C-terminal domain of the RNA chaperone Hfq drives sRNA competition and release of target RNA

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The bacterial Sm protein and RNA chaperone Hfq stabilizes small noncoding RNAs (sRNAs) and facilitates their annealing to mRNA targets involved in stress tolerance and virulence. Although an arginine patch on the Sm core is needed for Hfq's RNA chaperone activity, the function of Hfq's intrinsically disordered C-terminal domain (CTD) has remained unclear. Here, we use stopped flow spectroscopy to show that the CTD of *Escherichia coli* Hfq is not needed to accelerate RNA base pairing but is required for the release of dsRNA. The Hfq CTD also mediates competition between sRNAs, offering a kinetic advantage to sRNAs that contact both the proximal and distal faces of the Hfq hexamer. The change in sRNA hierarchy caused by deletion of the Hfq CTD in *E. coli* alters the sRNA accumulation and the kinetics of sRNA regulation in vivo. We propose that the Hfq CTD displaces sRNAs and annealed sRNA-mRNA complexes from the Sm core, enabling Hfq to chaperone sRNA-mRNA interactions and rapidly cycle between competing targets in the cell.

small noncoding RNA | RNA chaperone | intrinsically disordered protein | posttranscriptional regulation | ChiX

A member of the Sm protein family, Hfq was first identified as a host factor for phage Q beta. Hfq is found in at least 50% of sequenced bacterial genomes (1) and, in many bacteria, contributes to posttranscriptional regulation by small noncoding RNAs (sRNAs). Deletion of Hfq leads to pleiotropic effects, such as altered cellular morphology, slow growth, maladaptation to stress, and avirulence (2–5).

Escherichia coli Hfq comprises an Sm domain (amino acids 7–66) that assembles into a stable hexameric ring and an intrinsically disordered C-terminal domain (CTD) that projects from the rim of the hexamer (6–10). The Sm ring binds to both sRNAs and target mRNAs, stabilizing the sRNAs against turnover (11–13) and facilitating base pairing with complementary sequences in the mRNA (7, 14, 15). The conserved “proximal” face of the Hfq hexamer interacts with uridines at the sRNA 3' end (9, 16, 17), whereas the “distal” face of Hfq binds AAN triplet repeats (9, 16, 17) often found in the 5' UTRs of target mRNAs. These sequence-specific interactions recruit sRNAs and mRNAs to Hfq, allowing arginine-rich patches along the rim of Hfq to catalyze base pairing between complementary strands (18).

Although the functional importance of the Sm domain is established, the function of the disordered CTD has been unclear. The Hfq CTD varies greatly in length and sequence composition between bacterial families (1, 19), ranging from 7-residue stubs in *Bacillaceae* (20) to 100-residue tails in *Moraxellaceae* (21, 22) (Fig. S1). Previous studies reached conflicting conclusions about the importance of the Hfq CTD for sRNA regulation. In early studies, C-terminal deletions of *hfq* had no obvious phenotype in *E. coli* (23, 24) and little effect on sRNA binding (23, 25). By contrast, later studies found that the CTD was required for in vitro annealing and proper regulation by sRNAs and normal binding to long RNAs, such as the *spoS* mRNA (19, 26, 27). Moreover, Hfq from *Pseudomonas aeruginosa* and *Clostridium difficile*, which have much shorter CTDs than *E. coli* Hfq, functionally replace *E. coli* Hfq for certain sRNA-mRNA pairs but not others (28, 29).

This variability suggested to us that the CTD either serves an autoregulatory function or modulates sRNA regulation in different bacteria (30). As a result, the apparent importance of the CTD may depend on the sRNA being studied. This possibility became more apparent with the recognition that *E. coli* sRNAs can be divided into two classes based on their interactions with Hfq (31). The common Class I sRNAs typically bind the proximal face and rim of Hfq (14, 32–34), whereas a smaller number of Class II sRNAs contact the distal face of Hfq via an AAN motif near the 5' end of the sRNA in addition to the proximal face (31, 35). The CTD is not essential for sRNA recognition, because truncated Hfq variants lacking all or part of the CTD bind sRNAs in vitro and in the cell (10, 23–26, 28, 36). However, partial ordering of the CTDs in a recent structure of Hfq bound to RydC sRNA suggested that the CTDs help recruit sRNAs to Hfq (36).

To understand how the disordered CTD modulates Hfq's chaperone function, we compared the RNA binding and annealing activities of full-length *E. coli* Hfq (Hfq102) with a truncated protein lacking the entire CTD (Hfq65). Stopped flow spectroscopy and fluorescence anisotropy results show that the CTD is not required for RNA annealing as previously thought (19, 26) but rather, is needed for dissociation of the dsRNA product. We further show that the CTD enables kinetic competition between sRNAs, which can actively displace each other from Hfq (37). Deleting the *hfq* CTD in *E. coli* disrupts the hierarchy of sRNA accumulation and alters the kinetics of sRNA-mRNA interactions. We propose that the CTDs modulate Hfq's chaperone activity by stimulating the cycling of sRNAs and sRNA-mRNA complexes on the Sm ring.

Significance

The RNA chaperone Hfq binds hundreds of small noncoding RNAs (sRNAs) and facilitates their interactions with mRNAs, regulating bacterial stress responses and virulence. Hfq is limiting in the cell and must release RNAs after they base pair. Most bacterial Hfqs contain an intrinsically disordered C-terminal domain (CTD), with unknown function. Time-resolved assays now show that CTDs are needed to displace base-paired RNA, recycling Hfq. The CTDs also enable kinetic competition between different sRNAs in *Escherichia coli*, allowing some sRNAs to bind Hfq and accumulate, whereas other sRNAs are degraded. We propose that the CTDs sweep sRNAs from the surface of Hfq. This displacement allows Hfq to search among potential RNA partners and establishes a hierarchy of sRNA regulation.

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amount of Hfq102 hexamer equaled the concentration of molecular beacon (50 nM). By contrast, the observed annealing rate increased up to 110 times in 250 nM Hfq65 hexamer (○ in Fig. 1*B*). Thus, at high protein concentrations, Hfq65 anneals 16-nt Target RNA much faster than WT Hfq102, perhaps because of stronger binding of the molecular beacon to Hfq65 (Table 1). Hfq65 was also more active than Hfq102 when the target RNA contained a 3' U₆ tail (Target-U6) that interacts with the inner proximal surface of Hfq (Fig. 1*B*, *Middle*). By contrast, Hfq102 and Hfq65 were equally active on an RNA containing a 3' A₁₈ tail that binds the distal face (Target-A18) (Fig. 1*B*, *Bottom*). Both proteins increased the annealing rate for Target-A18 85 times above the no Hfq background.

These results showed the CTDs are not required to accelerate RNA base pairing but rather, inhibit annealing of RNA substrates that only interact with the rim (16-nt Target) or that also bind the inner proximal face (Target-U6). The three- to eightfold higher maximum velocity and approximately sixfold greater K_m^{app} for Hfq65 suggest that the CTDs limit the numbers of rim sites in each hexamer that are accessible to the RNA. These effects are analogous to autoinhibition in protein kinases and other nucleic acid binding proteins (42), in which partially disordered peptides block substrate recognition. By contrast, Target-A18 seems to be immune to the inhibitory effects of the CTD, being an equally good substrate for full-length Hfq102 and core Hfq65. The robust activity of Target-A18 agreed with our previous observation that Hfq is most effective when its distal face is recruited via an (AAN) motif in the target RNA (41).

Hfq CTD Increases the Yield of RNA Duplex. We next asked whether the CTD shifts the equilibrium of the RNA annealing reaction by stabilizing either the single-stranded reactants or the double-stranded product. We measured the amount of base pairing in the presence of 0–400 nM Hfq (Fig. 1*C*) in a separate equilibrium titration (*SI Materials and Methods*). Hfq102 had little effect on the amount of duplex formed by Target and Target-U6 (● and ■ in Fig. 1*C*), consistent with release of the target-beacon duplex from Hfq (39). However, Hfq65 lowered the amount of duplex formed (○ and △ in Fig. 1*C*), consistent with its stronger affinity for the molecular beacon compared with Hfq102 (Table 1). The combination of faster annealing kinetics (Fig. 1*B*) and low yield of duplex (Fig. 1*C*) may be explained by an increased rate of strand separation by Hfq65 (faster back reaction) or by a greater number of accessible rim sites (faster first turnover) plus a failure to release the target-beacon duplex after base pairing is complete (low yield). As previously observed (39), Hfq102 and Hfq65 limited the extent of base pairing by Target-A18 (▲ in Fig. 1*C*), presumably because of strong interactions with the A₁₈ tail.

Hfq CTD Is Required for Release of Annealed RNA Duplex. Because the perturbations to the annealing kinetics suggested that the CTDs participate in RNA binding or dsRNA release, we used stopped flow FRET to compare the kinetics of each step of the reaction by full-length Hfq102 or Hfq65 core (Fig. 2*A–C*). To measure interactions between the RNA and Hfq by FRET, we labeled the center of the Hfq hexamer by conjugating a single cysteine (V27C) with Cy3 (*Materials and Methods*). This labeling site avoids known RNA binding sites and is roughly equidistant from the arginine patch of each subunit. Association of D16-FAM with the proximal side of Cy3-labeled Hfq resulted in energy transfer from the FAM donor to the Cy3 acceptor, which was monitored over time (Fig. 2*A*). D16-FAM RNA bound Hfq102-Cy3 and Hfq65-Cy3 with similar rate constants, corresponding to $k_{on} \sim 8 \cdot 10^8 \text{ M}^{-1} \text{ s}^{-1}$.

Although the CTDs made no difference to the rate of RNA binding, we discovered that the CTD is essential for rapid release of the dsRNA product, which normally occurs after the two strands

Fig. 2. Hfq CTD is required for dsRNA release. (*A–C*) Stopped flow FRET of Hfq and RNA binding kinetics. Each plot shows the average of five replicates. (*A*) Hfq and D16 RNA binding kinetics. The Cy3 emission intensity at a given time after mixing (*F*) was adjusted to the starting fluorescence (*F*₀) and scaled by the relative labeling efficiency of each protein (*a*) (*SI Materials and Methods*); $k_{on} \sim 8 \cdot 10^8 \text{ M}^{-1} \text{ s}^{-1}$, about 10 times faster than previously observed with a different preparation of Hfq (39). Blue, (Hfq102:V27C)₆-Cy3; red, (Hfq65:V27C)₆-Cy3. (*B*) Release of dsRNA after addition of complementary R16 RNA to [(Hfq)₆-Cy3-D16-FAM] complex. We observe release from Hfq102 but not Hfq65. The rise in the fluorescence of the Hfq65-Cy3-D16-FAM complex may reflect a conformational change that brings the FAM donor closer to the Cy3 acceptor. (*C*) D16-FAM binds an [(Hfq)₆-Cy3-R16] complex, forming a high-FRET ternary complex, followed by release of D16-R16 duplex from Hfq102 but not Hfq65. (*D*) Fluorescence anisotropy assay for RNA binding and release. D16-FAM RNA (50 nM) was allowed to bind unlabeled 50 nM Hfq₆ (high anisotropy). The complex was challenged with 50 nM R16 RNA, which anneals with D16-FAM. The D16-R16 duplex was released from Hfq102 but not Hfq65 (medium-low anisotropy). The remaining RNA was displaced from Hfq by 400 nM ssDNA. The anisotropy of D16-FAM was recorded for 200 s after each addition to the cuvette (details are provided in *SI Materials and Methods*). Black, Hfq storage buffer; blue, Hfq102₆; red, Hfq65₆.

base pair (39) (Fig. 1*A*). When we challenged the Hfq102-Cy3-D16-FAM complex with complementary R16 RNA, we observed a rapid decrease in FRET as the D16-FAM-R16 duplex dissociated from Hfq102-Cy3 as expected (Fig. 2*B*). By contrast, when a complex of Hfq65-Cy3-D16-FAM was challenged with R16, the fluorescence increased slightly rather than decreased, suggesting that D16-FAM RNA remained bound to Hfq65. Because Hfq65 is an active chaperone and the increase in fluorescence occurs on the same timescale as duplex release from Hfq102-Cy3, it likely depends on annealing of the two RNAs (18, 39).

We observed a similar difference between Hfq102 and Hfq65 in a second experiment designed to simultaneously visualize substrate binding and product release (Fig. 2*C*). When a complex of Hfq102-Cy3-R16 was challenged with D16-FAM, FRET first increased as Hfq formed a ternary complex and then dropped as dsRNA was released (Fig. 2*C*). Conversely, when Hfq65-Cy3-R16 was challenged with D16-FAM, the same initial increase in FRET was followed by an additional slow increase in FRET, again suggesting that Hfq65-Cy3 cannot release its dsRNA product.

We next used steady-state anisotropy to verify that Hfq65 forms a ternary complex and that the inability to release dsRNA was not an artifact of the V27C mutation and Cy3 label. Although the anisotropy assay has poor time resolution, it tracks physical changes in the complexes as D16-FAM RNA binds Hfq and R16 cRNA (18). Hfq102 increased the anisotropy of D16-FAM as expected (blue in Fig. 2D). When the binary Hfq102-D16-FAM complex was challenged with R16 cRNA, the majority of D16-FAM was released in the form of D16-FAM-R16 in agreement with the stopped flow fluorescence results, whereas a minority of D16-FAM remained in a ternary complex. Addition of excess ssDNA chased the remaining D16-FAM-R16 duplex from Hfq102, resulting in very similar anisotropy as RNA duplex formed without Hfq (black in Fig. 2D).

Hfq65 also increased the anisotropy of D16-FAM (red in Fig. 2D), consistent with its similar affinity for RNA (Table 1). The FAM fluorescence was less polarized, owing to the smaller hydrodynamic drag of the Hfq core. In agreement with the stopped flow FRET results (Fig. 2B and C), however, addition of complementary R16 RNA did not cause a drop in anisotropy. Rather, we observed a small but measurable increase in anisotropy that may be caused by the increased restraint of FAM rotation in an Hfq-bound RNA duplex. Thus, all of the fluorescence assays showed that the Hfq65 core binds and anneals short RNAs but does not release the dsRNA product.

Hfq CTD Is Necessary for Discrimination Between Class I and Class II sRNAs. Because removing the CTD prevents Hfq from releasing dsRNAs (Fig. 2) and improves binding of structured RNAs to the rim (Table 1), we next asked whether the CTD influences competition between different sRNAs for Hfq. The competition of sRNAs for Hfq has been attributed to a combination of sRNA secondary structure and sequence (12, 35, 43), and the ability of one sRNA to outcompete another does not necessarily correspond to their relative binding affinities (35, 44), consistent with active displacement of bound sRNAs by other sRNAs in solution (37).

We investigated whether the Hfq CTD affects sRNA competition against ChiX, a Class II sRNA that interacts strongly with Hfq (Fig. 3A–C). The sRNA competitors included DsrA, a Class I sRNA that is a poor competitor (12, 35, 44), RyhB (Class I), RprA, which has an AAN sequence like Class II sRNAs but exhibits a mixed Class I/Class II phenotype in *E. coli* (31), and ChiX (Class II). The fraction of 32 P-ChiX bound to either Hfq102 (Fig. 3A) or Hfq65 (Fig. 3B) was monitored by native gel mobility shift at increasing concentrations of unlabeled DsrA, ChiX, RyhB, or RprA. As previously reported (35), 32 P-ChiX was efficiently displaced by excess unlabeled ChiX RNA but not by unlabeled DsrA. RyhB and RprA displayed an intermediate degree of competition against 32 P-ChiX (solid lines in Fig. 3C).

In contrast to the results with full-length Hfq102, all four sRNAs were able to displace 32 P-ChiX from Hfq65 over the concentration range 10–100 nM (dashed lines in Fig. 3C). We observed comparable differences between Hfq102 and Hfq65 when the same panel of unlabeled sRNAs was competed against 32 P-DsrA, the weakest member of the group (Fig. 3D–F). ChiX displaced DsrA about an order of magnitude better than RyhB from Hfq102. By contrast, these four sRNAs competed very similarly for Hfq65 (Fig. 3F).

Overall, the competition for Hfq65 was close to that predicted by the respective K_d values of the sRNAs tested (Table 1). By contrast, competition for binding to Hfq102 varied far more than predicted by the thermodynamic stabilities of the individual sRNA-Hfq102 complexes as seen previously (35). Although full-length Hfq102 prioritizes Class II sRNAs, Hfq65 binds most sRNAs equally. Because Hfq is limiting in the cell (46), this redistribution of Hfq could disrupt sRNA-mRNA regulatory networks or affect the accumulation of sRNAs when the CTD is absent (31).

Fig. 3. Hfq CTD establishes a hierarchy of sRNA competition. Competitive binding of 32 P-labeled ChiX or DsrA sRNA to Hfq was measured by native gel mobility shift. Unlabeled competitor sRNA is indicated along the top. Lane –, 32 P-labeled sRNA only; lane 0, +Hfq but no competitor. (A and B) 32 P-ChiX with (A) Hfq102 or (B) Hfq65. (C) The fraction of bound 32 P-ChiX vs. competitor [sRNA] was fit to Eq. 1 (Materials and Methods). Typical measurement error is 5%. Black symbols, Hfq102; white symbols, Hfq65. (D and E) 32 P-DsrA with (D) Hfq102 or (E) Hfq65 as in A and B. Slower bands likely represent ternary complexes of 32 P-DsrA, Hfq, and unlabeled sRNA (45) and were included in the bound fraction. (F) Fraction 32 P-DsrA bound as in C.

Hfq CTD Stabilizes Class II sRNAs in Vivo. We asked whether the loss of sRNA class discrimination observed in vitro (Fig. 3) is reflected by changes in the stability and behavior of Class I or Class II sRNAs in vivo. The *hfq65* mutation was introduced into the bacterial chromosome in place of the WT *hfq* gene (Materials and Methods). The level of Hfq65 was found to be 81% of that for Hfq102 (Fig. S6). These isogenic strains were then used to examine how loss of the Hfq C terminus affects the stability and behavior of sRNAs in vivo.

We first examined the effect of the Hfq CTD on sRNA degradation. sRNAs were overexpressed from plasmids under the control of a *lac* promoter for 15 min (Fig. 4A–E), sufficient time for a given sRNA to accumulate and load onto Hfq. In the first experiment (Fig. 4A), cells were transferred to fresh media lacking IPTG (isopropyl- β -D-1-thiogalactopyranoside) to halt sRNA transcription, whereas chromosomally derived sRNAs and mRNAs continued to be transcribed. The decrease in sRNA levels in this “washout” experiment (black symbols in Fig. 4C–E) can be attributed to displacement from Hfq by endogenous sRNAs, leading to degradation by cellular ribonucleases (31, 47). It can also result from base pairing with target mRNA, which leads to turnover of certain sRNA-mRNA pairs (48).

In general, Class I sRNAs are rapidly turned over in washout experiments, whereas Class II sRNAs vary, with ChiX being very stable and MgrR being relatively unstable (31). As expected, the Class I sRNA RyhB decreased rapidly in strains expressing either full-length Hfq102 (■ in Fig. 4C) or truncated Hfq65 (● in Fig. 4C). By contrast, both Class II sRNAs MgrR and ChiX (■ in Fig. 4D and E) were degraded more quickly in *E. coli* expressing Hfq65 (● in Fig. 4D and E). This result suggested that Class II sRNAs like ChiX are less able to compete against chromosomally encoded sRNAs for binding to Hfq when the CTD is missing.

Fig. 4. Hfq CTD increases Class II sRNA stability in vivo. (A and B) The degradation rate of overexpressed sRNAs analyzed by Northern blot. WT and an isogenic *hfq65* mutant deleted for the sRNA being examined and harboring a plasmid expressing the sRNA of interest under the control of *P_{lac}* were grown at 37 °C in the presence of IPTG. (A) Transcription of the sRNA expressed from a plasmid was stopped by washing out IPTG from cells. (B) sRNA turnover after inhibition of global transcription by rifampicin. In both cases, samples were taken from, at minimum, 0–20 min and normalized to an SsrA loading control (Fig. S7A). (C–E) Quantitation of sRNA lifetimes. All experiments were done in triplicate; error bars show the geometric SD. Black symbols, washout; white symbols, rifampicin. (F) Accumulation of endogenous sRNAs in cells expressing Hfq65 normalized to sRNA levels measured in WT Hfq102 cells. Error bars represent the SD of at least two measurements. RNA extracts were prepared from WT (DJS2690) and an isogenic *hfq65* mutant (KK01) grown in LB-Lennox medium at 37 °C to early stationary phase (OD₆₀₀ ~ 1.0). Total RNA (3 µg) isolated from each sample was subjected to Northern analysis using biotinylated oligonucleotides specific to the sRNA. Gray bars, Class I sRNAs; hatched bars, Class II sRNAs.

The intrinsic stabilities of the overexpressed sRNAs were compared in a second experiment by measuring the sRNA lifetime after transcription was globally inhibited by rifampicin (Fig. 4B). If sRNAs are not bound to Hfq, they are rapidly degraded; when bound to Hfq, they generally remain stable after rifampicin treatment (31). We observed much less variation in sRNA half-lives in cells expressing Hfq102 vs. Hfq65 (□ and ○, respectively, in Fig. 4C–E) than in the washout experiment. This result suggests that the CTD is not important for intrinsic sRNA stability, which is in agreement with the small effect of the CTD deletion on in vitro binding affinities.

The effect of the CTD on sRNA competition was also assessed from the steady-state accumulation of endogenously expressed *E. coli* sRNAs, which reflects both intrinsic stability and the rate of degradation after pairing. The amounts of Class I and Class II sRNAs (gray and hatched bars, respectively, in Fig. 4F) were measured by Northern blotting. The sRNA levels in cells expressing Hfq65 were normalized to sRNA levels in cells expressing WT Hfq102. In agreement with the in vitro sRNA competition and the in vivo sRNA turnover results, we found that Class I sRNA accumulation was largely unaffected for three of four sRNAs tested. By

contrast, four of four Class II sRNAs tested were twofold less abundant in cells expressing the truncated Hfq65. To further understand why one Class I sRNA, OmrB, showed lower accumulation in the Hfq65 strain (Fig. 4F), measurements of intrinsic stability (after rifampicin treatment) were done with both OmrB and the unaffected Class I Spot 42 sRNA (Fig. S7B and C). In this test, Spot 42 intrinsic stability was unchanged by removing the Hfq CTD, but OmrB intrinsic stability was decreased, consistent with decreased accumulation of this sRNA. Thus, although most sRNAs are unaffected in their binding by loss of the Hfq CTD, OmrB, for reasons that are currently unknown, is more sensitive to displacement from Hfq65, even in the absence of pairing.

The more rapid washout and lower accumulation of ChiX in cells lacking the Hfq CTD suggested that ChiX might be less effective in regulating a target, at least when ChiX was limiting. We tested the ability of chromosomally encoded ChiX to regulate a translational fusion to *chiP*, a target of ChiX (Fig. S8). As anticipated, *chiP* regulation was compromised in the strain expressing Hfq65 compared with the strain expressing Hfq102 (Fig. S8A). However, when ChiX was overexpressed, this difference was no longer seen, presumably because higher levels of ChiX overcame its increased turnover in cells expressing Hfq65 (Fig. S8B). This result supports our in vitro data, suggesting that Hfq65 is still an active RNA chaperone and that its defect in dsRNA release and sRNA discrimination is not debilitating when sRNA competition is less severe.

Discussion

Many nucleic acid binding proteins contain flexible extensions and loops thought to contribute to their chaperone activity, but these domains have been little studied owing to their weak sequence conservation and conformational disorder (49). Here, we show that the intrinsically disordered CTD of Hfq is not needed to stimulate RNA base pairing but rather, contributes to Hfq's chaperone activity by accelerating the release of dsRNA. By altering the dynamics of Hfq–RNA interactions, the CTDs also inhibit RNA binding to the arginine patch on the rim and increase kinetic competition between sRNAs (Fig. 3). We find that the CTDs are needed to maintain normal sRNA levels in the cell and tune the efficiency of specific sRNA regulatory pathways (Fig. 4 and Fig. S8).

These observations can be explained by a model in which the Hfq CTDs sweep RNAs from the rim and proximal surface of the Sm ring, shortening the lifetimes of complexes that rely on those interaction surfaces (Fig. 5). Displacement of RNA by the mobile CTDs provides a physical basis for sRNA cycling on the proximal face (37) and the release of annealed sRNA–mRNA complexes (15), which may be aided by binding of a second sRNA to the proximal face (gold in Fig. 5A). Although the mechanism of RNA release is not known, negatively charged patches on the CTDs (Fig. S1) may electrostatically repel RNAs bound to the rim. Alternatively, the CTDs could directly compete for binding with RNAs to the rim of Hfq, similar to the autoinhibitory effect of the CTDs from human T-cell leukemia virus type 1 nucleocapsid (HTLV-1 NC) (50) and ssDNA binding protein (51).

By contrast, we propose that RNAs bound to the distal face of Hfq (green in Fig. 5A) are immune to this sweeping action and able to remain in place over multiple cycles of sRNA binding and release. Deletion of the CTD has no effect on the affinity of A18 RNA for Hfq (Table 1) or the annealing of target RNA containing an A18 tail (Fig. 1B), which is a good substrate for both WT Hfq102 and Hfq65. This model is consistent with our earlier finding that the distal face of Hfq remains bound to an upstream AAN motif in *rpoS* mRNA after it base pairs with an sRNA (53, 54). It also helps explain why ternary complexes formed by MicC, *ompC* mRNA, and Hfq resist competition from other sRNAs (37): *ompC* mRNA also contains a strong AAN-rich Hfq binding site

Fig. 5. Model for RNA cycling and release by the Hfq CTD. (A) Side view of the Hfq core (cyan), proximal side up, with the CTDs (violet) extruding from the rim near the arginine patch (blue). During annealing, the intrinsically disordered Hfq CTDs promote cycling of sRNAs on the proximal face, while inhibiting indiscriminate RNA binding to the rim of Hfq. When a cycling sRNA engages a cRNA bound to the distal face of Hfq, arginine patches on the rim facilitate initiation and zippering of the antisense base pairs (18, 52). The CTDs stimulate release of the double helix from the rim either directly or through sRNA cycling (gold; incoming sRNA). (B) Aerial view of Hfq-ChiX complex (proximal face up). The Hfq core and RNAs are roughly to scale, but the CTDs are depicted shorter, and the N termini are omitted for clarity. (C) The CTD enhances sRNA competition by enabling rapid cycling of RNAs on the proximal face and rim. The mRNAs and Class II sRNAs, such as ChiX, form persistent complexes by virtue of an AAN motif (green) bound to the distal face, which is proposed to be immune to displacement by the CTDs. Class I sRNAs (red) bind the proximal face but are displaced by local competition with the 3' end of ChiX (gold).

(55) that we suggest keeps the sRNA-mRNA duplex tethered to Hfq. Putative AAN binding sites occur in other targets of Hfq, such as *ompA* and *ompF*. Although our minimal RNAs are unstructured, the secondary structures present in natural Hfq substrates may also lengthen the lifetimes of specific Hfq-RNA complexes by reinforcing interactions with multiple surfaces of Hfq (54).

The unequal effects of the CTDs on RNAs bound to the proximal and distal faces of the Sm core explain several aspects of Hfq's chaperone activity that were previously difficult to understand. First, continuous displacement of RNAs from the rim of Hfq102 is consistent with Hfq65's higher affinity for short stem loops (Table 1) and three- to eightfold higher $V_{\max}^{\text{app}}$ for annealing the 16-nt Target RNA compared with Hfq102 (Fig. 1B). The volume excluded by the CTDs may also limit access to the six arginine patches along the rim of Hfq102. Fast release of dsRNA by Hfq102, however, disfavors the reverse reaction, explaining why Hfq102 yields more annealed product than Hfq65 (Fig. 1C).

Second, as illustrated in Fig. 5C, the kinetic advantage of RNAs possessing AAN motifs over RNAs that only bind the proximal surface (Fig. 3) explains the superior competition for Hfq by ChiX sRNA (12, 35) and *flhA* mRNA (56). Our results suggest that removing the CTD nullifies the advantage of distal face binding, collapsing the range of competition between different sRNAs (Fig. 3) and reducing the accumulation of Class II sRNAs in the cell by increasing their degradation after pairing (Fig. 4). Additionally, this model predicts that Class II sRNAs will remain bound to Hfq via distal face contacts for multiple annealing cycles, whereas the Class II mRNA targets, which bind the rim of Hfq (31), cycle off—the reverse of Class I sRNAs and their targets.

Our results show that the Hfq CTD is important for directing the limited quantity of Hfq to specific targets in the cell (12, 43). The CTDs likely reduce the sequestration of Hfq in unproductive

complexes and recycle Hfq after an sRNA anneals with its target. Because most sRNAs are stabilized in vivo if they bind Hfq (12, 31, 47), competition among sRNAs for Hfq determines their accumulation (Fig. 4). Class II sRNAs, such as ChiX and MgrR, are more affected by deletion of the CTD, because this mutation undermines the kinetic advantage of their interactions with Hfq's distal face. The observation that the CTD deletion has a greater effect on sRNA turnover after IPTG washout (Fig. 4) suggests that the CTD is more important for competition against other RNAs than the intrinsic stability of the Hfq-sRNA binding. The interplay of autoinhibition, sRNA competition, and sRNA accumulation could be the source of the conflicting results on the importance of the CTD (23, 24, 26, 27).

The CTD has been proposed to stabilize RNA-Hfq complexes via direct interactions with sRNAs (19, 36, 57) owing to the slightly weaker binding of sRNAs in the absence of the CTD (10, 25), which we have recapitulated (Table 1). However, the data presented in this paper do not support this stabilization model because the CTDs do not increase the affinity of RNA oligomers (A18-FAM and D16-FAM) (Table 1) and actually decrease the affinity of RNA stem loops (beacon and minRCRB) (Table 1). Although the CTDs may transiently interact with RNAs anchored to either face of Hfq because of their proximity and the large space that the six CTDs must occupy, these interactions do not seem to be very stabilizing on their own. We also find no evidence that the intrinsically disordered CTDs speed up association of RNA oligomers with Hfq as proposed by “fly-casting” models, in correspondence with research in other systems (58). The Hfq CTD was previously stated to be required for annealing RNA oligomers in vitro (19). However, these reactions were done under conditions in which the majority of the annealing reaction could have been missed, in particular a high Hfq:RNA ratio combined with a mixing delay before measurements were started (19, 26, 59).

The autoinhibitory activity exhibited by the Hfq CTD may be generally important in nucleic acid binding proteins. The intrinsically disordered CTDs of HTLV-1 NC (50), *E. coli* ssDNA binding protein (51), and mammalian high-mobility group B1 (60) proteins inhibit binding of their N termini to their respective nucleic acid substrates. Furthermore, the CTD of HTLV-1 NC also accelerates RNA dissociation (50), which may suggest a general mechanism for RNA chaperone turnover.

The CTD of Hfq is highly variable across all bacterial sequences and only weakly conserved within bacterial families. The expansion of the *Enterobacteriales* Hfq CTD after the split from other γ -proteobacteria was previously noted to have occurred simultaneously with the acquisition of new transacting sRNAs and the evolution of mRNA targets (30). Therefore, the long CTD was hypothesized to be important for the function of sRNA-mRNA pairs in these bacteria (30). It remains to be seen if this is generally true. For example, there is currently a limited number of known sRNA-mRNA pairs in the *Moraxallaceae* family (21, 61–64), despite the large expansion of its Hfq CTD to about 100 residues. Identification of residues involved in a putative Hfq core-CTD interface could enable us to determine whether there are unifying features among the disparate CTD sequences from different bacterial families.

Materials and Methods

Hfq Purification and Cy3 Labeling. Untagged Hfq102 and Hfq65 were over-expressed in *E. coli* BL21 (DE3) Δ hfq::cat-sacB cells grown in 1 L LB-Miller media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 100 μ g/mL ampicillin (Amp). The plasmid for Hfq65 was created by site-directed mutagenesis of the plasmid pET21b-Hfq (14). The purification method in ref. 19 was modified as detailed in *SI Materials and Methods*. In brief, resuspended cell lysates were clarified by heat denaturation or ammonium sulfate precipitation (Hfq65) and captured via Ni^{2+} affinity (Hfq102) or hydrophobic interaction chromatography (Hfq65) followed by cation exchange chromatography to remove nucleic acids (Fig. S8). Hfq102:V27C and Hfq65:V27C purified in the same manner were treated with Cy3-maleimide (GE Healthcare) according to the manufacturer's protocol and repurified by ion exchange chromatography

as for the unlabeled protein. The extent of labeling was estimated from the absorbance at 280 and 552 nm, and it was less than one dye per hexamer on average.

RNA Preparation. Target RNAs (Dharmacon) and R16 (Invitrogen) have been previously described (39, 41) and were purified by denaturing PAGE. The molecular beacon, D16-FAM, and A18-FAM were obtained from Trilink Biotechnologies and purified by reverse-phase HPLC. The ssDNA competitor (IDT) was used without additional purification. minRCRB RNA (IDT) was reduced with TCEP [tris(2-carboxyethyl)phosphine HCl] and purified by denaturing PAGE before labeling with Cy3-maleimide (GE Healthcare) according to the manufacturer's protocol. The extent of labeling was estimated from the absorbance at 260 and 552 nm. RNA and DNA sequences are provided in Table S1.

Hfq Binding Assays. RprA, DsrA, RyhB, and ChiX were transcribed in vitro as previously described (15). The affinities of Hfq102 and Hfq65 for ³²P-labeled sRNAs at 30 °C were measured by native gel mobility shift as previously described (15) and fit to a partition function for two independent sites (15). Binding constants for D16-FAM, A18-FAM, molecular beacon, or Cy3-minRCRB (5 nM) were measured in TNK buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM KCl) at 30 °C by FAM fluorescence anisotropy as described before (65).

RNA Annealing. The annealing kinetics between the molecular beacon (50 nM) and target RNA (100 nM) in the presence of 0–250 nM Hfq hexamer in TNK buffer at 30 °C was measured by stopped flow fluorescence spectroscopy as described previously (52, 66) and in *SI Materials and Methods*. MgCl₂ was not included in the annealing buffer, because it is not required for annealing or Hfq binding and because it can lead to RNA aggregation (15). Progress curves were fit to single- or double-exponential rate equations. End points for annealing reactions were measured separately by titrating complexes of 50 nM target RNA and 50 nM molecular beacon with Hfq as outlined in *SI Materials and Methods*. Titrations were performed in duplicate.

Hfq–RNA Binding Kinetics. Association of 83.3 nM Cy3-labeled Hfq with 50 nM D16 RNA in TNK buffer at 30 °C was measured by stopped flow spectroscopy as previously described (39), except that the change in emission of the Cy3 acceptor was monitored with a 570-nm cutoff filter, with excitation of the FAM donor at 490 nm. The data from six trials were averaged, and the increase in Cy3 fluorescence was fit to a single-exponential rate equation. Hfq release was measured as previously described (39) by rapidly mixing 100 nM R16 RNA with preincubated 100 nM D16-FAM plus 83.3 nM Hfq-Cy3 or rapidly mixing 100 nM D16-FAM with a preformed complex of 100 nM R16 and 83.3 nM Hfq-Cy3. This assay is further detailed in *SI Materials and Methods*.

To measure RNA binding and release from unlabeled Hfq102 and Hfq65 by anisotropy, the polarization of D16-FAM was recorded every 20 s for ≥3 min after each addition (Horiba Fluorolog-3 L-format). Samples were examined with single excitation and emission monochromators at 495 and 515 nm, respectively, and 5-nm slit widths. Samples were prepared in a 500-μL cuvette containing 50 nM D16-FAM in TNK buffer at 30 °C with additions of 50 nM Hfq102, 50 nM Hfq65, or an equal volume of HB buffer [50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 250 mM NH₄Cl, 10% glycerol (v/v)], 50 nM R16 RNA, and 400 nM ssDNA competitor.

sRNA Binding Competition. Competitive sRNA equilibrium binding experiments were carried out by native gel shift assays as described above. For each experiment, the concentration of either Hfq102 or Hfq65 was chosen, such that most of the labeled RNA was bound in the absence of unlabeled competitor RNA. These concentrations were 37.9 nM (³²P-ChiX) and 60.6 nM (³²P-DsrA) Hfq102 hexamer and 68.2 nM (³²P-ChiX) and 90.9 nM (³²P-DsrA) Hfq65, respectively; 5× Hfq stocks in HB buffer (2 μL) were first dispensed to the bottom of 1.5-mL microcentrifuge tubes. On the wall of each tube was

placed 1 μL 10× unlabeled competitor RNA (0.11–11.1 μM) in 1× TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), so that the final concentration of competitor RNA would range from ~0.1 to 1 μM. Next, 8 μL reaction mixture [1 μL ³²P-labeled RNA, 1 μL 30% (vol/vol) glycerol loading dye, 2 μL 5× TNK buffer, 2 μL 5× TE buffer, 2 μL water] was first mixed with the drop of competitor RNA, holding the tube at an angle to keep the solution on the wall of the tube. Finally, the solution containing labeled and unlabeled RNA was mixed with the Hfq stock at the bottom of the tube. The reactions were incubated at 30 °C for 1 h before resolving the complexes on 8% (wt/vol) (29:1 mono:bis) polyacrylamide gels in 1× TBE (Tris-borate EDTA). Gels were dried and quantified using a PhosphorImager. The fraction of ³²P-labeled RNA bound to Hfq, including any ternary complexes, was fit to the four-parameter empirical model,

$$f_B = f_{\min} + \frac{f_{\max} - f_{\min}}{1 + \left(\frac{[sRNA]}{IC_{50}}\right)^n}, \quad [1]$$

in which $f_{\min}$ and $f_{\max}$ are the minimum and maximum fractions bound, respectively, $[sRNA]$ is the concentration of competitor RNA, and n is a Hill coefficient.

In Vivo Measurement of sRNA Half-Life and Accumulation. For in vivo experiments, bacterial strains used were derived from *E. coli* PM1205; *hfq65* was introduced into bacterial strains in place of the native *hfq* gene by λ-Red-mediated recombineering and P1 transduction as described previously (31). Strains used in this study are listed in Table S2. sRNAs RyhB, OmrB, Spot42, MgrR, and ChiX were expressed from derivatives of pBR-plac, a pBR322-based plasmid, under control of the *lac* promoter (67); pBR-RyhB (68), pBR-ChiX (69), pBR-MgrR (70), pBR-OmrB (68), and pBR-Spot42 (68) were used for washout and rifampicin experiments. All strains were grown in LB-Lennox media (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37 °C. Liquid and solid media were supplemented with 50–100 μg/mL Amp, 25 μg/mL kanamycin, and 25 μg/mL chloramphenicol where appropriate for selection.

To assess sRNA half-life, cells transformed with the appropriate plasmid were grown in 50 mL LB-Lennox media with 100 μg/mL Amp to an OD₆₀₀ of ~0.4, induced for 15 min with 100 μM IPTG, and treated as previously described (31), except that quantitation of RNA was done with biotinylated probes. In brief, to measure intrinsic stability, culture aliquots were treated with 250 μg/mL rifampicin, and samples were removed at times indicated and processed for Northern blots. In parallel, for washout experiments, aliquots of cultures were filtered and resuspended in LB-Lennox media with 100 μg/mL Amp without IPTG to stop induction of the sRNA, and samples were removed at appropriate times during growth and processed for Northern blots at the times indicated. Total RNA was isolated by the hot phenol method (71), and RNA samples dissolved in diethylpyrocarbonate-treated water were stored at –80 °C until used. Total RNA of each sample (3 μg) was run on a 10% TBE-Urea Gel (Bio-Rad) and transferred onto Zeta-Probe GT Membrane (Bio-Rad) followed by UV cross-linking of RNA with the membrane and hybridization with appropriate biotinylated probes (Table S1). Detection of probes was performed using the Bright-Star Biotect Kit (Ambion) as per the manufacturer's instructions. RNA levels were quantified from the Northern blot using Image Studio Lite, version 4.0 software normalized to levels of SsrA from the same blot (Fig. S7A).

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