Analysis of σ^{32} mutants defective in chaperone-mediated feedback control reveals unexpected complexity of the heat shock response

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Protein quality control is accomplished by inducing chaperones and proteases in response to an altered cellular folding state. In *Escherichia coli*, expression of chaperones and proteases is positively regulated by σ^{32}. Chaperone-mediated negative feedback control of σ^{32} activity allows this transcription factor to sense the cellular folding state. We identified point mutations in σ^{32} altered in feedback control. Surprisingly, such mutants are resistant to inhibition by both the DnaK/J and GroEL/S chaperones *in vivo* and also show dramatically increased stability. Further characterization of the most defective mutant revealed that it has almost normal binding to chaperones and RNA polymerase and is competent for chaperone-mediated inactivation *in vitro*. We suggest that the mutants identify a regulatory step downstream of chaperone binding that is required for both inactivation and degradation of σ^{32}.

heat shock transcription factor | proteolysis | GroEL | DnaK | stress response

The heat shock response is a major homeostatic mechanism for controlling the state of protein folding and degradation in all cells (1–3). Upon heat stress, a set of highly conserved heat shock proteins (hsps), including chaperones and proteases, is rapidly and transiently induced. Hsps maintain optimal states of protein folding and turnover during normal growth and also minimize cellular damage from stress-induced protein misfolding and aggregation (4, 5). The level of hsps is controlled primarily by heat shock transcription factors that sense the cellular folding environment through negative feedback control mediated by chaperones (6–9). Understanding this mode of regulation is central to our understanding of protein quality control as well as cellular stress responses. Here, we report the determinants required for chaperone regulation of σ^{32}, the heat shock transcription factor in *Escherichia coli* (10–12).

σ^{32} regulon members control both the activity and stability of σ^{32}. The DnaK/J/GrpE and GroEL/S chaperone machines each constitute a negative feedback loop that couples σ^{32} activity to cellular protein folding state: overexpression of either chaperone machine decreases σ^{32} activity; conversely, chaperone depletion or overexpression of chaperone substrates increases σ^{32} activity (13–16). The chaperones are likely to act directly on σ^{32} because they bind to σ^{32} and inhibit its activity in a purified *in vitro* transcription system (17–19). Regulated degradation of σ^{32} is mediated by the FtsH protease and facilitated by DnaK/J/GrpE and GroEL/S *in vivo*, but this process has not been completely recapitulated *in vitro*, where degradation of σ^{32} by FtsH is slow and not facilitated by chaperones (20, 21).

We selected and characterized feedback-resistant mutants of σ^{32}. The residues altered by the mutations map to a small patch in σ^{32}. Surprisingly, most mutants simultaneously diminished all three negative feedback loops operative *in vivo*; the most severe mutant essentially eliminated chaperone-mediated activity control and degradation by FtsH protease. In striking contrast to the *in vivo* phenotype, this strong mutant exhibited few defects when tested either for binding or chaperone-mediated inactivation *in vitro*. The implications of these findings for the mechanism of chaperone-mediated negative feedback control will be discussed.

Results

Isolation of σ^{32} Mutants That Are Not Fully Responsive to Chaperone-Mediated Inactivation. When σ^{32} is overexpressed, the resultant accumulation of chaperones triggers a negative regulatory loop that inactivates σ^{32} (13). The features in σ^{32} that permit chaperone-mediated inactivation are unknown. To identify σ^{32} mutants defective in this process, we overexpressed σ^{32} and selected mutants with higher σ^{32} activity than expected if the inactivation loop were operative. A high-copy plasmid carrying mutagenized *rpoH* driven from an isopropyl β-D-thiogalactoside (IPTG)-inducible promoter was transformed into cells with two σ^{32}-dependent reporters, one driving expression of *cat* (chloramphenicol resistance) and a second driving expression of *lacZ* (Fig. 1*A*). Cells were selected for higher than normal σ^{32}-dependent transcription of *cat* by plating on LB/chloramphenicol/IPTG plates having a concentration of chloramphenicol that inhibits growth of cells with wild-type (WT) σ^{32} activity. To eliminate mutations in the *cat* promoter, all candidates were then screened for increased σ^{32}-dependent transcription of *lacZ* on IPTG-containing lactose indicator plates (red color). Sequencing the entire *rpoH* gene of each mutant revealed five independent single-base substitutions, four of which were located in conserved region 2.1 (Fig. 1*B*, above bar). Three other mutants obtained from a slightly different screen (22) that mapped in the same region were included for analysis (Fig. 1*B*, below bar).

We compared the activity of the plasmid-encoded σ^{32} mutants by quantifying the differential rate of β-galactosidase synthesis in strains carrying the σ^{32}-dependent P_{htpG}-*lacZ* reporter at 30°C. All mutants exhibited somewhat higher σ^{32} activity than their WT counterparts when *rpoH* expression was not induced (1.5- to 2.5-fold; data not shown) and severalfold higher activity when induced with IPTG (3- to 6-fold; Table 1, first column). These mutants also had increased σ^{32} levels (data not shown) and stability (Table 1, fourth column). Thus, these mutants were altered both in the activity and stability of σ^{32}.

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Abbreviations: hsp, heat shock protein; IPTG, isopropyl β-D-thiogalactoside.

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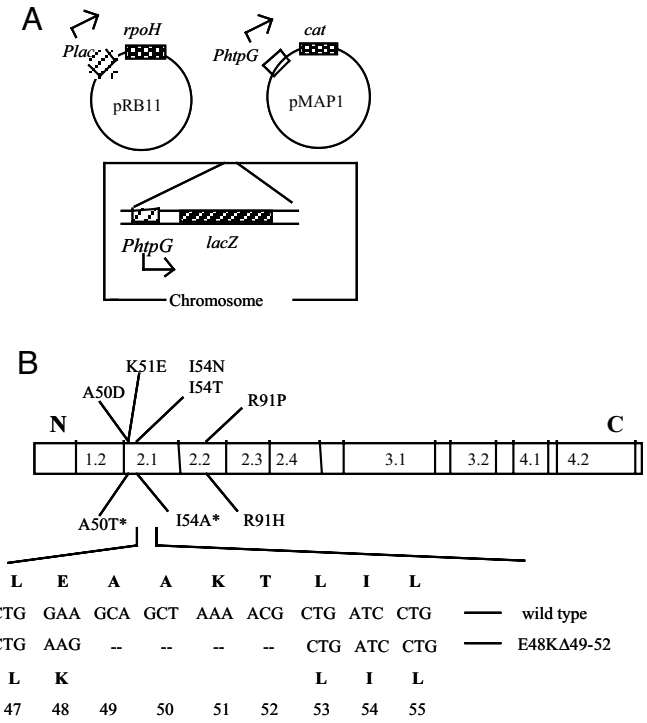

Fig. 1. Selection for σ^{32} mutations on a high-copy *rpoH* plasmid. (*A*) Plasmid and chromosomal constructs of the parental strain used for selection. (*B*) Location of σ^{32} mutations. Region 2 mutants obtained in this selection are shown above the bar, which illustrates nine conserved regions of the bacterial σ factor; the three mutants indicated below the bar were identified from a slightly different selection described in ref. 19. *, mutants reported previously (19). The E48KΔ49–52 deletion mutant, shown at the bottom, was obtained during transfer of the plasmid-encoded A50D mutation onto the chromosome (see *Results*).

Effect of Chaperone Overexpression on σ^{32}-Dependent Transcription. The activity of WT σ^{32} decreases after overexpression of GroEL/S or DnaK/J chaperones because excess chaperones inactivate σ^{32}. We investigated whether the σ^{32} mutants had decreased susceptibility to chaperone overexpression as expected if they were defective in this feedback loop. These experiments were performed in a strain with the chromosomal GroEL/S or DnaK/J operons under control of an inducible σ^{70} promoter so that chaperone expression could be initially adjusted to that of the WT strain despite differences in the activity of σ^{32} mutants. Overexpression of σ^{32} (Table 1, first column) and GroEL/S (Table 1, second column) were from plasmids encoding, respectively, *rpoH* or *groEL/S* under control of an inducible σ^{70} promoter; DnaK/J overexpression (Table 1, third column) was from the chromosomal locus by increasing inducer concentration. GroEL/S or DnaK/J overexpression markedly inhibited the activity of WT σ^{32} (≈3-fold), as expected from previous studies (15, 16). In contrast, all region 2.1 mutants and one of the region 2.2 mutants exhibited varying levels of resistance to excess GroEL/S and DnaK/J. In particular, I54N was essentially resistant to inhibition. Thus, these mutants are less sensitive to negative feedback inhibition by both GroEL/S and DnaK/J chaperones.

Table 1. The σ^{32} mutants are altered in activity, stability, and response to chaperone overexpression

σ^{32}	Relative σ^{32} activity*			Relative σ^{32} stability†
	Control	GroEL/S	DnaK/J	
WT	1.0	0.33	0.27	1.0
A50D	6.1	4.9	5.0	5.4
A50T	3.2	2.6	2.0	
K51E	4.2	3.3	2.6	5.2
I54N	4.9	4.9	3.8	47.0
I54T	3.0	1.8	1.7	3.7
I54A	5.4	3.5	3.4	
R91P	3.2	2.2	2.1	2.2
R91H	3.6	1.2	1.5	

*σ^{32} activity is taken as the differential rate of β-galactosidase synthesis from P_{htpG}-*lacZ* reporter determined starting 30 min after induction of σ^{32} only (first column), σ^{32} and GroEL/S (second column), or σ^{32} and DnaK/J (third column) (see *Materials and Methods*). All activities are normalized to that of the WT control shown as 1.0. Results of a representative experiment are shown; variation among two to four experiments was <20%.

†The amount of σ^{32} remaining after the addition of 200 μg/ml chloramphenicol was determined by immunoblotting (see *Materials and Methods*). Stability relative to WT from a typical experiment is shown. The variation among two to four experiments was <20%.

Activity Control of Chromosomally Located I54N and E48KΔ49–52 Mutants. We performed detailed characterization of two mutants in the physiologically relevant genomic context. Our goal was to compare the phenotypes of a strong mutant (I54N) with those of a somewhat weaker mutant (A50D). We were unsuccessful in transferring A50D to the chromosome, but fortuitously we isolated a chromosomal mutant consisting of both a small deletion (Ala^{49} to Thr^{52}) and an E48K substitution, hereafter called E48KΔ49–52 (Fig. 1*B*) while trying to transfer A50D (see *Materials and Methods*). Chromosomal I54N-σ^{32} showed 7-fold higher activity and E48KΔ49–52 σ^{32} ≈4-fold higher activity than WT σ^{32} both in LB and M9 medium (Table 2, first two columns), but they had almost normal growth at 30°C, 37°C, or 42°C (data not shown).

We used two different assays to characterize the effect of chaperone overexpression on σ^{32} activity. Measuring the differential rate of β-galactosidase synthesis from the σ^{32}-dependent P_{htpG}-*lacZ* reporter (Fig. 2) indicated that I54N cells were completely resistant and E48KΔ49–52 cells partially resistant (30% inhibited) to inhibition by excess GroEL/S (Fig. 2 *A–C*); both mutants were also more resistant to inhibition by excess DnaK/J than cells with WT σ^{32} (Fig. 2 *D–F*). Because this assay measures accumulation of β-galactosidase, it is inherently insensitive to small changes in rates of synthesis. Therefore, to characterize the instantaneous extent of inhibition, we determined the rate of HtpG synthesis at different times after overexpression of either GroEL/S (Fig. 3*A*) or DnaK/J (Fig. 3*B*) with a pulse-chase–immunoprecipitation protocol. After 10 min of GroEL/S overexpression, WT σ^{32} activity was severely inhibited (≈10-fold), the E48KΔ49–52 mutant was inhibited somewhat less (≈5-fold), and the I54N mutant was essentially resistant to inhibition (<30% inhibition). Indeed, the level of inhibition exhibited by I54N is equivalent in magnitude to the nonspecific effects of GroEL/S overexpression manifest also at σ^{70} and σ^{E} promoters (16). After 30 min of DnaK/J overexpression, WT σ^{32} activity was inhibited 80%, the E48KΔ49–52 mutant was inhibited ≈60%, and I54N was almost completely resistant (20% inhibition). Taken together, these results indicate that σ^{32} activity of the "strong" I54N mutant is more resistant to both GroEL/S and DnaK/J overexpression than the "moderate" E48KΔ49–52 mutant.

We used these same strains to deplete GroEL/S or DnaK/J, to determine whether I54N and E48KΔ49–52 σ^{32} increase their activity under these conditions. Whereas the activity of WT σ^{32} increased 3- to 10-fold after depletion of GroEL/S or DnaK/J over the time course of 2–3 h, the mutants showed little (E48KΔ49–52) or no (I54N) response to depletion during most of this period [supporting information (SI) Table 5). Thus, when

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Table 2. Relative σ^{32} activity, level, and stability in chromosomal σ^{32} mutants

	σ^{32} Activity		σ^{32} Level		Specific activity (activity/level)		Stability
Strain	LB	M9	LB	M9	LB	M9	LB
WT	1.0	1.0	1.0	1.0	1.0	1.0	1.0
I54N	6.9 ± 0.6	6.8 ± 0.8	10	15	0.7	0.5	42.0
E48KΔ 49–52	3.8 ± 0.4	3.9 ± 0.5	4	6	1.0	0.6	2.7
SfhC	1.5		1.2	1.3	1.3		
sfhC Δ *ftsH*	3.0	2.3	25	35	0.12	0.07	100

Isogenic WT (CAG48238) and σ^{32} mutant strains, and *sfhC* (suppressor of Δ *ftsH* lethality) and its isogenic Δ *ftsH* strain were grown at 30°C. Activity was determined by measuring the differential rate of β-galactosidase activity (LB; first column) or by pulse-labeling experiments measuring HtpG synthesis (M9; second column); the σ^{32} level (third and fourth columns) was determined by immunoblotting (level in M9 was about one-third of that in LB); stability data (seventh column) were taken from SI Fig. 6.

expressed in single copy from the chromosome, these two σ^{32} mutants are less sensitive than WT σ^{32} to sudden change (both increase and decrease) in cellular chaperone levels.

Level and Stability of Mutant σ^{32}. Quantification of the level of σ^{32} revealed that I54N is much more abundant (10- to 15-fold) and E48KΔ49–52 is somewhat more abundant (4- to 6-fold) than WT σ^{32} (Table 2, third and fourth columns). Assessment of σ^{32} stability with immunoblotting after the addition of chloramphenicol in LB medium revealed that an increased σ^{32} level resulted from increased stability of the mutants, with E48KΔ49–52 showing an ≈3-fold increase and I54N exhibiting a ≈40-fold increase in stability compared with WT (Table 2, seventh column, and SI Fig. 6). The increase in stability of the mutants is sufficient to explain their increase in level. σ^{32} synthesis was unaffected by the I54N mutation, as expected (data not shown).

As an independent assessment of feedback inhibition of the mutant σ^{32}s, we compared the specific activity of the mutant proteins with that of WT σ^{32} (σ^{32} activity/σ^{32} level; Table 2, fifth and sixth columns). Setting the activity of WT σ^{32} as 1.0 (first line), the specific activity of the mutant proteins is very close to that of WT (between 0.5 and 1.0), depending on the mutant and the medium used (second and third lines) even though the levels of the mutant proteins are sufficiently high that they should be feedback-inhibited. For comparison, when the level of WT σ^{32} is increased by the absence of the FtsH protease, its specific activity decreases to ≈0.1 (fifth line), consistent with previous reports (23).

Behavior of the Mutants upon Temperature Upshift. After shift from 30 to 42°C, synthesis of hsps transiently increases ≈10-fold as a consequence of increased translation of σ^{32} and its transient stabilization (24). In contrast, neither I54N nor E48KΔ49–52 strains exhibited a heat shock response as demonstrated for both strains by using the reporter assay for σ^{32}-dependent transcription (data not shown) and for I54N by examining HtpG synthesis (Fig. 4). Lack of a heat shock response was somewhat unexpected because the translational response of the mutant remains intact (data not shown). We therefore considered the possibility that the mutant proteins were destabilized at 42°C. Indeed, both mutant σ^{32}s were destabilized compared with 30°C, exhibiting $t_{1/2}$ of 2.5 min (I54N) and 0.5 min (E48KΔ49–52). Thus, a lack of a

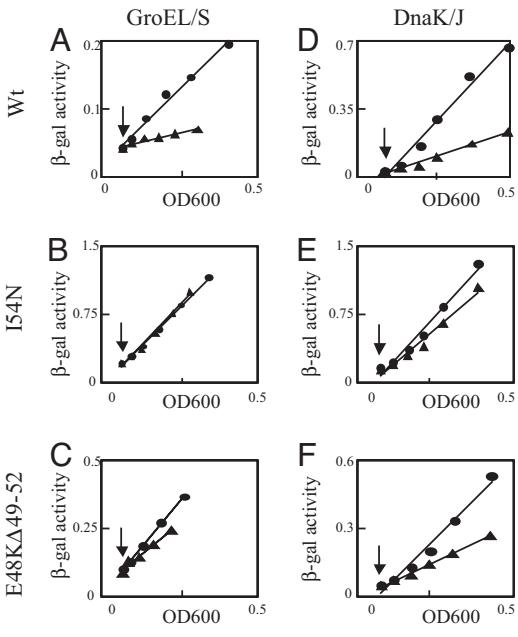

Fig. 2. Effect of chaperone overexpression on σ^{32}-dependent transcription in the chromosomal σ^{32} mutants. WT and σ^{32} mutants carrying the P_{htpG}-*lacZ* reporter and either P_{ara}-*groESL* (*Left*) or $P_{A1/lacO-1}$-*dnaKJ* (*Right*) on the chromosome were grown at 30°C in LB medium containing, respectively, 0.2% arabinose or 5 μM IPTG to maintain normal chaperone levels. (*A–C*) GroEL/S was overexpressed from pGro11 by addition of 25 ng/ml anhydrotetracycline. (*D–F*) DnaK/J was overexpressed by addition of 1 mM IPTG. ●, control cultures; ▲, chaperone overexpression cultures. Arrows, time of induction. Western blots taken at the last time point indicated an ≈5- to 8-fold increase in GroEL and ≈4- to 5-fold increase in DnaK.

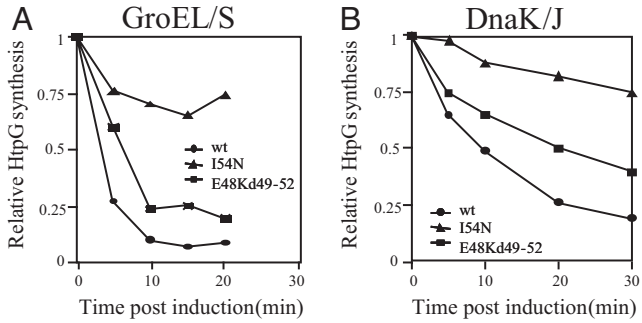

Fig. 3. Effect of chaperone overexpression on HtpG synthesis in the chromosomal σ^{32} mutants. WT and mutant strains with a chaperone expression plasmid (pGro11 or pKJE8) were grown in M9 medium containing all amino acids except methionine and cysteine at 30°C. At time 0, GroEL/S was induced by the addition of 40 ng/ml anhydrotetracycline (*A*), or DnaK/J/GrpE was induced by addition of 0.2% L-arabinose (*B*). At the times indicated, samples were pulse-labeled for 1 min with [^{35}S]Met/Cys, chased with unlabeled methionine for 1 min, and immunoprecipitated to determine HtpG synthesis.

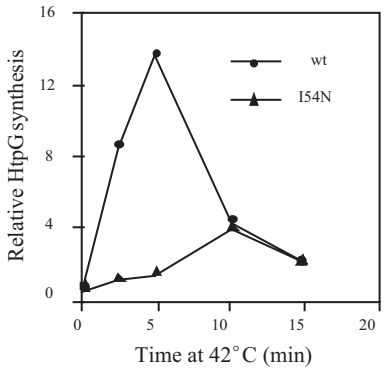

Fig. 4. Heat shock response of WT and I54N mutant. Cells were grown to log phase in M9 medium at 30°C and shifted to 42°C. Samples were taken at the times indicated to determine HtpG synthesis by immunoprecipitation as described in Fig. 3.

classical heat shock response is explained by the fact that the σ^{32} level in the mutant strains decreased upon heat shock rather than transiently increasing (data not shown). Enhanced degradation at 42°C is not a reflection of complete protein unfolding because the specific activity of the mutant proteins at 42°C (1.0 for I54N and 1.4 for E48KΔ49–52) was similar to that at 30°C (0.5–1.0) (data not shown).

Analysis of I54N σ^{32} *in vitro*. Altered binding of I54N σ^{32} to either chaperones or to RNA polymerase could explain why I54N is resistant to chaperone-mediated feedback inhibition *in vivo*. We quantified binding to GroEL and DnaK by using fluorescence anisotropy and to DnaJ by using surface plasmon resonance (see *Materials and Methods*). Surprisingly, I54N σ^{32} bound as well as WT σ^{32} to DnaK and GroEL and bound only 2-fold less tightly to DnaJ than WT σ^{32} (Table 3). The measurements for WT σ^{32} reported here are consistent with previously reported values (16, 18). Thus, decreased binding to chaperones cannot explain the observed feedback resistance. Because chaperones are believed to compete with RNA polymerase for binding to σ^{32}, another explanation for feedback resistance is that I54N σ^{32} binds more tightly to RNA polymerase than WT. We used fluorescence anisotropy to measure binding of mutant and WT σ^{32} to RNA polymerase. Our determined value for binding of WT σ^{32} is consistent with the previously reported value (25); I54N exhibited a slightly lower affinity than WT for RNA polymerase (Table 3). Thus, resistance to chaperone-mediated inhibition cannot be explained by increased affinity for RNA polymerase.

We examined the transcriptional capacity of I54N σ^{32} *in vitro* by using a multiround transcription assay conducted at 30°C. Titration of both WT and mutant σ^{32} (His-tagged and untagged) with a constant level of RNA polymerase core revealed that both proteins have similar transcriptional activity *in vitro* (data not shown). We then tested chaperone-mediated inhibition of σ^{32}-dependent transcription. Surprisingly, addition of either DnaK/J/GrpE or GroEL/S to the transcription reaction inhibited both WT and mutant σ^{32} to a similar extent (Table 4). Addition of either DnaK/J or GroEL alone to the reaction also inhibited mutant and WT proteins similarly (data not shown). Finally, a similar extent of inhibition of WT and I54N σ^{32} was observed with a single-round transcription protocol or when the reaction was carried out at 37°C or 42°C rather than 30°C (data not shown).

Table 3. Binding of σ^{32} to its relevant partners *in vitro*

	Dissociation constants, K_d			
σ^{32}	GroEL, μM	DnaK, μM	DnaJ, nM	RNA polymerase, nM
WT	2.72 ± 0.22	5.70 ± 2.39	39.6 ± 4.10	5.38 ± 1.41
I54N	3.83 ± 0.66	4.38 ± 1.51	86.6 ± 21.7	13.64 ± 4.00

The affinity of σ^{32} for its binding partners was determined by using fluorescence anisotropy (GroEL, DnaK, and RNA polymerase) or surface plasmon resonance (DnaJ) using purified proteins. Averages from four measurements ± SD are presented.

Table 4. Chaperone-mediated inhibition of σ^{32}-dependent transcription *in vitro*

	Relative activity		
σ^{32}	Control	+GroEL/S	+DnaK/J/GrpE
WT	1.0	0.3 ± 0.02	0.53 ± 0.06
I54N	1.0	0.28 ± 0.13	0.55 ± 0.07

σ^{32}-Dependent transcription of *htpG* was determined essentially as described previously (16) using purified His-tagged σ^{32}, core RNA polymerase, template DNA, and chaperone proteins as indicated. Multiround transcription reactions were run at 30°C, and ^{32}P-labeled *htpG* transcript was analyzed by a PhosphorImager scanning system. Averages from four measurements with standard deviation are presented.

Discussion

In the present work, we report the characterization of σ^{32} mutants selected to be resistant to chaperone-mediated feedback inhibition. Our work casts doubt on several accepted features of σ^{32} regulation. First, chaperone-mediated sequestration of σ^{32} from RNA polymerase was believed to underlie inactivation. However, the present results indicate that the only mutants thus far defective in this process affect a step not consistent with this simple model. Second, chaperone-mediated inactivation and regulated degradation of σ^{32} were believed to be linked only by a requirement for binding chaperones. However, the present results suggest that the two pathways are functionally interconnected in an additional manner. Finally, these mutants do not recapitulate their inactivation defective phenotype *in vitro*.

The prevalent model for chaperone-mediated inactivation is that chaperone binding to σ^{32} sequesters it from RNA polymerase (6, 7). Therefore, we expected our inactivation-defective mutants to be altered either in interaction with chaperones or RNA polymerase. Our demonstration that I54N σ^{32} is almost completely defective in inactivation without appreciably altering binding to chaperones or RNA polymerase argues against the sequestration model in its simplest form. Instead, these results argue that there is an unanticipated step required for chaperone-mediated inactivation. Because I54N is defective in feedback regulation mediated by both GroEL/S and DnaK/J without significantly affecting chaperone binding, it most probably affects a step downstream of chaperone binding.

Surprisingly, all of our σ^{32} mutants exhibited a dual phenotype: although they were selected for defects in inactivation, they also exhibited a defect in degradation control. Importantly, a screen that selected σ^{32} mutants based solely on their increased stability identified mutants in the same or nearby residues as our selection (26). These residues could be essential for both inactivation and degradation because (*i*) they have two different functions, one necessary for activity control and the other necessary for degradation control; or (*ii*) they have a single function, necessary for both processes. Although we cannot eliminate the first possibility, our data are most consistent with the proposition that these residues define a single function. First, mutant defects are of similar severity for activity and degrada-

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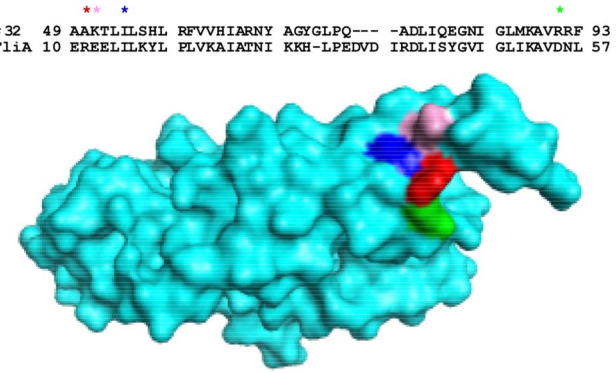

Fig. 5. Sequence alignment of σ^{32} and FliA illustrates that the mutations define a cluster of surface-exposed residues. Regions 2.1 and 2.2 of Ec σ^{32} and Aa FliA were aligned (ClustalW; DNASTAR, Madison, WI). Residues corresponding to σ^{32} mutations were identified on the FliA structure (27) and mutated to the naturally occurring residues of σ^{32} (asterisks) in PyMOL. Shown are Arg^{11} mutated to Ala (Ala^{50} in σ^{32}; red), Glu^{12} mutated to Lys (Lys^{51} in σ^{32}; pink), Ile^{15} (Ile^{54} in σ^{32}; blue), and Asp^{55} mutated to Arg (Arg^{91} in σ^{32}; green). These residues form a surface-exposed patch.

tion control. Thus, E48KΔ49–52 is moderately defective, and I54N is severely defective in both control processes. Such a correlation is consistent with the expectations of a model in which the mutations disrupt a single function. Second, homology modeling with FliA (27) (like σ^{32}, FliA is a group 3 σ) reveals that the mutants define a cluster of surface-exposed residues centered in σ region 2.1 (Ala^{50}, Lys^{51}, Ile^{54}, and Arg^{91}), suggestive of a patch with a single function (Fig. 5). We note that alterations in adjacent residues not on the same surface (Glu^{48}, Ala^{49}, Thr^{52} and Leu^{53}) have no phenotype (22), reinforcing the idea that these mutations define a small patch with a crucial function in both degradation and inactivation. Chaperone binding is the only currently known commonality between degradation and inactivation, and these mutations have little effect on that process (Table 3). We suggest that the mutants identify a regulatory step that operates downstream of chaperone binding and is required for both inactivation and degradation of σ^{32}.

It was surprising to find that the *in vivo* and *in vitro* phenotypes of I54N σ^{32} were quite discrepant: I54N is almost completely resistant to chaperone-mediated inactivation *in vivo* (Figs. 2 and 3) but indistinguishable from WT σ^{32} in both GroEL/S and DnaK/J chaperone-mediated inactivation *in vitro* (Table 4). It is conceivable but unlikely that the *in vivo* phenotype is an indirect consequence of the mutational alteration in the protein. Both the strength of the *in vivo* phenotypes and the fact that selections for both activity and degradation control identify this tight cluster of residues argue against this possibility. Alternatively, the *in vitro* system may only partly recapitulate inactivation *in vivo*. For example, the mutant σ may lack a conformational change that is required for inactivation *in vivo* but is not necessary for inactivation under *in vitro* conditions. Alternatively, the mutant may be defective in interacting with an additional factor that is bypassed in the current *in vitro* system. In this regard, it is interesting that degradation of σ^{32} by FtsH protease *in vitro* does not mimic degradation *in vivo*, possibly because of a missing factor (21). It is unclear whether the missing factor is the same as the one postulated to be missing from our *in vitro* inactivation system or yet an additional factor. Resolving this discrepancy is crucial for understanding how the chaperone-mediated control adjusts the activity of σ^{32} to a level appropriate to maintain protein-folding homeostasis in the cell.

Finally, it is interesting to note that σ region 2.1 is commonly used for controlling σ activity. In addition to the region 2.1 cluster identified here, *E. coli* σ^{E} uses a residue comparable with Ile^{54} to contact its anti-σ factor RseA (28). Likewise, *Rhodobacter sphaeroides* σ^{E} interacts with its zinc-containing anti-σ (ChrR) by using residues in region 2.1 (29). Indeed, structural homology modeling suggests that many σ–anti-σ pairs may interact in a similar manner (30). Region 2.1 of σ has many surface-exposed residues and does not itself contact either core RNA polymerase or DNA. This region may be free to evolve regulatory capacities so that the activity of σ can be controlled appropriately.

Materials and Methods

Strains. All strains used were derivatives of *E. coli* K-12 strain MG1655. σ^{32} mutants originally isolated on pRB11 or slightly different *rpoH* plasmids were placed under the control of an IPTG-inducible promoter in strain CAG48238 carrying Δ*lacX74* and prophage λJW2 (P_{htpG}-*lacZ*) (31) and its Δ*rpoH* derivative.

Chaperone Overexpression Studies. For chaperone overexpression experiments, chromosomal P_{ara}-*groEL/S* (32) or $P_{A1/lacO-1}$-*dnaK/J-lacI*^{q} (15) was transduced into CAG48238 to obtain CAG48239 or CAG48275, respectively. For Table 1, derivatives of CAG48239 carrying a plasmid-encoded *rpoH* mutations and a pACYC184-based plasmid pGro11 (P_{tet}-*groESL*) (33) were grown in LB medium containing L-arabinose (0.2%) at 30°C to obtain the WT level of GroEL/S; *rpoH* was induced by the addition of 1 mM IPTG, and GroEL/S was overexpressed by the addition of 25 ng/ml anhydrotetracycline. For DnaK/J overexpression, derivatives of CAG48275 carrying plasmid-encoded *rpoH* mutations were grown in 5 μM IPTG at 30°C to achieve the WT level of DnaK/J from the chromosomal $P_{A1/lacO-1}$-*dnaK/J-lacI*^{q} locus. DnaK/J and σ^{32} were simultaneously overexpressed by the addition of 1 mM IPTG; alternatively, DnaK/J was overexpressed from another compatible plasmid pKJE8 (P_{ara}-*dnaK/J/grpE*) by the addition of L-arabinose (33). The differential rate of β-galactosidase synthesis was determined over a 2-h time course immediately after chaperone induction.

Media and Antibiotics. LB medium and M9 medium were prepared as described (34) except that M9 medium was supplemented with 0.2% glucose/1 mM $MgSO_4$/all amino acids (40 μg/ml) except methionine and cysteine/vitamin mixture. When required, antibiotics were added to the medium as follows: 100 μg/ml ampicillin, 30 μg/ml kanamycin, and 20 μg/ml chloramphenicol.

λ*red*-Mediated Recombination. Synthetic deoxyoligonucleotides (30–70 bp) containing a specific *rpoH* mutation were electroporated into cells carrying pKD46 (λ*red*) essentially as described (35, 36), and dark blue colonies were screened on LB agar medium containing X-Gal. The *rpoH* mutant candidates were confirmed by β-galactosidase assay, linkage to a nearby tetracycline resistance (Tn*10*) marker, and by sequencing. The chromosomal *rpoH* mutations thus obtained were transduced into CAG48238 by selecting for the nearby *tet* marker.

β-Galactosidase Assay. Overnight cultures (LB medium) were diluted 200- to 500-fold and grown to exponential phase (OD_{600} = 0.05–0.3). Samples were taken at intervals starting at OD_{600} = 0.05, and σ^{32} activity was monitored by measuring β-galactosidase activity expressed from the σ^{32}-dependent *htpG* promoter by the standard procedure (37).

Pulse Labeling and Immunoprecipitation. Cells were grown in supplemented M9 medium and pulse-labeled with [^{35}S]methionine for σ^{32} synthesis, or with EasyTag EXPRESS^{35}S protein labeling mix (PerkinElmer, Waltham, MA) for HtpG synthesis and immunoprecipitated as described previously (16).

Immunoblotting. Cells were treated with cold 5% tricholoroacetic acid, kept on ice for 30 min, precipitated by centrifugation, washed in acetone, and resuspended in Laemmli buffer. Serial dilutions of WT and mutant samples were loaded onto a polyacrylamide gel, and the proteins were transferred to nitrocellulose membranes. The blots were first probed with rabbit primary antibodies and then with anti-rabbit horseradish peroxidase-conjugated secondary antibody. Immunoblots were developed with chemiluminescence and exposed to film. Fold increase (level experiments) was estimated by comparison with a dilution series of samples from the WT. Fold decrease after addition of chloramphenicol (stability experiments) was determined by direct scanning and analyzing bands with ImageJ software (National Institutes of Health, Bethesda, MD).

Protein Purification. The following proteins were purified essentially as described: RNA polymerase (38), His-tagged σ^{32} (18), GroEL, GroES (39), DnaK, DnaJ, and GrpE (40). Misfolded proteins were removed from chaperone preparations as described (16). σ^{32} (untagged) was purified by using pQE30-Xa vector, as suggested by the manufacturer (Qiagen, Valencia, CA). The protein was affinity purified as a His-tagged protein and treated with protease Xa to cleave off the His tag, leaving the intact σ^{32} with no additional amino acid attached. Xa was removed by using Xa removal resin. Fluorescently labeled σ^{32} was prepared by purifying His-tagged L118C σ^{32} from a *slyD* mutant strain (41). The L118C mutation allows specific labeling at 118 because σ^{32} does not have any endogenous cysteines and SlyD is a contaminant labeled by the fluorophore. Alexa Fluor 488 C5-maleimide (Molecular Probes, Eugene, OR) was used to label σ^{32} according to the manufacturer's instructions.

Fluorescence Anisotropy. Fluorescence data were collected on an ISS K2 multifrequency phase fluorometer running on Vinci software. For GroEL binding, 100 nM labeled σ^{32} was incubated in 50 mM Tris, pH 7.5/100 mM KCl/0.002% Tween 20/0.2 mM β-mercaptoethanol/10% (vol/vol) glycerol. For RNA polymerase binding, 10 nM labeled σ^{32} was incubated in 50 mM Tris, pH 7.5/500 mM KCl/5% glycerol/0.05% Nonidet P-40/0.001% Tween 20/0.01 mM β-mercaptoethanol. For DnaK binding, 100 nM N-terminal FITC-labeled peptide QRKLFFNLRKTKQ (Tufts University Core Facility, Boston MA), previously shown to bind to DnaK, was incubated in 50 mM Tris, pH 7.5/100 mM KCl/10% (vol/vol) glycerol (42). In each case, competition experiments were performed by using either unlabeled WT σ^{32} or unlabeled I54N σ^{32}. The equilibrium binding constant was then calculated (43).

Surface Plasmon Resonance. Data were collected by using a Biacore 1000. Untagged WT or I54N σ^{32} was flowed over an nitrilotriacetic acid chip containing immobilized His-tagged DnaJ in 10 mM Hepes, pH 8.3/500 mM NaCl/350 mM EDTA/0.05% Tween 20. Each binding experiment used duplicate samples of at least four different concentrations of σ^{32} spanning at least 27× change in concentration. Data were analyzed by using a 1:1 binding with drifting baseline model and BiaEvaluation software.

***In Vitro* Transcription.** Multiround *in vitro* transcription was carried out as reported previously (16), and single-round transcription was done as described (44).

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