The Role of the DIF Motif of the DnaJ (Hsp40) Co-chaperone in the Regulation of the DnaK (Hsp70) Chaperone Cycle

Gordana Cogelja Cajo, B. Erin Horne, William L. Kelley, Françoise Schwager, Costa Georgopoulos, and Pierre Genevaux

From the Département de Microbiologie et Médecine Moléculaire, Centre Médical Universitaire, 1, rue Michel-Servet, CH-1211, Geneva, Switzerland and the Department of Biochemistry, Tulane University Health Sciences Center, New Orleans, Louisiana 70112

To perform effectively as a molecular chaperone, DnaK (Hsp70) necessitates the assistance of its DnaJ (Hsp40) co-chaperone partner, which efficiently stimulates its intrinsically weak ATPase activity and facilitates its interaction with polypeptide substrates. In this study, we address the function of the conserved glycine- and phenylalanine-rich (G/F-rich) region of the Escherichia coli DnaJ in the DnaK chaperone cycle. We show that the G/F-rich region is critical for DnaJ co-chaperone functions in vivo and that despite a significant degree of sequence conservation among the G/F-rich regions of Hsp40 homologs from bacteria, yeast, or humans, functional complementation in the context of the E. coli DnaJ is limited. Furthermore, we found that the deletion of the whole G/F-rich region is mirrored by mutations in the conserved Asp-Ile/Val-Phe (DIF) motif contained in this region. Further genetic and biochemical analyses revealed that this amino acid triplet plays a critical role in regulation of the DnaK chaperone cycle, possibly by modulating a crucial step subsequent to DnaK-mediated ATP hydrolysis.

Molecular chaperones assist the folding of proteins, mainly by protecting them from intracellular aggregation (1). Of these, the highly conserved Hsp70 chaperone machine plays a central role in a plethora of cellular processes related to protein folding. Hsp70 assists the folding of newly synthesized polypeptides, the assembly and disassembly of protein complexes, as well as protein translocation, disaggregation, and targeting for degradation (1, 2). All of these Hsp70 functions depend on the ability of Hsp70 to interact in an ATP-dependent manner with extended hydrophobic stretches of polypeptide substrates (2). To actively perform as a bona fide chaperone, Hsp70 necessitates the assistance of its Hsp40 co-chaperone partners, which efficiently stimulate its ATPase activity and facilitate its interaction with polypeptide substrates (2–4). Some Hsp70 family members additionally require other co-chaperone partners, such as GrpE, Bag-1, or HspBP1, to regulate substrate release and nucleotide exchange (5–7).

All of the members of the Hsp40 family are characterized by a highly conserved sequence of ~70 amino acids, called the J-domain. This domain is absolutely necessary for the stimulation of Hsp70 ATPase activity (3, 8–11). Active J-domains contain a canonical His-Pro-Asp (HPD) triplet (in some cases Asp is replaced by Glu) that is essential for their activity. The members of the Hsp40 proteins family can be grouped in three classes with respect to their additional conserved domains (9, 10). Type I members, such as the well studied DnaJ protein from the bacterium Escherichia coli, possess adjacent to their N-terminal J-domain (i) a glycine- and phenylalanine-rich (G/F-rich) (3, 11) sequence of unknown function, (ii) a central cysteine-rich domain containing two zinc-binding sites with apparently distinct functions, one related to a DnaK-independent chaperone function and the second most likely involved in a mechanism associated with the stabilization of DnaK-substrate complex (12–15), and (iii) a less conserved C-terminal domain involved in substrate binding and homodimerization (16–20). Although the type II Hsp40 family members lack the central zinc-binding domain, the type III members only share the J-domain signature sequence with their related type I and II members (9, 10).

The role of the conserved G/F-rich region of both major type I and II family members has remained enigmatic. This region was originally proposed to be a flexible link between the J-domain and the central zinc-binding domain, but later evidence suggested different functions. For example, in E. coli, the N-terminal J-domain together with the flanking G/F-rich region of the type I Hsp40 member DnaJ were necessary and sufficient to activate DnaK (3, 21). A DnaJ construct where the G/F-rich region was replaced by an unrelated sequence (HMGS/HM hexapeptide) could efficiently stimulate DnaK ATPase activity and bind the σ22 substrate but could not efficiently activate DnaK itself to bind σ22 (22). These results suggested that, in contrast to the N-terminal J-domain, the G/F-rich region does not directly trigger the stimulation of DnaK ATPase activity per se but may be rather involved in a mechanism that modulates substrate binding by DnaK. In the yeast Saccharomyces cerevisiae, this region seems to confer a certain degree of specificity. Indeed the G/F-rich region of the S. cerevisiae type II Hsp40 protein Sis1 could not be replaced by the G/F-rich region of the S. cerevisiae type I Ydj1 Hsp40 homolog, when complementation for the lethal phenotype of a Δsis1 strain was monitored (23). An additional central aspect of the Sis1 G/F-rich region is that, although not essential for yeast growth, this region was specifically required for the maintenance of the yeast prion [RNQ+ (24, 25). Interestingly, prion maintenance was specifically dependent upon the presence of a short stretch of amino acid residues in the Sis1 G/F-rich region not present in the Ydj1 homolog (24).

The NMR solution structure of the J-domain and the flanking G/F sequence of E. coli DnaJ revealed that in contrast to the compact structure of the J-domain, the G/F-rich region adopts an extended disordered...
conformation (26). However, a short stretch of amino acids in the G/F-rich region presents reduced local flexibility when compared with the rest of the region and undergoes rapid exchange between favored conformations (26). This short segment corresponds to conserved Asp-Ile/Val-Phe triplets of amino acids in the DnaJ G/F-rich region, referred to as the DIF motif (see Fig. 1A) (22). In this study, we have used in vivo and in vitro approaches to examine the role of the G/F-rich region and its DIF motif in the biology of DnaJ.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Bacteriophages**—All of the bacterial strains used in this study are derivatives of *E. coli* K-12. The strain Δ is an arabinose-resistant derivative of MC4100 dnaJ::Tn10-42 cbpA::kan Δthr::thr::Tn10(Tet) cbpA::kan Δthr::Tn10 (27). The strain Δ is MC4100 araA714 araD1KdnA::kan the thr::Tn10(Tet) cbpA::kan Δthr::Tn10 and was constructed as follows. The ΔaraA714::kan allele from PK101 (28) tightly linked to the thr::Tn10(Tet) marker (laboratory collection) was introduced at 30 °C by P1 transduction into the arabinose-resistant strain MC4100 ΔaraA714 cbpA::kan Δthr::Tn10 (29) by selecting for tetracycline resistance. Subsequently, transductants were screened for temperature sensitivity and were further verified for the loss of DnaK and DnaJ by Western blot analysis. MG1655 dnaJ::Tn10-42 cbpA::kan (27), referred to as Δ, was used to test bacterial motility and bacteriophage λ growth using bacteriophages λcl and the dnaJ transducing phage λclDNA+ (laboratory collection), essentially as described (27).

**Plasmid Constructs**—Plasmids pBAD22 and pBAD33 (30), pSE380ΔNcol and p29SEN (31), pWK900 (pBAD22-dnaJ), pWK900KPN (pBAD22-dnaJ-H71T), pWK900 (pBAD22-dnaJ) and pWK900KPN (pBAD22-dnaJ-H71T) (32), pWKG52 (pBAD22-djF’ATM) (33), and pDM38 (dnaKΔdnaJ) (34) have been described previously.

Alanine substitution mutants in the DIF motifs of DnaJ and DnaJ12 were constructed by PCR-based, site-directed mutagenesis using the appropriate primers and plasmids pWK900 or pWK1000. The entire deletion of the G/F-rich region (Δ72–108) of DnaJ was constructed by two-step PCR using pWK900 and the primers: ΔF’ for, 5′-ggaggtcatcaacggtgcgcggcgtgctg-3′ and ΔG-F’, 5′-gcagctgtgacatctgcatagtgtgctg-3′. The resulting 2596-bp fragment was digested with KpnI and ligated into pSE380ΔNcol digested with the same enzymes. The chimera comprising the DnaJ J-domain was first PCR-amplified using primers Mdj1G/F-for (5′-gctgcagcagcagcatctgtgctgctg-3′) and Hdj2G/F-rev (5′-gtgtcgagatccagctgctgctgctgctgctg-3′) and cloned into pBR322. The resulting 1167-bp fragments (containing dnaJ linked in frame with six additional C-terminal histidines) were cleaved with EcoRI-BglII and cloned into pSE380ΔNcol vector digested with the same enzymes. The dnaJ (H33Q) mutant was constructed using the same strategy, except that the EcoRI-BglII fragment was inserted in pBAD22. Control experiments indicated that the C-terminal addition of His, to DnaJ did not noticeably alter DnaJ function.

The pBAD33 DnaJ and DnaJ-3DIF derivatives were obtained by sub-cloning the EcoRV-SphI-digested fragment of pWK900 containing dnaJ wild type or dnaJ (3DIF) into the EcoRV-SphI-digested pBAD33 vector. All of the constructs obtained by PCR were sequenced-verified using the appropriate primers.

**In Vivo DnaJ Activity Assays**—Bacterial motility and bacteriophage λ plating assays were performed at 30 °C as described previously (27). To assess bacterial viability, fresh plasmid transformants were grown overnight at 30 °C, serially diluted, and spotted on LB agar plates supplemented when necessary with the appropriate antibiotics (100 μg/ml ampicillin, 20 μg/ml chloramphenicol) and various l-arabinose inducer concentrations. The plates were incubated at 30 and 40 °C for 20 h and at 14 °C for 6 days.

**Immunoblot Analysis**—For steady state protein expression, whole cell extracts were prepared as described previously (32). DnaJ proteins were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes (Schleicher & Schuell), probed with anti-DnaJ rabbit polyclonal antibodies, and developed by enhanced chemiluminescence using goat anti-rabbit horseradish peroxidase-conjugated IgG as secondary antibodies (Amersham Biosciences).

**Protein Purifications**—To avoid a toxic effect of protein overexpression in wild type *E. coli*, DnaJ and DnaJ-3DIF were purified from the Δ strain. Fresh overnight cultures were diluted 1:100 in 500 ml of LB broth supplemented with 100 μg/ml ampicillin and grown with vigorous shaking at 30 °C. At an *A* of 0.5, 1 mM IPTG was added, and the cultures were incubated for an additional 3 h. For DnaJ-H33Q-His, overexpression was replaced by 0.5% of l-arabinose. The cells were harvested at 5000 rpm for 30 min in a Sorvall GSA rotor and resuspended in 10 ml of J1 buffer (50 mM Tris, pH 8.0, 10% (w/v) sucrose, 10 mM DTT, 1 mM EDTA, 1 mg/ml lysozyme, and 0.6% (w/v) Brij 58). The cells were incubated on ice for 45 min and then sonicated twice for 10 s to reduce
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viscosity. After centrifugation at 28,000 rpm for 1 h in a Beckman 35 Ti rotor, the pellets were resuspended in 10 ml of J2 buffer (1 M NaCl, 50 mM Tris, pH 7.3, 2 mM β-mercaptoethanol, 10% sucrose, 0.5% (v/v) Triton X-100, 500 μl of protease inhibitor mix; Sigma) and then gently tumbled at 4 °C for 1 h. Insoluble material was removed by centrifugation at 28,000 rpm for 90 min in a Beckman 35 Ti rotor. Supernatants (5 ml) were applied to 2 ml of nickel-nitrilotriacetic acid columns pre-equilibrated with 5 volumes of J2 buffer (without protease inhibitors). The following steps were performed as described in the procedure from Qiagen for purification of His6-tagged proteins from E. coli using nickel-nitrilotriacetic acid superflow under native conditions, using buffer J2 supplemented with 20 mM imidazole as washing buffer and with 250 mM imidazole as elution buffer. The proteins were stored at −80 °C in buffer containing 25 mM HEPES, pH 7.6, 0.4 M KCl, 1 mM DTT, 10% (v/v) glycerol.

DnaK with a C-terminal His6 tag was purified from strain ΔA. The cells were grown at 30 °C to mid-exponential phase, protein expression was induced with 1 mM IPTG for 3 h, and purification was performed as described in the Qiagen protocol. The proteins were stored at −80 °C in TEG buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.1 M EDTA, 1 mM DTT, 20% (v/v) glycerol).

Purified GrpE protein was a kind gift of Dr. Krzysztof Liberek (University of Gdansk, Gdansk, Poland). Protein concentrations were routinely measured by a Bradford assay using bovine serum albumin as standard and estimated by SDS-PAGE analysis.

Luciferase Aggregation and Refolding Assays—Luciferase aggregation was performed as follows. 25 μM luciferase (Promega) was denatured in 6 M guanidinium hydrochloride, 30 mM Tris–HCl, pH 7.6, 5 mM DTT for 2 h at 22 °C. The reactions were initiated by a 100-fold dilution of denatured luciferase into a premix containing the indicated concentrations of DnaJ proteins in 30 mM HEPES, pH 7.5, 40 mM KCl, 50 mM NaCl, 7 mM magnesium acetate, 1 mM DTT. Aggregation kinetics were followed at 22 °C by measuring light scattering at 320 nm. Luciferase refolding was essentially assessed as described (35), with minor modifications. Specifically, luciferase 25 μM (Sigma) was denatured as described above and diluted 100-fold into a premix containing 10 mM MOPS, pH 7.2, 50 mM KCl, 5 mM MgCl2, 0.015% (w/v) bovine serum albumin, 0.1 mg/ml creatine kinase, 20 mM creatine phosphate, 5 mM ATP, 2 μM DnaK, and 0.5 μM DnaJ. After 10 min, the indicated concentration of GrpE was added. The luciferase activity was monitored at 22 °C by using the luciferase assay system from Promega (E1500) and a Turner luminometer (TD-20/20).

Co-immunoprecipitation—The co-immunoprecipitation experiments were carried out as described previously (36), except that Pansorbin was replaced by IgGorb (The Enzyme Center, Malden).

Steady State and Single-turnover ATPase Assays—The steady state ATPase assay was performed essentially as described, with minor modifications (4). The standard reaction conditions were 300 mM HEPS, pH 7.6, 40 mM KCl, 50 mM NaCl, 7 mM magnesium acetate, 2 mM DTT, 0.29 mg/ml bovine serum albumin, 100 μM ATP, 1 μCi/ml [γ-32P]ATP (3000 Ci/mmol), 1 μM DnaJ, 1 μM GrpE, and DnaJ or DnaJ mutants in the range 0–0.8 μM. When necessary, 250 nM of luciferase (Promega) denatured with 6 M guanidinium hydrochloride was added in the reaction mix. Aliquots were removed from the reaction at the specified time points and spotted onto polyethyleneimine thin layer chromatography plates (Merck). The plates were resolved with a 1:1 solvent mixture 1 M formic acid, 1 M LiCl. The amount of liberated γ-phosphate was quantified using phosphorimaging.

Single-turnover ATPase assay was performed as described by Mayer et al. (37) with minor modifications. Briefly, 15 μM DnaJ was incubated with 800 μM ATP and 12 μCi [α-32P]ATP (3000 Ci/mmol) in buffer A (25 mM HEPES, 50 mM KCl, 10 mM MgCl2) for 2 min on ice. DnaK-[α-32P]ATP complexes were then rapidly separated from free nucleotides with a Sephadex G50 Nick-column (Amersham Biosciences). The aliquots were flash frozen in liquid nitrogen and stored at −80 °C. The reactions were initiated by adding DnaK-[α-32P]ATP complex to a premix containing 0.4 μM DnaJ in buffer A maintained at 25 °C. Aliquots (2 μl) were spotted onto polyethyleneimine thin layer chromatography plates (Merck) and separated with 150 mM formic acid, 0.150 mM LiCl, and the ADP was quantified using phosphorimaging.

RESULTS

Functional Conservation of the G/F-rich Region among Hsp40 Family Members—The G/F-rich region of Hsp40 family members is characterized by a relatively high frequency of glycine and phenylalanine residues, with one universally conserved sequence motif DIF (Fig. 1A). To gain insight into the function of this region, we first asked whether functional conservation could be observed among type I Hsp40 G/F-rich regions from several organisms. To this end, we constructed a series of chimerae where the G/F-rich region of E. coli DnaJ was replaced by the corresponding region from either yeast (cytosolic Ydj1 and mitochondrial Mdj1) or human (cytosolic Hdj2) type I Hsp40s. To minimize complexity, these chimerae were constructed in the stringent context of DnaJ12, which comprises the N-terminal amino acids 1–108 containing the J-domain and the G/F-rich region and is the smallest known part of E. coli DnaJ sufficient and necessary to partially support its DnaK co-chaperone function (21). These constructs were then tested in vivo for their ability to complement the various phenotypes of the E. coli strain Δ3, which lacks all three Hsp40 co-chaperones of DnaJ, namely DnaJ, CbpA, and DjaK (29). We observed that none of the yeast and human G/F-rich regions tested are able to fully functionally replace the E. coli G/F-rich region (Fig. 1B). Specifically, although all the chimerae efficiently support bacterial growth at high temperature and λ propagation in the Δ3 strain, no complementation for bacterial growth at low temperature was observed for any of the chimerae (Fig. 1B; compare DnaJ12 to DnaJ12-Ydj1 G/F, DnaJ12-Mdj1 G/F, and DnaJ12-Hdj2 G/F). Steady state accumulation levels of the chimerae at permissive temperature (30 °C) were similar to those of DnaJ12 (data not shown). Among the chimerae tested, the DnaJ12-Hdj2 G/F construct exhibited the weakest complementation activity and, in contrast to the yeast chimerae, even exerted a negative effect on E. coli growth and λDNA* transducing phage propagation at a permissive temperature (30 °C).

We next asked whether addition of the G/F-rich region of DnaJ could convert a non-type I Hsp40 homolog into a functional DnaJ-like co-chaperone in vivo. To answer this, we grabbed the G/F-rich region of DnaJ to the C-terminal part of the E. coli type III Hsp40 Dja protein, a bona fide DnaK co-chaperone that does not efficiently complement DnaJ function in vivo for either bacterial growth at high and low temperatures or λ propagation (29, 35). Interestingly, the chimera (DjaΔΔM G/F) was able to rescue all of the phenotypes of the E. coli Δ3 mutant strain tested, including bacterial growth at low temperature (Fig. 1B). Taken together, these results indicate that the G/F-rich region of DnaJ retains a substantial degree of specificity and is critical for DnaJ function in E. coli.

The DIF Motif of the G/F-rich Region Is Critical for DnaJ Function in Vivo—The G/F-rich region of the Hsp40 proteins includes the conserved Asp-Ile/Val-Phe triplet of amino acids, referred to as DIF (Fig. 1A). The E. coli DnaJ G/F-rich region possesses three such repeats. To investigate whether this DIF motif is important for DnaJ function,
the DIF Amino Acid Substitutions Exert a Toxic Effect on *E. coli* Growth and Motility (Fig. 2, C and D). Similar observations were made for each of the three repeats and resulted in a triply mutant (see constructs 3D, 3I/V, and 3F). As found with the 3DIF mutant, these constructs exert a minor effect on DnaJ propagation at high temperature but not at low temperature nor for cellular motility (Fig. 2, B and C; see constructs D1, D2, and D3). Furthermore, a single alanine substitution of any residue of the first DIF motif exerts a major inhibitory effect on DnaJ function at low temperature, but not at high temperature nor for cellular motility (Fig. 2, B and C; see constructs D1, D2, and D3). As with the 3DIF mutant, these constructs exert a minor effect on DnaJ propagation (data not shown). As shown in the *bottom panel* of Fig. 2B, all of the mutant constructs are well expressed at the permissive temperature of 30 °C, and their steady state level is comparable with that of wild type DnaJ. Taken together, our results indicate that the DIF motif plays a subtle, yet crucial role in the ability of DnaJ protein to function as a *bona fide* DnaK co-chaperone.

The DIF Mutants Exhibit a DnaK-dependent Toxic Effect—Besides their inability to functionally substitute for the wild type DnaJ protein, we observed that the DIF substitutions, in both the DnaJ12 and full-length DnaJ contexts, exert a toxic effect on *E. coli* growth. As seen in Figs. 1B and 3A, expression of the mutant proteins completely blocked the ability to form colonies at the otherwise permissive temperature of

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we first replaced all of the nine amino acid residues comprising the DIF motif by alanines in the stringent context of DnaJ12 (DnaJ (1–108)) and tested the resulting mutant for complementation in our *in vivo* assays. As judged by the inability of the DnaJ12–3DIF mutant to support propagation of bacterial growth and motility (Fig. 2, A and B), we observed that the DIF substitutions, in both the DnaJ12 and full-length DnaJ contexts, exert a toxic effect on *E. coli* growth at permissive temperatures (see below) and even partially block the propagation of *dnaJ*+ transducing phage.

To further assess the critical role of the DIF motif, we asked whether its substitution in the context of the full-length DnaJ protein exerts an effect similarly to that observed in the DnaJ12 context. To do so, we generated both a full-length DnaJ with the three DIF repeats substituted by alanines (3DIF) and a DnaJ lacking the whole G/F-rich region (ΔJ(72–108)) and tested for their activity *in vivo* (Fig. 2). As found with DnaJ12, both the 3DIF and the ΔG/F mutations completely abolish complementation of bacterial growth and motility (Fig. 2, B and C; data not shown). The steady state expression levels of both DnaJ and DnaJ-3DIF are comparable at permissive temperature (30 °C) in the ΔJ mutant strain (Fig. 2B). The steady state expression levels of these proteins at the nonpermissive temperatures for ΔJ (16° and 40 °C) are also indistinguishable when the mutant protein was expressed in the *dnaJ* single mutant able to grow at such temperatures (data not shown). Furthermore, the same inhibitory effect was observed when the DIF motif was replaced by glycines instead of alanines (data not shown).
30 °C. In contrast, the overexpression of either wild type DnaJ or DnaJ-H33Q (substitution His33 to Gln in the J-domain), known to abolish all DnaJ co-chaperone functions, exerts no such toxic effect. A level of DnaJ-3DIF/H11011 3-fold higher than endogenous DnaJ at 30 °C was sufficient to severely impair bacterial growth (data not shown). A similar effect on *E. coli* growth was also observed in the *dnaJ* single mutant or the *dnaJ cbpA* or the *dnaJ djlA* double mutants. However, the simultaneous co-expression of the wild type DnaJ significantly reduces DnaJ-3DIF toxicity (data not shown).

To investigate whether toxicity of the mutant is due to an impaired interaction with its chaperone partner DnaK, we tested the effect of DnaJ-3DIF in a strain lacking DnaK plus its three Hsp40 co-chaperone partners, namely DnaJ, DjlA, and CbpA (strain called Δ4). Consistent with a possible impaired interaction with DnaK, the toxicity of the DnaJ-3DIF mutant is totally abolished in the absence of DnaK (Fig. 3A). A typical set of results using 0.02% of L-arabinose inducer is presented. C, complementation for bacterial motility in strain Δ4 (dnaJ cbpA double mutant) using the L-arabinose-inducible pBAD22-based DnaJ constructs depicted in A. A typical set of results using 0.02% of L-arabinose inducer is presented. D, complementation for bacteriophage λ plaque forming ability in strain Δ4 using the L-arabinose-inducible pBAD22-based DnaJ and DnaJ-3DIF constructs. A typical set of results using 0.05% of L-arabinose inducer is presented.

It is known that the J-domain of DnaJ efficiently stimulates the weak ATPase activity of DnaK, a necessary step for DnaK-substrate stable complex formation (40). To assess whether DnaJ-mediated stimulation of DnaK ATPase activity occurs prior to the DIF motif signal, we generated a double mutant that combines the DIF substitution together with the well characterized, disabling H33Q substitution in the J-domain and tested the construct for toxicity in a Δ4 strain. As seen in Fig. 3 (see construct H33Q-3DIF), the 3DIF-mediated toxicity is totally abolished when the DIF substitution is combined with the J-domain H33Q disabling mutation. These results suggest that under wild type conditions, the DIF motif plays a role at a step subsequent to ATP hydrolysis by DnaK.
that the DnaJ-3DIF mutant protein protects both proteins from aggregation in a DnaK-independent manner. Using the purified DnaJ-3DIF protein to bind and protect denatured substrates firefly luciferase and bovine rhodanese, we observed that the DnaJ-3DIF mutant protein protects both proteins from aggregation in a DnaK-independent manner. Using the purified DnaJ-3DIF protein to bind and protect denatured substrates firefly luciferase and bovine rhodanese, we observed

motif, we carried out several in vitro studies. We first tested the ability of the purified DnaJ-3DIF protein to bind and protect denatured substrates from aggregation in a DnaK-independent manner. Using the model substrates firefly luciferase and bovine rhodanese, we observed that the DnaJ-3DIF mutant protein protects both proteins from aggregation, in a manner indistinguishable from wild type DnaJ (Fig. 4; data not shown). These results suggest that the DIF motif of DnaJ is not involved in substrate binding and are in agreement with previous published work (22, 42). Indeed, replacement of the whole G/F-rich region of DnaJ by the random HGSHM amino acid segment had no effect on its affinity for $\sigma^{2}$ (22). Likewise, in yeast, the deletion of the whole G/F-rich region of Sis1 had no effect on Sis1 binding to luciferase or to Rnp1 (42). In addition, other experimental evidence suggests that the polypeptide-binding sites of the type I and type II Hsp90 homologs Ydj1 and Sis1 are localized at the C-terminal domain (16–18).

The DIF Motif Does Not Directly Participate in the Stimulation of DnaK ATPase Activity—The in vivo data suggest that the DIF motif of DnaJ regulates the DnaK chaperone cycle at a post-ATP hydrolysis step, such as the proper locking in of substrate or the timely release of DnaJ from DnaK. To investigate this possibility, we tested the effect of the DIF substitutions on the stimulation of DnaK-mediated ATP hydrolysis, both under single-turnover and steady state conditions.

First, we tested the ability of purified DnaJ-3DIF to stimulate DnaK-mediated ATP hydrolysis under single-turnover conditions, where the kinetics of only a single round of DnaK-mediated ATP hydrolysis is measured (37). Under such conditions, the DIF substitutions in DnaJ do not exert a noticeable effect on the stimulation of DnaK ATPase activity, compared with wild type DnaJ (Fig. 5A). This result confirms that DIF involvement is at a step subsequent to that of the stimulation of DnaK ATP hydrolysis.

Considering that the DIF motif does not influence the stimulation of DnaK ATP hydrolysis but is absolutely needed for DnaJ co-chaperone functions and that the DnaJ-3DIF mutant exhibits a DnaK-dependent toxic effect on E. coli growth at permissive temperature. A, the $\lambda$-arabinose (ara)-inducible pBAD22-based full-length DnaJ constructs were expressed at 30 °C in strain $\Delta$ (dnaJ/cbpA djlA triple mutant) in the absence or in the presence of 0.5% of $\lambda$-arabinose inducer. The $\lambda$-arabinose-inducible pBAD33-based full-length DnaJ constructs were expressed with or without inducer at 30 °C in strain $\Delta$ (dnaJ/cbpA djlA quadruple mutant), in the simultaneous presence of either empty p29SEN vector or the p29SEN-based dnaJ gene under the control of its native promoters. B, the $\lambda$-arabinose-inducible pBAD33-based full-length DnaJ constructs were expressed with or without inducer at 30 °C in strain $\Delta$ (dnaJ/cbpA djlA triple mutant), in the simultaneous presence of either empty pSE380 vector or the IPTG-inducible pSE380-based grpE gene. The various inducer concentrations are shown.
The DIF Motif Regulates the DnaK Chaperone Cycle—To determine the involvement of the DIF motif in the regulation of the DnaK chaperone cycle, we tested whether DnaJ-3DIF could function as a DnaK co-chaperone in vitro. To do so, we tested the ability of the purified DnaJ-3DIF protein to assist DnaK in the refolding of chemically denatured firefly luciferase (Fig. 6A). We observed that in contrast to wild type DnaJ, DnaJ-3DIF does not activate DnaK for the refolding of denatured luciferase. Increased concentrations of DnaJ-3DIF (up to 5 μM) does not allow luciferase reactivation (data not shown). These results indicate that the DIF motif is critical for the activation of the DnaK chaperone function in vitro.

To our knowledge, the DIF mutations in DnaJ are the only DnaJ mutant proteins known to exhibit such a DnaK-dependent toxic effect in vivo. To test whether DnaJ-3DIF could function as a DnaK co-chaperone in vitro, we performed co-immunoprecipitation of DnaK and its luciferase substrate in the presence of either DnaJ or DnaJ-3DIF. As control, we performed the same experiment with DnaJ-H33Q. This well-characterized mutation in the J-domain does not affect DnaJ chaperone functions (like DIF mutations) but totally abolishes all known DnaK-dependent co-chaperone functions, including the stimulation of DnaK ATPase activity and the efficient substrate transfer to DnaK (10). Furthermore, in contrast to DIF, this mutation does not exhibit any DnaK-dependent toxic effect (27) (Fig. 3A). As expected, the DnaJ-H33Q protein does not activate DnaK in the refolding of denatured luciferase (data not shown). These results indicate that the DIF motif is critical for the activation of the DnaK chaperone function in vitro.

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FIGURE 5. Influence of the DIF motif on DnaK ATPase cycle. A, stimulation of DnaK ATPase activity under single-turnover conditions. Shown are DnaK alone and in the presence of DnaJ (A) or DnaJ-3DIF (C). The percentage of hydrolyzed ATP is normalized to the maximal value obtained with wild type DnaJ. B, stimulation of DnaK ATPase activity under steady state conditions. DnaK (1 μM) and GrpE (1 μM) in the presence of the indicated concentrations of DnaJ, DnaJ-H33Q, or DnaJ-3DIF. The percentage of hydrolyzed ATP/min is plotted as a function of the final DnaJ concentration used in the reaction mix.

FIGURE 6. Influence of the DIF motif on DnaK activities in vitro. A, refolding of denatured luciferase (250 nM) by DnaK (250 nM) in the presence of GrpE (1 μM) and 0.5 μM of DnaJ (DnaJ, DnaJ-H33Q (+), or DnaJ-3DIF (●)). The values of luciferase refolding were normalized to the maximal value obtained with wild type DnaJ. B, co-immunoprecipitation of DnaK and firefly luciferase. Denatured luciferase (250 nM) and DnaK (250 nM) were incubated with DnaJ, DnaJ-3DIF, or DnaJ-H33Q (250 nM) for 10 min at 22 °C in the presence of 1 mM ATP. DnaK and luciferase were co-immunoprecipitated with anti-luciferase antibodies, and both luciferase and DnaK were visualized by Western blotting using their respective antibodies. C, influence of GrpE on DnaK-mediated luciferase refolding in the presence of DnaJ or DnaJ-3DIF. The experimental conditions were as described for A, except that various concentrations of GrpE were added. The luciferase activity was measured after 60 min, and the concentrations (in μM) of GrpE used for each experiment are indicated on top of each bar.

steady state conditions. For both DnaJ and DnaJ-3DIF, the addition of GrpE in the co-immunoprecipitation reaction identically reduces the yield of DnaK-bound luciferase (data not shown).

Because GrpE overexpression alleviates the DnaK-dependent toxicity of the DnaJ-3DIF mutant in vivo, we asked whether increased GrpE concentrations could partially restore luciferase refolding in the presence of DnaJ-3DIF. Although a wide range of GrpE concentrations was tested, no detectable improvement in luciferase refolding was observed (Fig. 6C). In a similar manner, increased GrpE concentrations do not
improve the steady state ATPase activity of DnaK in the presence of DnaJ-3DIF (data not shown). These results are in agreement with the in vivo observation that GrpE overexpression does not support bacterial growth at a nonpermissive temperature in the presence of DnaJ-3DIF. In turn, this suggests that accelerating substrate release from DnaK or inhibiting its interaction with DnaK prevents the formation of toxic forms of DnaK-substrate complexes in the presence of DnaJ-3DIF.

**DISCUSSION**

In this study, we have investigated the role of the DnaJ G/F-rich region and the DIF motif in the regulation of the DnaK chaperone cycle in *E. coli*. We first show that the G/F-rich region retains an exquisite specificity that is required for full *E. coli* DnaJ activity in vivo. Specifically, our chimerae study revealed that although functional complementation by the G/F-rich region from either human or yeast type I Hsp40 homologs is observed during bacterial growth at high temperatures and for bacteriophage λ propagation, none of these chimerae can functionally replace the DnaJ G/F-rich region for bacterial growth at low temperatures. Such specificity has been observed among G/F-rich regions of types I and type II Hsp40 homologs in yeast (23, 24, 42).

Indeed, the G/F-rich region of the Sis1 type II Hsp40, which is critical for [RNQ] prion maintenance and cell survival in a *sis1* mutant, could not be replaced by the G/F-rich region of the Ydj1 type I Hsp40 (24). The specificity of action of the G/F-rich region is further emphasized by our results obtained with the *E. coli* type III Hsp40 homolog DjlA. Indeed, DjlA, which possesses no G/F-rich region and by itself does not complement for DnaJ functions in vivo (29, 35), could efficiently replace DnaJ when the G/F-rich region of DnaJ was grafted to its C-terminal region.

In addition, we showed that deletion of the whole G/F-rich region of DnaJ (DnajΔ(72–108)) exerts a noncomplementing phenotype in vivo and that this phenotype is also seen with single amino acid alterations in a stretch of amino acids that we previously named the DIF motif (22). An alteration of any of the repeats of the DIF motif mimics the deletion of the whole G/F-rich region, both in the context of the full-length DnaJ and of the smallest active DnaJ construct (DnaJ1–108), which contains only the J-domain and the flanking G/F-rich region. These results suggest that these motifs may, at least in part, be responsible for the specificity exhibited by the G/F-rich region.

In this respect, it is interesting to note that the NMR solution structure of the J-domain and the G/F-rich region of DnaJ shows that although the whole G/F-rich region is overall highly unstructured and does not form a globular core, amino acids 90–103 exhibit reduced local flexibility and thus tend to adopt a preferential conformation (26). Remarkably, this short segment contains all the 11 amino acids of the critical DIF motif. The *in vitro* analysis of the DnaJ-3DIF mutant shows that the DIF motif neither participates in substrate binding (Refs. 14, 16–18, 22, and 42 and this work) nor directly stimulates DnaK ATPase activity. However, substitutions in the DIF motif significantly slow down the steady state ATPase cycle of the DnaK/DnaJ/GrpE chaperone machine and inhibit its ability to refold luciferase substrate *in vitro*. In addition, DIF mutations exert a remarkable DnaK-dependent poisonous effect on *E. coli* growth at otherwise permissive temperatures but do not block the activation of the formation of DnaK-substrate complexes *in vitro*. This suggests that the poisonous effect may be due to the formation of kinetically trapped DnaK-substrate or DnaJ-DnaK-substrate complexes. Some of the trapped substrates may be essential *E. coli* proteins, and the failure to properly fold or release these proteins in a timely fashion may inhibit bacterial growth. This situation may be analogous to the previously observed lethality of a *grpE* deletion seen only in the presence of functional DnaK (41). Such a possible scenario is further supported by the fact that either overexpression of GrpE or a mutation in the J-domain of DnaJ, known to block the stimulation of DnaK ATPase activity, alleviates DIF toxicity.

While our study was in progress, Aron et al. (42) observed a relatively similar situation in yeast. The deletion of the G/F-rich region of the type II Hsp40 homolog Sis1 also exerted a poisonous effect on wild type yeast growth. Additional experiments indicated that the absence of a 13-amino acid segment of the G/F-rich region was the cause of the toxicity. The same segment was also shown to be necessary for Sis1-mediated [RNQ] prion maintenance (24, 42). Interestingly, the toxicity of Sis1 ΔG/F was suppressed by point mutations in the Sis1 C-terminal domain, which weakened the overall interaction of Sis1 with its Hsp70 partner Ssa1 (42). However, the specific motif of the G/F-rich region of the yeast type II Hsp40 Sis1 involved is also necessary for [RNQ] prion maintenance and is not present in the type I Hsp40 homologs, Ydj1 in yeast and DnaJ in *E. coli*.

Based upon these observations, a model for the role of the DIF motif of DnaJ in the function of the DnaK/DnaJ/GrpE chaperone machine is proposed in Fig. 7. Both DnaJ wild type (Fig. 7A) and DnaJ-DIF mutant (Fig. 7B) bind similarly to substrate and equally stimulate DnaK ATPase activity. Upon ATP hydrolysis by DnaK and substrate transfer from DnaJ to DnaK, DnaJ wild type is rapidly released from DnaK, leaving the DnaK-substrate complex accessible to interact with the GrpE dimer, thus accelerating nucleotide exchange and the subsequent release of the substrate from DnaK. In the case of the DnaJ-DIF mutant, we propose that more stable DnaK-substrate or DnaJ-DnaK-substrate complexes are formed, perhaps trapped in an erroneous conformation. The formation of such a kinetically trapped complex could either delay or block the folding or disaggregation of some essential substrate(s). In the absence of DnaK, this putative essential substrate(s) may be eligible for folding with the help of the other *E. coli* chaperone machines. Clearly, more experiments are warranted to validate such a model.

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