jmb Article

Mutations in Interaction Surfaces Differentially Impact *E. coli* Hfq Association with Small RNAs and Their mRNA Targets

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http://dx.doi.org/10.1016/j.jmb.2013.01.006
Edited by M. Gottesman

Abstract

The RNA chaperone protein Hfq is required for the function of all small RNAs (sRNAs) that regulate mRNA stability or translation by limited base pairing in *Escherichia coli*. While there have been numerous *in vitro* studies to characterize Hfq activity and the importance of specific residues, there has been only limited characterization of Hfq mutants *in vivo*. Here, we use a set of reporters as well as co-immunoprecipitation to examine 14 Hfq mutants expressed from the *E. coli* chromosome. The majority of the proximal face residues, as expected, were important for the function of sRNAs. The failure of sRNAs to regulate target mRNAs in these mutants can be explained by reduced sRNA accumulation. Two of the proximal mutants, D9A and F39A, acted differently from the others in that they had mixed effects on different sRNA/mRNA pairs and, in the case of F39A, showed differential sRNA accumulation. Mutations of charged residues at the rim of Hfq interfered with positive regulation and gave mixed effects for negative regulation. Some, but not all, sRNAs accumulated to lower levels in rim mutants, suggesting qualitative differences in how individual sRNAs are affected by Hfq. The distal face mutants were expected to disrupt binding of ARN motifs found in mRNAs. They were more defective for positive regulation than negative regulation at low mRNA expression, but the defects could be suppressed by higher levels of mRNA expression. We discuss the implications of these observations for Hfq binding to RNA and mechanisms of action.

Introduction

The Sm-like Hfq binds more than 30% of the known small, regulatory RNAs (sRNAs) in *Escherichia coli*[1] and *Salmonella*.[2] The protein both stabilizes these sRNAs and is required for their base pairing with mRNA targets (reviewed in Refs. 3 and 4). For some targets (e.g., the DsrA target *rpoS* and the McaS target *flhD*), base pairing can lead to increased translation, due to the opening of a secondary structure that blocks translation of the mRNA. However, for the majority of targets (e.g., the ArcZ target *flhD* and the RyhB target *sodB*), base pairing leads to decreased expression by inhibiting ribosome binding and/or promoting mRNA degradation (reviewed in Ref. 5). Some of the effects on mRNA stability may be due to the reported association of Hfq with components of the degradosome complex, including RNase E and polynucleotide phosphorylase.[6,7]

Given its central role in the functions of base-pairing sRNAs, Hfq has been subject to numerous *in vitro* studies. Structural analysis of Hfq showed that the protein forms a hexameric ring with proximal and

distal faces similar to Sm proteins. The solution of the first crystal structure of a bacterial Hfq with a 5′-AUUUUUG oligoribonucleotide revealed that the RNA bound in a circular conformation around the pore on the proximal face of the *Staphylococcus aureus* protein.[8] Subsequent mutational studies suggested that *E. coli* Hfq has distinct interaction surfaces for DsrA and poly(A).[9,10] Mutations of the proximal face residues and a charged amino acid on the rim led to decreased binding to the DsrA RNA *in vitro*; none of the proximal face mutations had significant effects on poly(A) binding. In contrast, mutations of distal face residues led to decreased binding to poly(A) but had little effect on binding to DsrA. Recent crystallographic studies of *E. coli* Hfq bound to an A18 oligoribonucleotide showed that the poly(A) RNA binds to the distal face.[11] This structure, together with oligonucleotide binding assays, led to the proposal that the distal face of Hfq binds repeats of an ARN motif (where R is adenine or guanine and N is any nucleotide). This proposal was consistent with the earlier finding that the upstream $(AAN)_4$ sequence motif in the *rpoS* mRNA contributes to tight Hfq binding and formation of stable ternary complexes between Hfq, *rpoS*, and DsrA.[12] Similar ARN sequences were also shown to be important for sRNA-dependent regulation for other sRNA/mRNA pairs.[13–15] Recent studies revealed that the 3′ end terminal poly(U) tail found in Hfq-binding sRNAs[2] significantly contributes to the recognition of sRNA by Hfq and is essential for the ability of sRNA to bind to the central cavity of the Hfq hexamer and regulate mRNA targets.[16,17] Finally, a charged patch at the outer rim of the hexamer also has been implicated in sRNA binding.[18] Together, these data suggest that the Hfq ring has at least three RNA binding surfaces: a proximal face for U-rich sRNAs, the distal face for A-rich mRNA targets, and a rim region that may provide additional binding sites.

Only a few studies have examined the effects of specific amino acid substitutions on the *in vivo* function of Hfq. Proximal mutants Q8A, F42A, and K56A, expressed from a plasmid, showed significant defects for Hfq-dependent activation of *rpoS.*[9] In another study, a plasmid-expressed V43R mutant abrogated *rpoS* activation but both plasmid-expressed and chromosomally expressed V43R retained the ability to repress *oppA*.[19]

Despite the extensive *in vitro* studies and limited *in vivo* studies, many questions regarding Hfq binding to mRNAs and the mechanism by which Hfq facilitates the interaction between sRNAs and mRNAs remain. Furthermore, most of the *in vitro* studies have been focused on one model system, activation of *rpoS* by DsrA and RprA; thus, it is not clear if the lessons learned with this system will extrapolate to negative regulation or even other sRNA-based positive regulation. To contribute to answering these questions and learn more about the mechanism of Hfq action *in vivo*, we constructed isogenic sets of strains in which 18 mutant Hfq derivatives were expressed from the chromosome. We then examined the effects of the 14 alleles expressed at significant levels on *in vivo* activity in a selected set of assays and on sRNA accumulation. Association of specific sRNAs and mRNAs with the Hfq mutants also was analyzed by co-immunoprecipitation (co-IP). In addition, we examined the effects of the Q8A, R16A, and K31A mutations on RNA association with Hfq on a whole transcriptome scale by probing tiling arrays with total RNA and co-IP RNA isolated from these strains. The implications of these findings for the mechanism of Hfq binding to RNAs and facilitating base pairing are discussed.

Results

Most Hfq mutants are well expressed from the chromosomal locus

To assay the effects of Hfq mutants expressed at endogenous levels, we replaced the wild-type (wt) *hfq* gene with derivatives carrying alanine substitutions of the amino acids around the central pore on the proximal side of the hexamer (Q8, D9, F39, D40, F42, Y55, K56, and H57, Fig. 1a), alanine, cysteine, or aspartic acid substitutions of charged amino acids on the outer rim (R16, R17, and R19, Fig. 1c) and alanine or aspartic acid substitutions of amino acids on the distal face (Y25, G29, I30, and K31, Fig. 1e). The mutations were introduced into the chromosome at the native *hfq* locus and moved between strains as described in Materials and Methods.

The levels of the different mutant proteins were then examined by Western blot analysis under denaturing conditions (Fig. 1b, d, and f). The levels of the D40A, Y55A, R16D, and R19A derivatives were low and were eliminated from further study. The levels of the Q8A, F42A, H57A, R16A, R17A, R19D, and Y25D proteins were similar to those of the wt protein, while D9A and G29A levels were slightly lower. The F39A, K56A, R16C, and K31A levels were somewhat higher and the I30D levels were significantly higher than the wt protein.

Additionally, we always observed a higher-molecular-weight band consistent with the size of a hexamer for three of the rim mutants (R16A, R16C, and R17A) and two of the distal face mutants (I30D and K31A), suggesting that these mutants might form particularly stable rings recalcitrant to denaturation. The oligomerization state of the mutants was further examined using semi-native gels previously used to distinguish between Hfq monomers and oligomers[20] (Fig. S1). In these gels, the wt Hfq migrates primarily as an oligomer, with barely