is controlled in vivo. Using these approaches, more chaperones are likely to be discovered, previously isolated ones may be optimized for specific substrates, and new chaperones may be generated using in vitro design followed by in vivo selections to optimize chaperone activity.

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