Chapter 15
Substrate Interaction Networks of the *Escherichia coli* Chaperones: Trigger Factor, DnaK and GroEL

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Abstract In the dense cellular environment, protein misfolding and inter-molecular protein aggregation compete with protein folding. Chaperones associate with proteins to prevent misfolding and to assist in folding to the native state. In *Escherichia coli*, the chaperones trigger factor, DnaK/DnaJ/GrpE, and GroEL/ES are the major chaperones responsible for insuring proper de novo protein folding. With multitudes of proteins produced by the bacterium, the chaperones have to be selective for their substrates. Yet, chaperone selectivity cannot be too specific. Recent biochemical and high-throughput studies have provided important insights highlighting the strategies used by chaperones in maintaining proteostasis in the cell. Here, we discuss the substrate networks and cooperation among these protein folding chaperones.

Keywords Molecular chaperones • Trigger factor • GroEL/GroES • DnaK/DnaJ/GrpE • Protein folding • Protein aggregation • Chaperone interaction network

15.1 Introduction

Amongst the most renowned tenets in the study of protein folding, Anfinsen's thermodynamic principle states that the native conformation of a protein is achieved to attain the structure with minimum free energy for the respective polypeptide sequence (Anfinsen 1973). This is found to be true for many small proteins that have been experimentally studied and which have been observed to have a funnel-like energy landscape folding pathway to reach the lowest free energy native state (Mayor et al. 2003; Hartl et al. 2011). Burial of hydrophobic residues in the interior of the protein is a major driving force for the folding of soluble proteins. However, large proteins often seem to have a ragged pathway where protein folding can

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be inefficient and error prone. Folding intermediates in this situation can fall into kinetic traps and may expose hydrophobic residues or unstructured elements, which lead to misfolding of the protein and/or its aggregation (Dill and Chan 1997; Ellis 2006).

Molecular chaperones are a class of proteins that function to prevent such misfolding and aggregation that may occur in the chaotically dense medium that is the cellular environment. Chaperones bind, stabilize, fold and remodel proteins in healthy and stressed cells (Hartl et al. 2011). Many chaperones are present in the cell. De novo protein folding in a model bacteria such as *Escherichia coli* is mainly performed by three highly conserved chaperone systems: trigger factor (TF), DnaK/DnaJ/GrpE (Hsp70/Hsp40/nucleotide exchange factor) system and the GroEL/GroES (Hsp60/Hsp10) system (Horwich et al. 1993; Hesterkamp et al. 1996; Hartl and Hayer-Hartl 2002; Mayer and Bukau 2005).

The number of proteins that require chaperone assistance in the cell, the extent of the required assistance and the structural properties allowing for this assistance are actively being studied by many groups. Furthermore, the chaperone interaction networks of these different chaperones are also being investigated as this can provide answers to many pertinent questions about protein biogenesis, post-translational protein regulation and protein evolution.

15.2 Trigger Factor

15.2.1 Trigger Factor Structure and Substrate Recognition

Trigger factor (TF) is the only ribosome-associated bacterial chaperone (Hesterkamp et al. 1996). It is a 48 kDa protein that can be divided into three structural domains: namely, the N-terminal domain (NTD), the C-terminal domain (CTD) and the PPIase domain (Fig. 15.1a) (Ferbitz et al. 2004). The NTD and PPIase domains are connected by an extended linker, which allows the TF to have an elongated three-dimensional structure where the CTD is in the middle with the NTD and PPIase domain at opposite ends (Martinez-Hackert and Hendrickson 2009). The CTD contains helical-extensions that mimic protruding arms. CTD and PPIase domain form a cleft-like concave binding pocket for potential substrates (Merz et al. 2006; Mashaghi et al. 2013) (Fig. 15.1a).

Within the cell, TF can be found freely in the cytosol or attached to ribosomes. It is estimated that there is two to threefold molar excess of TF over ribosomes (Lill et al. 1988). TF associates transiently in a 1:1 stoichiometry with the ribosome, binding and acting on most nascent polypeptides emerging from the ribosome polypeptide exit tunnel (Kramer et al. 2002; Ferbitz et al. 2004; Raine et al. 2006; Rutkowska et al. 2008). TF associates with a vacant ribosome with a K_d of about 1–2 μM and a mean residence time of 10 s (Patzelt et al. 2001; Maier et al. 2003; Kaiser et al. 2006; Hoffmann et al. 2010). Nascent polypeptides increase TF's

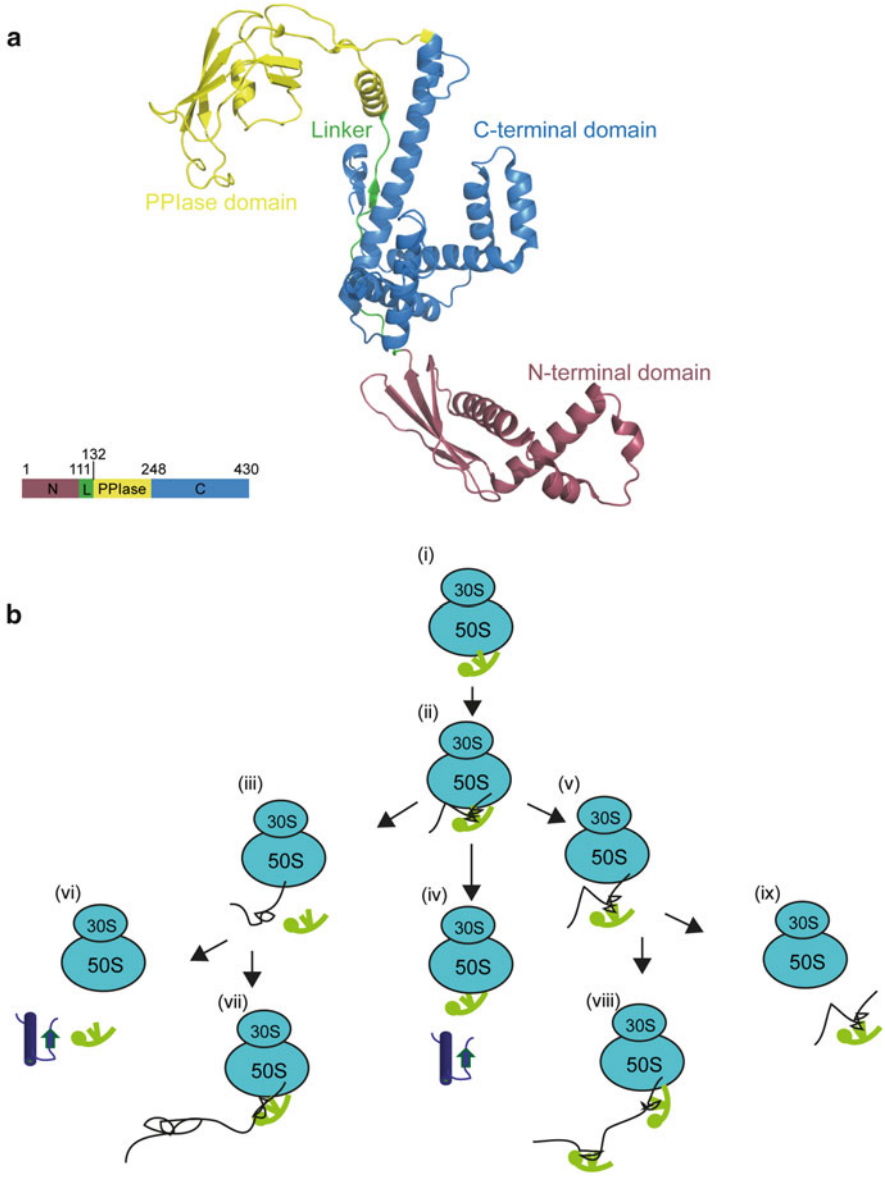

Fig. 15.1 Structure and function of trigger factor. (**a**) Structure of TF [PDB ID 1W26 (Ferbitz et al. 2004)] with N-terminal domain (*violet*), C-terminal domain (*blue*), PPIase domain (*yellow*), and linker (*green*) highlighted. Structures were drawn using the PyMOL molecular graphics system (DeLano 2002). A bar graph of TF domain arrangement is shown below the structure. (**b**) Mechanism of TF (*in green*) function is shown at various states of substrate interaction on and off the ribosome (*blue*)

affinity for ribosomes by 2–30-fold, based on their size, folded state and amino acid composition (Raine et al. 2006; Hoffmann et al. 2010). This enables the chaperone to differentiate between translating ribosomes and vacant ones (Rutkowska et al. 2008). Binding polypeptides on ribosomes also increases the half-life of the TF-ribosome association (Rutkowska et al. 2008).

TF associates promiscuously with polypeptides as they exit the ribosome during translation, protecting hydrophobic elements of emerging polypeptides from the hydrophilic environment of the cytoplasm through direct interactions with these elements (Hesterkamp et al. 1996; Hoffmann et al. 2006; Kaiser et al. 2006; Lakshmipathy et al. 2007; Rutkowska et al. 2008). Approximately eight amino acid-long sequences rich in hydrophobic and aromatic residues with a positive net charge are thought to be responsible for TF-substrate recognition (Patzelt et al. 2001; Saio et al. 2014). These sites occur regularly in most polypeptides, approximately once every 32 residues (Bukau et al. 2000; Patzelt et al. 2001).

The X-ray crystal structure for TF from *Thermatoga maritima* in complex with the ribosomal small subunit protein S7 has been solved (Martinez-Hackert and Hendrickson 2009) and provides some clues as to the basis of substrate recognition by this chaperone. The TF–S7 interaction was found to be a non-specific interaction, as would be expected for an interaction between a promiscuous chaperone and one of its many substrates. The interaction interface was very large, poorly packed, dominantly polar and sharing low shape complementarity. The interaction between these two proteins depicts a non-specific association and offers insights into the promiscuity displayed by the chaperone, which is necessary for TF function.

More recently, NMR-based techniques were used to map the interaction of TF with an unfolded substrate, *E. coli* alkaline phosphatase (PhoA) (Saio et al. 2014). The authors show that three TF molecules bind to one unfolded PhoA molecule. At least four substrate binding sites were identified in TF: one in the PPIase domain and three in the CTD. TF was found to use these four sites to bind to several regions of PhoA primarily through hydrophobic contacts. The TF–PhoA interaction was found to be highly dynamic, however, a more stable complex was formed as the length of the substrate protein and the number of regions recognized by TF increased.

Approximately 70 % of proteins are thought to fold to their native structures after association with TF. Other proteins can be passed onto downstream chaperone systems, DnaK and GroEL for further folding. Indeed, DnaK can compensate for the loss of TF in the cell (Deuerling et al. 1999; Teter et al. 1999) (discussed further below).

15.2.2 Trigger Factor Functional Cycle

The mechanism of TF action has been described as dynamic, consisting of a series of substrate binding and release events on and off the ribosome (Fig. 15.1b) (Kaiser et al. 2006; Hoffmann et al. 2010; Saio et al. 2014). TF is assumed to contact most polypeptides upon their exit from the ribosome, but many of these interactions

are transient and weak (Valent et al. 1995). TF can bind the vacant ribosome (i) but the association is enhanced upon interaction of TF with an emerging nascent polypeptide (ii). Once on the ribosome, TF remains bound for a minimum of about 10 s, which is enough time for the ribosome to translate a polypeptide chain of up to 200 residues. Following this, TF can be released from the nascent chain and ribosome (iii, vi) or the completed nascent chain might be released to fold to the native state with TF remaining bound to the ribosome (iv). A released TF is free to rebind the ribosome at the exit tunnel and assist in the folding of another (or same) emerging polypeptide (vii). Alternatively, TF might be released from the ribosome but remain bound to the growing nascent chain (v). For a long polypeptide sequence, multiple TFs on or off the ribosome may bind the chain (viii) (Agashe et al. 2004). Finally, TF may also assist in folding of a polypeptide recently released from the ribosome (ix) (Hoffmann et al. 2010).

15.2.3 Identification of Trigger Factor Substrates

Attempts have been made to identify protein substrates of TF using either co-purification with His-tagged TF or by identifying proteins that aggregate in the absence of TF but not in its presence in a Δ*dnaKJ* background strain (Martinez-Hackert and Hendrickson 2009). A total of 178 substrates were identified. Co-purification led to the identification of 42 substrates and 110 were identified by analysis of protein aggregation, while 26 substrates were identified by both techniques. Many of the identified proteins were ribosomal proteins or were part of multimeric complexes. The size distribution of proteins associating with TF was similar to the *E. coli* cytoplasmic proteome having a size range from 8 to 118 kDa with a mean of 36.5 kDa, again highlighting the promiscuity of this chaperone for its substrates.

15.3 DnaK/DnaJ/GrpE System

15.3.1 DnaK Structure and Function

DnaK is the major bacterial ortholog of the eukaryotic ATP-dependent Hsp70 chaperone. Substrates of DnaK include unfolded, misfolded and aggregated proteins (Schlecht et al. 2011). The chaperone is primarily involved in protein folding and protein disaggregation, but also has overlapping function with TF in promoting cotranslational protein folding (Deuerling et al. 1999, 2003; Teter et al. 1999; Rosenzweig et al. 2013). Structurally, like other Hsp70s, DnaK is composed of two domains (Mayer and Bukau 2005; Bertelsen et al. 2009): the N-terminal ATPase domain and the C-terminal substrate binding domain (Fig. 15.2a). DnaK function

depends on a bidirectional allosteric communication between these two domains (Ung et al. 2013). The enzymatic cycle of DnaK alternates between ATP-bound open state and ADP-bound closed state (Mayer and Bukau 2005). The ATP-bound state is characterized by low affinity and fast exchange rate for substrates, while the ADP-bound state is characterized by high affinity and slow exchange rate for substrate. The hydrolysis of ATP to ADP triggers the closing of the substrate binding site resulting in locking the associated substrate to DnaK.

The functional cycle of DnaK is dependent upon DnaJ cochaperone, an Hsp40 ortholog, and the GrpE nucleotide exchange factor (Liberek et al. 1991; Szabo et al. 1994; Hartl et al. 2011). DnaJ is the major cochaperone for DnaK in *E. coli* and generally acts to stimulate the ATPase activity of DnaK. Also, DnaJ binds substrates and then transfers them to DnaK (Fig. 15.2b). The ATPase activity of DnaK is low when no substrate is bound and is stimulated two to tenfold in the presence of a substrate (Mayer and Bukau 2005). The ATPase activity is further enhanced by DnaJ. DnaJ and the DnaK-bound substrate synergistically enhance the ATPase activity of DnaK by greater than 1000-fold (Liberek et al. 1991; Karzai and McMacken 1996; Laufen et al. 1999). ATP hydrolysis then allows for a tight complex to form between the DnaK–ADP and its substrate (Kampinga and Craig 2010). The release of ADP from DnaK is slow (Brehmer et al. 2001), hence, the need for the nucleotide exchange factor GrpE which catalyzes the release of ADP from DnaK. GrpE itself dissociates from the chaperone when DnaK binds ATP, which also results in the release of the substrate protein. The substrate protein can then attempt to fold to the native state, if unsuccessful, the protein can be rebound by DnaJ or DnaK and the cycle repeated (Fig. 15.2b).

15.3.2 Interaction Network of DnaK

In order to identify how DnaK differentially recognizes its substrates in the cellular environment, the DnaK substrate binding motif was analyzed using a library of overlapping 13-mer peptides arrayed on cellulose membranes (Rudiger et al. 1997). The binding motif was found to consist of a hydrophobic core of about seven residues, enriched in leucines, flanked by basic amino acids. Based on the solved crystal structure of DnaK with a substrate peptide, the link between DnaK structure and its preferential substrates was further illustrated (Zhu et al. 1996; Mayer and Bukau 2005). Substrates interact with the substrate binding domain of the chaperone, which consists of a β-sandwich subdomain and an α-helical lid subdomain (Fig. 15.2a). The binding pocket in DnaK is composed of hydrophobic residues flanked by acidic residues contributed by both subdomains, which is consistent with the preferential DnaK binding motif identified by the peptide array analysis described above. This DnaK substrate binding motif is estimated to generally occur once every 36 residues in proteins (Rudiger et al. 1997). Indeed, it is estimated that 98 % of the *E. coli* annotated proteome would harbor potential DnaK binding sites (Srinivasan et al. 2012).

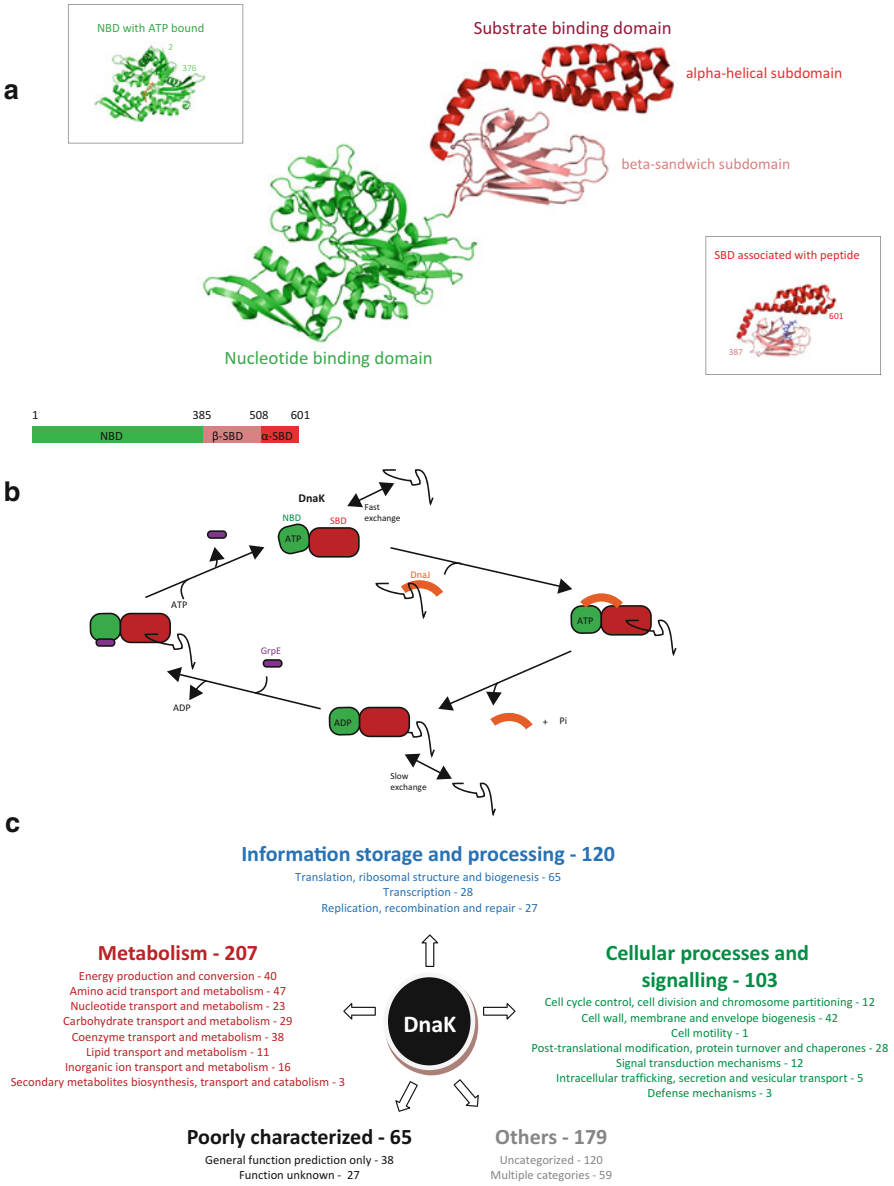

Fig. 15.2 Structure and function of DnaK. (**a**) Structure of full length DnaK [PDB ID 2KHO (Bertelsen et al. 2009)] with its nucleotide binding domain (NBD) and substrate binding domain (SBD) indicated. A bar graph of DnaK domain arrangement is shown below the structure. The inset on the *right* shows the SBD residues 387-601 with bound NRLLLTG peptide (*in blue*) [PDB ID 1DKZ (Zhu et al. 1996)]. The inset on the *left* shows residues 2-376 of NBD of DnaK with bound ATP (in *orange*) [PDB ID 4B9Q (Zhu et al. 1996; Kityk et al. 2012)]. (**b**) The functional cycle of DnaK (*red*, *green*) is shown depicting its action on its substrates with assistance from its cochaperone DnaJ (*orange*) and the nucleotide exchange factor GrpE (*violet*). As shown, the ATP-bound state of DnaK is characterized by weak binding of substrate and fast exchange rates while the ADP-bound state is characterized by strong substrate binding and slow exchange rates. (**c**) Functional categories based on Cluster of Orthologous Group (COG) for the 674 DnaK interacting proteins are shown (Tatusov et al. 2001, Calloni et al. 2012). Numbers of proteins belonging to each functional group and to each category are indicated beside the COG category name

Different approaches have been used to identify potential DnaK substrates. In one approach, protein aggregates in DnaK (and DnaJ) depleted cells with or without trigger factor, were resolved by two-dimensional gel electrophoresis (Deuerling et al. 2003). Regardless of the presence of TF, 340 major spots were identified that are representative of potential DnaK substrates. It should be noted that the levels of aggregated proteins in cells with TF were much lower, indicative of functional overlap among the two chaperones. The size range for these proteins was 16–160 kDa, but proteins larger than 60 kDa in size were found to be enriched in the aggregates compared to soluble cytoplasmic proteins. Of the distinct spots on the 2-D gel, 94 were identified by mass spectrometry (Deuerling et al. 2003). The identified proteins were all cytoplasmic and involved in different cellular processes. Though no secondary structural features or chemical features were identified to distinguish the substrates from other proteins, it was observed that a majority (∼72 %) of aggregated proteins tended to be thermolabile.

More recently, in another approach to identify DnaK substrates, DnaK-substrate complexes were isolated from wild type cells or cells either lacking TF or depleted of GroEL (Calloni et al. 2012). Using endogenously expressed His-tagged DnaK, immobilized metal affinity chromatography (IMAC) was used to pulldown DnaK-interacting proteins, which were then identified and quantified by mass spectrometry. Both DnaK cochaperones, DnaJ and GrpE, were isolated in these pulldowns. In total, 674 DnaK interactors were identified belonging to diverse functional groups (Fig. 15.2c). A majority of these were predicted to be cytoplasmic (∼80 %) with a significant minority of inner membrane, outer membrane and periplasmic proteins as well. Many of the interactors were involved in metabolic and cell signaling pathways (Fig. 15.2c).

Several features were observed for substrates enriched on DnaK. They were found to be more aggregation prone upon translation than less enriched DnaK substrates (Calloni et al. 2012; Niwa et al. 2012). Additionally, though enriched DnaK substrates were not more hydrophobic than the average soluble cellular protein, they were observed to be less effective in burying their hydrophobic residues from solvent (Tartaglia et al. 2010; Calloni et al. 2012). DnaK-enriched substrates were generally of low cellular abundance and of large size (Calloni et al. 2012). The negative correlation between cellular abundance and aggregation propensity was previously observed (Tartaglia et al. 2007, 2010) as folding states for abundant proteins are thought to have been evolutionarily optimized to prevent overloading chaperones. Proteins that interact extensively with DnaK were more likely to be part of hetero-oligomeric complexes. Partially structured regions of proteins that form hetero-oligomeric complexes can be shielded from the dense cellular environment through chaperone assistance (Schlecht et al. 2011). Thus, through shielding of hydrophobic charges, DnaK allows proper folding of numerous proteins.

15.3.3 DnaJ Structure and Function

DnaJ is the major cochaperone for DnaK in *E. coli* and generally acts to stimulate the ATPase activity of DnaK. DnaJ has a conserved domain of approximately 70 residues located at the N-terminus, called the J domain, that is required for DnaJ to associate with DnaK (Wall et al. 1994). There is a highly conserved His-Pro-Asp motif present in a loop between the second helix and third helix of the J domain (Fig. 15.3a), which is found to be crucial for stimulation of DnaK ATPase activity by DnaJ (Cheetham and Caplan 1998). Following the N-terminal J-domain, DnaJ is composed of a glycine/phenylalanine rich region followed by a linker region, a zinc-binding domain and a C-terminal domain (Fig. 15.3a). The C-terminus of DnaJ is known to associate with substrates in a transient fashion, binding to hydrophobic sequences that contain motifs similar to those recognized by DnaK; DnaJ then presents these substrates to DnaK (Gamer et al. 1996; Rudiger et al. 2001; Srinivasan et al. 2012) (Fig. 15.2b). Related to its importance for DnaK function, temperature sensitivity has been observed for *dnaJ* null mutant strains, as well as, a loss of bacterial motility (Sell et al. 1990; Shi et al. 1992).

DnaJ is only one of the Hsp40 paralogs present in *E. coli*; others include CbpA, DjlA, DjlB, DjlC and HscB (Ueguchi et al. 1994; Genevaux et al. 2001; Gur et al. 2005; Chenoweth et al. 2007). DjlB and DjlC are membrane associated proteins that do not associate with DnaK but interact with HscC, a specialized DnaK paralog (Kluck et al. 2002). Similarly, HscB (or Hsc20) acts as a cochaperone for another DnaK paralog termed HscA (or Hsc66) (Silberg et al. 1998; Hennessy et al. 2005; Fuzery et al. 2008). HscA/B are involved in the iron–sulfur cluster assembly pathway. Apart from DnaJ, CbpA and DjlA are the only cochaperones observed to have a significant association with DnaK (Genevaux et al. 2007). CbpA has been observed to act as a multicopy suppressor of Δ*dnaJ* mutants, though no phenotype is observed in cells lacking just CbpA (Ueguchi et al. 1994; Gur et al. 2004). DjlA, a membrane associated protein with its N-terminal anchored to the inner-membrane, has been shown to substitute for DnaJ in vitro as a cochaperone for DnaK (Genevaux et al. 2001). However, among these Hsp40 cochaperones of DnaK, DnaJ is the best characterized and functions as the premier regulator of the various DnaK activities (Kelley 1998; Gur et al. 2004).

15.3.4 GrpE Structure and Function

Along with DnaJ, DnaK works in concert with the nucleotide exchange factor GrpE. GrpE associates with DnaK and catalyzes the otherwise slow release of ADP (Packschies et al. 1997). GrpE functions as a homodimeric protein (Schonfeld et al. 1995). Structurally, the first 33 N-terminal residues of the protein are disordered, followed by a long α-helix, a short α-helix and a compact β-sheet domain (Fig. 15.3b) (Harrison et al. 1997). Much of the long α-helix forms a tail,

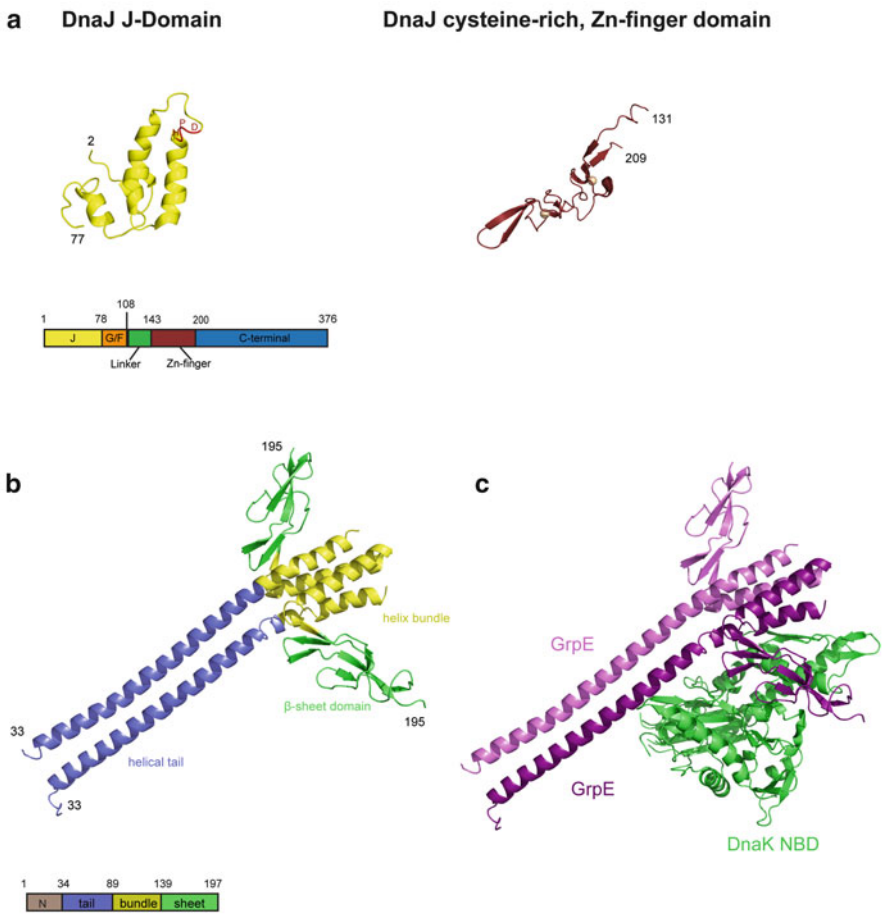

Fig. 15.3 Structure and function of DnaJ and GrpE. (**a**) Structure of DnaJ J-domain residues 2-77 (in *yellow*) with the conserved HPD motif highlighted in *red* is shown on the *left* [PDB ID 1BQ0 (Huang et al. 1999)]. DnaJ residues 131-209 (in *brown*) containing the zinc-finger domain with bound zinc depicted as *light orange spheres* is shown on the *right* [PDB ID 1EXK (Martinez-Yamout et al. 2000)]. A bar graph of DnaJ domain arrangement is shown below the structures. (**b**) Structure of GrpE [PDB ID 1DKG (Harrison et al. 1997)] highlighting its domains is shown. N-terminal amino acids 1-32 are disordered and are not shown. A bar graph of GrpE domain arrangement is shown below the structure. (**c**) Structure of the DnaK nucleotide binding domain in complex with GrpE [PDB ID 1DKG (Harrison et al. 1997)]. The GrpE subunit associating with the NBD-domain of DnaK is depicted in *dark purple* while the other GrpE in the dimer is presented in a lighter shade

with the tails in the dimer positioned parallel to each other. The remaining part of the longer helix and the shorter helix combine with their counterparts from the other GrpE in the dimer to form a helical bundle. The β-sheet domains protrude outward from the helical bundle (Fig. 15.3b). Only one of the GrpE molecules within the

dimer takes part in associating with DnaK (Fig. 15.3c). The β-sheet domain makes the majority of the contacts with DnaK by binding the nucleotide binding cleft, while the helix bundle and the top of the helical tail contribute additional contacts (Harrison et al. 1997; Harrison 2003).

The interaction of GrpE with DnaK is very strong, with a K_d of 1–30 nM (Harrison et al. 1997; Packschies et al. 1997). On binding DnaK, GrpE has been shown to reduce the affinity of ADP for DnaK by 200-fold and as a result accelerating nucleotide exchange by 5000-fold (Packschies et al. 1997). This is accomplished by an associative displacement mechanism whereby the binding of the nucleotide and GrpE is not competitive and the binding sites are either distinct or only partially overlapping (Packschies et al. 1997). GrpE binding leads to a conformational change in DnaK, disrupting the contacts between the DnaK and ADP (Packschies et al. 1997; Harrison 2003).

Apart from its generalized role as a nucleotide exchange factor for DnaK, GrpE has also been found to assist in polypeptide release from the substrate binding domain of the chaperone (Mally and Witt 2001; Brehmer et al. 2004). The importance of GrpE for DnaK activity is highlighted by its temperature-dependent control of nucleotide exchange from DnaK. The long helix of GrpE was shown to undergo a reversible thermal transition above about 40 °C that leads to a decrease in the rate of ADP/ATP exchange on DnaK (Grimshaw et al. 2003). Thus, GrpE provides a thermal regulation of DnaK activity by shifting the DnaK-substrate complexes towards the ADP-associated, slow substrate exchange state at high temperatures. The slow rate of substrate release at such temperatures prevents unfolded polypeptides from accumulating in the cytoplasm where they might be susceptible to misfolding and aggregation.

15.4 The GroEL/ES Chaperone System

15.4.1 GroEL/ES Structure and Function

The GroEL protein (also called chaperonin) is the bacterial ortholog of the eukaryotic Hsp60 present in the mitochondria. GroEL (57 kDa) along with its cofactor GroES (10 kDa, Hsp10) is the only chaperone system in *E. coli* that is essential under all growth conditions (Fayet et al. 1989; Goloubinoff et al. 1989; Horwich et al. 1993). GroEL is a cylindrically-shaped oligomer composed of two rings, arranged back to back, of seven subunits each (Fig. 15.4a) (Braig et al. 1994; Horwich et al. 2006). Each GroEL subunit consists of three domains (Fig. 15.4a): the apical domain, the intermediate domain and the equatorial domain. The equatorial domain at the ring–ring interface contains the ATP-binding pocket and has been recently proposed to assist in orientation of substrate proteins as they enter the GroEL ring (Fenton et al. 1994; Weaver and Rye 2014). The apical domain at the ends of the cylinder harbors hydrophobic residues required for substrate and GroES

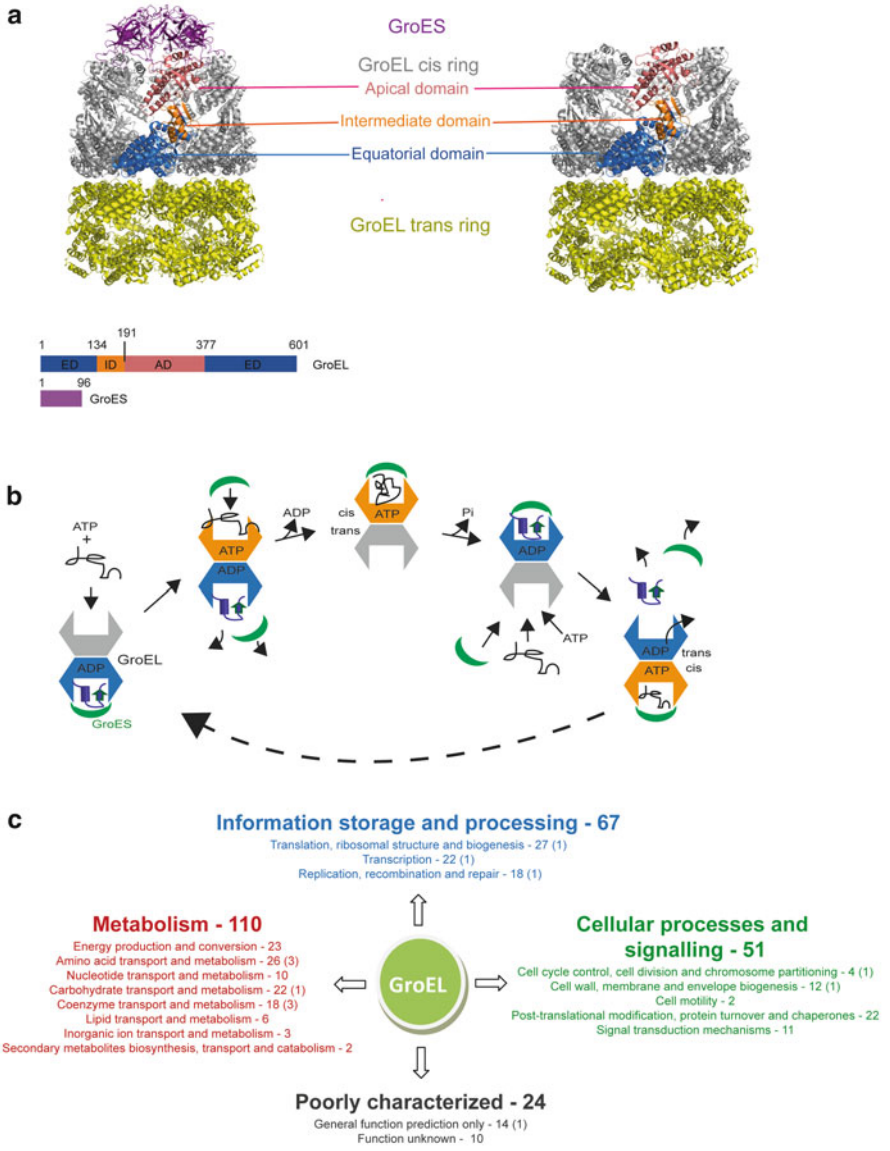

Fig. 15.4 Structure and function of the GroEL/ES chaperone system. (**a**) Shown are the structures of the GroEL–GroES complex [*left*, PDB ID 1PCQ (Chaudhry et al. 2003)] and the GroEL tetradecamer [*right*, PDB ID 1PCQ (Chaudhry et al. 2003)]. One heptameric ring is in *yellow*, while the opposite ring is in *gray* with the domains of one of the subunits colored as follows: equatorial domain in *blue*, intermediate domain in *orange* and the apical domain in *red*. GroES heptamer capping the *cis* heptameric GroEL ring is shown in *purple*. Bar graphs of GroEL and GroES domain arrangement are shown below the structures. (**b**) A cartoon representation of the nucleotide-dependent GroEL/ES functional cycle. Refer to the text for further details. (**c**) Shown are Cluster of Orthologous Group (COG) functional categories for the 252 GroEL interacting proteins (Tatusov et al. 2001; Kerner et al. 2005). The numbers between brackets indicate the GroEL substrates of the respective categories that are essential for the cell and are also obligate GroEL substrates

binding (Xu et al. 1997; Farr et al. 2000; Chaudhry et al. 2003). The intermediate domain acts as a linker between the other two domains. Each GroEL ring has a large central cavity (Fig. 15.4a).

GroES is a heptameric protein whose subunits form a ring dome-like structure (Fig. 15.4a) (Hunt et al. 1996). The sevenfold symmetry of the GroES protein complements that of GroEL and, upon association with GroEL, forms a structure analogous to a lid for the central cavity of GroEL (Langer et al. 1992; Chaudhry et al. 2003). The GroEL–GroES interaction results in the doubling of the size of the GroEL central cavity (Fig. 15.4a) due to large conformational changes in the intermediate and apical domains (Xu et al. 1997). The intermediate domain physically and functionally connects the equatorial and apical domains by transferring the energy of ATP hydrolysis in the equatorial domain with conformational changes in the apical domain (Ranson et al. 2001, 2006; Saibil et al. 2013).

The GroEL/ES functional cycle is shown schematically in Fig. 15.4b. The GroEL open ring captures non-native but compact forms of a polypeptide substrate that is exposing a hydrophobic surface, thus, GroEL prevents the substrate from misfolding or forming irreversible aggregates (Goloubinoff et al. 1989; Braig et al. 1994; Horwich et al. 2006). Mutational studies indicate that this primary association is based on hydrophobic interactions with the apical domain of the chaperone (Fenton et al. 1994; Farr et al. 2000). Binding to GroEL might result in unfolding of non-native states allowing for subsequent refolding (Lin et al. 2013). Subsequently, ATP binds cooperatively to the equatorial domains of seven subunits of one GroEL ring (Yifrach and Horovitz 1995). This allows for the association of GroES to GroEL (Chandrasekhar et al. 1986) due to large conformational shifts that release the bound substrate from its hydrophobic association with GroEL since GroES competes for the same binding sites on GroEL as the substrate. The formation of the GroEL–GroES complex results in the formation of an enclosed hydrophilic chamber that traps the substrate and promotes folding in an environment isolated from the cellular milieu (Chaudhry et al. 2003). Following ATP hydrolysis, a stable GroEL(ADP)–GroES complex is formed containing the trapped substrate (Fig. 15.4b). Subsequently, ATP binds to the opposite ring of the tetradecamer that does not contain the substrate, and, due to the negative cooperativity in nucleotide binding between the two GroEL rings (Rye et al. 1999), this leads to the release of GroES, ADP and bound substrate allowing for a new substrate interaction cycle to occur (Saibil et al. 2013).

15.4.2 The GroEL Interaction Network

Based on immunoprecipitation of GroEL and its bound substrates in pulse-chase type experiments, 10–15 % of all newly translated cytoplasmic proteins were estimated to transit through the GroEL chaperone under normal cellular conditions (Ewalt et al. 1997; Houry et al. 1999); this number increased to 30 % under heat stress of 42 °C. Combining immunoprecipitation with 2-D gel electrophoresis and

mass spectrometry, 52 of the most abundant GroEL substrates were identified (Houry et al. 1999). These proteins included members of the transcription and translation machineries as well as many metabolic enzymes. To more comprehensively identify GroEL substrates, affinity chromatography was utilized to pull down proteins trapped inside the GroEL/ES chamber. Kerner et al. (2005) attempted to isolate GroEL/ES complexes formed with *E. coli* GroES-His_6. However, such complexes were not stable, which led the authors to replace *E. coli* GroES with GroES from *Methanosarcina mazei* (Mm). MmGroES was shown to functionally replace *E. coli* GroES but was found to bind more stably to GroEL in the presence of ADP allowing for the isolation of stable GroEL/ES complexes containing trapped substrates. Pull downs followed by mass spectrometry led to the identification of 250 GroEL/ES substrates (Fig. 15.4c). Most proteins were cytoplasmic with only eight being either periplasmic or outer membrane. Of the 250 recognized GroEL interactors, 83 were defined as obligate GroEL/ES interactors, which included 13 essential proteins (Gerdes et al. 2003; Kerner et al. 2005). Based on cellular abundance of the proteins and their GroEL dependence, about 75–80 % of cellular GroEL molecules were estimated to be occupied by 83 obligate substrates.

The identified substrates were divided into three classes based on their dependence on the GroEL/ES system for folding (Ewalt et al. 1997; Kerner et al. 2005). Class I substrates require minimal chaperone assistance to fold. Class II substrates are those that required the presence of both GroEL and GroES for folding at 37 °C but these substrates do not require GroES at lower temperatures. Furthermore, Class II substrates are not solely dependent on GroEL, as DnaK can also assist in their folding at 37 °C. Class III proteins are obligate GroEL/ES substrates (Kerner et al. 2005). Substrates belonging to Class III fail to refold in the absence of GroEL/ES even if DnaK is present; however, DnaK may be able to bind these proteins and prevent their aggregation.

Few salient characteristics were identified that differentiate a GroEL substrate from other cytosolic proteins. The GroEL-associated proteins spanned a range of sizes from 10 to 150 kDa, but they typically were of molecular weight around 20–60 kDa (Houry et al. 1999; Kerner et al. 2005), especially for class III proteins. The size range is consistent with the fact that the chamber formed upon association of GroEL with GroES can hold globular proteins with an upper size limit of 50–60 kDa (Chen et al. 1994). Considering that class I and II substrates may be assisted during their folding by chaperones other than GroEL, a size preference was not found among these proteins.

In addition to a size preference, obligate substrates had pI values around 5.5–6.5, leading to a lower net charge at physiological pH in comparison to other cytosolic proteins (Kerner et al. 2005). A lower net charge is correlated with an increased propensity to aggregate, providing an additional clue to their chaperone requirement (Chiti et al. 2002). No difference in hydrophobicity was observed for obligate GroEL substrates compared to other cytosolic proteins. Structurally, αβ domains were enriched in GroEL substrates over all-α or all-β domains with a special partiality towards the $(\beta\alpha)_8$ TIM-barrel fold belonging to SCOP class c1 (Houry et al. 1999; Kerner et al. 2005; Georgescauld et al. 2014). Recently, it has

been suggested the GroEL/ES can accelerate the rate of TIM-barrel domain folding (Georgescauld et al. 2014). The TIM-barrel is a common structural fold and not all proteins with such TIM-barrel need GroEL to reach their native state. Additionally, the observation that class I substrates with TIM-barrel are unable to displace class II or III substrates suggests that intermediate folded states rather than the final native state of TIM-barrel proteins may share features that favorably associate with GroEL.

15.5 Overlapping Functional Roles of the Chaperones

With trigger factor, GroEL and DnaK each responsible for the correct folding of hundreds of cellular proteins, it is interesting to note that only the GroEL/ES system is essential at all temperatures (Fayet et al. 1989). Loss of TF does not affect cell viability but DnaK is required at growth temperatures above 37 °C and below 15 °C (Bukau and Walker 1989; Deuerling et al. 1999). The indispensability of GroEL has been linked to the requirement of the chaperone to fold one or all of 13 characterized obligate GroEL substrates essential for survival of the organism (Kerner et al. 2005). The lack of essentiality for the DnaK and TF chaperones is a little more complicated, but is likely due to compensatory mechanisms and overlapping functional roles among these chaperones and GroEL.

It is well known that the viability of a Δ*tig*Δ*dnaK* mutant (*tig* is the gene for TF) can be rescued by expression of either TF or DnaK alone (Genevaux et al. 2004). Hence, the two chaperones are able to compensate for each other. In Δ*tig* cells, the DnaK interactome was found to increase by about 48 % with the chaperone associating with 998 proteins compared to 674 in wild type cells (Calloni et al. 2012). Indeed, in the absence of TF, DnaK and GroEL levels were found to increase by up to threefold compared to steady state levels in wild type cells (Deuerling et al. 2003). Also, 77 % of TF-bound peptides showed affinity for DnaK, likely based on the similarity in the binding motifs for the two chaperones, which comprise a hydrophobic core flanked by basic residues (Deuerling et al. 2003). Similarly, in the absence of DnaK and TF, an additional 150 proteins were observed to interact with GroEL compared to WT cells at 30 °C (Kerner et al. 2005). The extra burden upon the GroEL/ES system is mitigated by upregulation of its protein levels (Calloni et al. 2012).

Despite some overlap, the chaperone systems are not perfectly complementary to each other. TF has a specific role in transportation of outer membrane proteins; that role cannot be substituted by DnaK (Oh et al. 2011). As a consequence, cells lacking TF are more sensitive to the detergent deoxycholate and antibiotic vancomycin, a symptom of a weaker outer membrane (Nichols et al. 2011; Calloni et al. 2012). Similarly, when searching for their individualized importance, TF or GroEL cannot replace the function of DnaK in resolving protein aggregates in Δ*dnaK* cells (Calloni et al. 2012). Hence, while the functional overlap among chaperones ensures efficiency under stress conditions or when one of the chaperone systems is overwhelmed, each chaperone system also shows some degree of specialization in its activity.

15.6 Chaperone–Chaperone Interactions

Chaperones can be divided into two functional groups. TF and GroEL/ES belong to the group primarily involved in de novo protein folding, while a second group that is involved in refolding and protein disaggregation includes ClpB, ClpX, and some small heat shock proteins not discussed in this review (Haslbeck et al. 2005; Barends et al. 2010; Baker and Sauer 2012). ClpB acts as a disaggregase, while ClpX acts as an unfoldase and targets proteins to the ClpP protease for degradation. DnaK seems to link the two groups as it plays a major part in de novo folding and in aggregation prevention (Deuerling et al. 1999; Mogk et al. 1999; Calloni et al. 2012).

Using FRET-based analyses, utilizing fluorescence transfer from CFP to YFP, direct and indirect interactions among *E. coli* chaperones were observed (Kumar and Sourjik 2012). Consistent with the inference of DnaK as the 'chaperone' hub of the cell, it was observed that DnaK and DnaJ interact with many other chaperones including TF, the small heat shock proteins IbpA and IbpB, the Hsp100 family ATPase ClpB and Hsp90 ortholog HtpG. TF was shown to interact with DnaJ, confirming previous observation showing substrate transfer from TF to DnaJ and then DnaK (Deuerling et al. 1999; Teter et al. 1999; Kumar and Sourjik 2012). Interestingly, the DnaK nucleotide exchange factor GrpE was observed to be in close vicinity in the cell to HtpG and ClpB proteins (Genest et al. 2011; Miot et al. 2011; Kumar and Sourjik 2012). Addition of the translation inhibitor chloramphenicol, abolished these interactions suggesting that chaperone–chaperone interactions are not direct but rather are mediated by substrates. Interactions among chaperones involved in de novo folding were unaffected by similar treatment, indicative of the fact that they are substrate-independent, direct inter-chaperone interactions.

Protein–protein interactions among different chaperones have also been identified using pulldown methods. Chaperones pulled-down with GroEL included TF, DnaK, DnaJ and the redox-related chaperones Hsp33 and YegD (Kerner et al. 2005). Indeed, DnaK, DnaJ as well as TF have been noted to associate with and to deliver substrates to GroEL (Buchberger et al. 1996; Calloni et al. 2012). YegD is a member of the Hsp70/DnaK family of proteins, although its cellular function and the physiological significance of its association with GroEL is not clear. The association between GroEL and Hsp33 was previously found to occur during heat and oxidative stress and was speculated to allow GroEL to fold proteins initially interacting with Hsp33 (Echave et al. 2002; Hoffmann et al. 2004; Genevaux et al. 2007).

Among numerous proteins pulled down with DnaK were the small heat shock proteins IbpA and IbpB, whose function includes stabilization of aggregating proteins under heat stress (Laskowska et al. 1996; Calloni et al. 2012). Other chaperones identified in the pulldown with DnaK include the DnaJ paralog CbpA, the cytoplasmic chaperones TF, ClpB, HtpG, SecB, HscA, the periplasmic acid stress chaperones HdeA and HdeB, and the oxidative stress response chaperone Hsp33 (Calloni et al. 2012). ClpB and DnaK are known to act synergistically to reverse protein aggregation (Mogk et al. 1999). SecB, involved in protein export

through the general secretory (Sec) system, is known to act as a chaperone in modulating folding and aggregation states of its substrates before their export (Randall and Hardy 2002; Ullers et al. 2004). DnaK and SecB might have some complementary functions but may act cooperatively as well (Wild et al. 1992; Ullers et al. 2004).

15.7 Sequence of Substrate–Chaperone Interactions

Trigger factor, DnaK and GroEL function as the main chaperone hubs for de novo protein folding. Based on the relative roles of the three chaperone systems, the most basic of models suggests a simple sequential functionality for the three chaperones (Fig. 15.5). According to such a model, TF would be the most upstream of the three systems, assisting in the folding of proteins as they emerge from the ribosome, while GroEL is thought to be utilized for proteins requiring the most chaperone assistance. Many observations support the sequential nature of the function of these chaperones. Primarily, TF is the only prokaryotic chaperone associated with the ribosome. Many GroEL substrates are directly transferred either from TF or DnaK (Kerner et al. 2005; Fujiwara et al. 2010; Calloni et al. 2012). In the absence of TF, the number of DnaK-bound GroEL substrates increases from 119 to 152 (Calloni et al. 2012). The increase suggests a shift from TF-assisted folding to DnaK-assisted folding prior to interaction with GroEL. Furthermore, although little aggregation of GroEL substrates was observed in the absence of either DnaK or TF, 70 % of GroEL substrates were found aggregated in cells lacking both DnaK and TF despite the upregulation of GroEL (Kerner et al. 2005; Calloni et al. 2012). Thus, many GroEL substrates are dependent on the 'upstream' TF and DnaK chaperones.

Such a simplistic model of Fig. 15.5 is sufficient for the description of the general de novo folding machinery based on our current knowledge of the mechanism of function of these chaperones and their interaction networks. However, as has been shown for luciferase folding, DnaK and GroEL may sometimes compete for binding to a substrate and do not always act in succession (Buchberger et al. 1996). Such cases imply that, while the simple sequential model of TF to DnaK to GroEL might be true for chaperone-assisted folding for many proteins, there are a subset of proteins that may be acted on competitively or laterally by these chaperones.

15.8 Conclusion

Numerous newly synthesized proteins rely on chaperone assistance for folding in the crowded cellular environment. Trigger factor, DnaK/DnaJ/GrpE and GroEL/ES are the three major systems that assist newly synthesized proteins. Though their mechanisms of function are well understood, their proteomic contributions are less so. Recent biochemical and structural analyses have attempted to define the

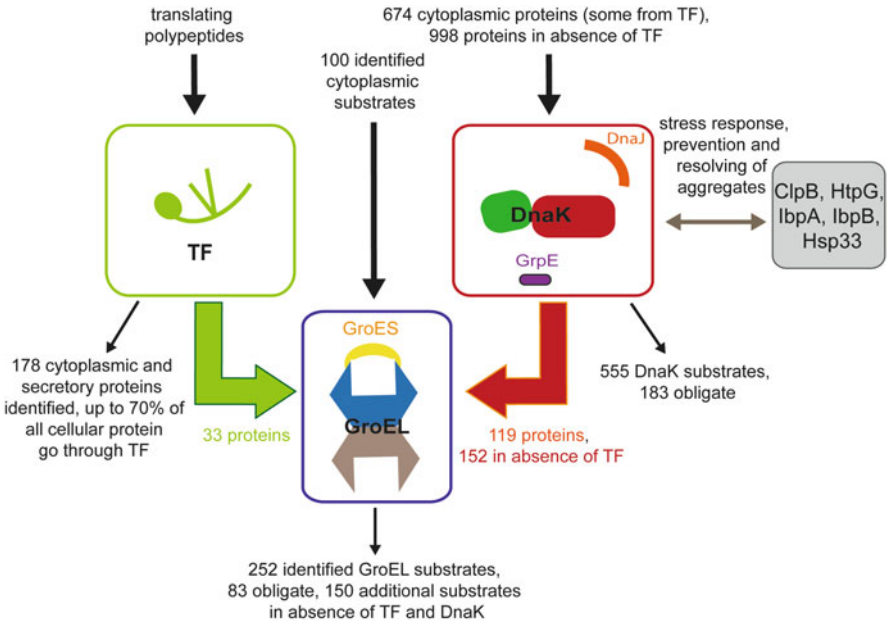

Fig. 15.5 Substrate–chaperone interaction network in *E. coli*. A schematic showing the number of substrates identified to associate with each of the three chaperone systems. Also depicted is how these substrates flux through TF, DnaK and GroEL systems. *Black arrows* leading into chaperones indicate proteins that bind to chaperones and *colored arrows* refer to the transfer of substrates between chaperone systems. TF interacts with proteins directly emerging from the ribosome. Up to 70 % of all cellular proteins are estimated to be folded by TF (Vabulas et al. 2010). Among these, 178 proteins have been identified (Martinez-Hackert and Hendrickson 2009). 674 DnaK substrates have been identified using pulldown assays (Calloni et al. 2012). Though some proteins are known to be transferred to DnaK from TF, the exact number remains unknown (Deuerling et al. 2003). TF and DnaK are thought to deliver 33 and 119 proteins to GroEL, respectively, with DnaK delivering 152 proteins in the absence of TF (Calloni et al. 2012). An additional 100 substrates are known to associate with GroEL (Ewalt et al. 1997; Kerner et al. 2005). Known increases in the number of different substrates for DnaK and GroEL are also indicated when either TF, DnaK or both are missing from the cell

interactome for each of these systems. In doing so, a better understanding is gained of the function of these chaperones in maintaining proteostasis. An understanding of the interaction networks of these chaperones can help in drug discovery efforts for pathogenic bacteria as well as in providing clues on the regulation, biogenesis and evolution of cellular proteins and protein complexes.

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