Structural basis for the CsrA-dependent modulation of translation initiation by an ancient regulatory protein

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Regulation of translation is critical for maintaining cellular protein levels, and thus protein homeostasis. The conserved RNA-binding protein CsrA (also called RsmA; for carbon storage regulator and regulator of secondary metabolism, respectively; hereafter called CsrA) represents a well-characterized example of regulation at the level of translation initiation in bacteria. Binding of a CsrA homodimer to the 5'UTR of an mRNA occludes the Shine–Dalgarno sequence, blocking ribosome access for translation. Small noncoding RNAs (sRNAs) can competitively antagonize CsrA activity by a well-understood mechanism. However, the regulation of CsrA by the protein FliW is just emerging. FliW antagonizes the CsrA-dependent repression of translation of the flagellar filament protein, flagellin. Crystal structures of the FliW monomer reveal a novel, minimal β -barrel-like fold. Structural analysis of the CsrA/FliW heterotetramer shows that FliW interacts with a C-terminal extension of CsrA. In contrast to the competitive regulation of CsrA by sRNAs, FliW allosterically antagonizes CsrA in a noncompetitive manner by excluding the 5'UTR from the CsrA–RNA binding site. Our phylogenetic analysis shows that the FliW-mediated regulation of CsrA regulation is the ancestral state in flagellated bacteria. We thus demonstrate fundamental mechanistic differences in the regulation of CsrA by sRNA in comparison with an ancient regulatory protein.

translation | CsrA | flagellum | FliW | crystallography

The ability of ribosomes to access mRNAs at the ribosome-binding site (RBS) is crucial for productive translation and presents an important element of translational control during bacterial gene expression. The Shine–Dalgarno sequence is part of the RBS and marks the starting point of translation, as it allows base pair interaction of the mRNA with the 3'-end of the 16S ribosomal RNA (1, 2). Translation is inhibited if an RBS is occluded either by base-pairing RNAs or by regulatory proteins.

CsrA/RsmA proteins (for carbon storage regulator and regulator of secondary metabolism, respectively; hereafter called CsrA) are widely distributed regulators of bacterial gene expression that bind their target mRNAs at the RBS (3). Members of this family mediate the posttranscriptional regulation of a broad variety of bacterial genes and have pleiotropic effects on numerous processes, such as central carbon metabolism, secondary metabolite metabolism, motility, biofilm formation, and the expression of virulence factors in pathogenic bacteria (summarized in refs. 3–5).

CsrA proteins from different bacterial species form highly similar homodimers that are composed of a five-stranded, domain-swapped β -sheet with a barrel-like architecture (6–9). CsrA homodimers contain two RNA binding sites that establish optimal contacts with an A_UCANGGANGU_A sequence motif present in the 5'UTRs (8, 10). When bound by CsrA, the ANGGAN core folds into a loop stabilized by a 3-bp stem of the flanking nucleotides. In this clamp-like structure, the Shine–Dalgarno is sequestered and translation is repressed.

Small noncoding RNAs (sRNAs; e.g., CsrB/C or RsmX/Y/Z) antagonize CsrA binding to mRNA in a competitive manner (11–13). These sRNAs act as “protein sponges,” which contain multiple binding sites that permit sequestration, storage, and release of CsrA (13–15). Although the underlying mechanisms of the sRNA-

dependent regulation of CsrA are well understood, modulation of CsrA activity by the regulatory protein FliW lags behind.

FliW regulates CsrA to homeostatically restrict translation of the flagellar filament protein flagellin (encoded by the *hag* gene) (reviewed in refs. 16 and 17). In *Bacillus subtilis*, CsrA inhibits translation of flagellin by binding to two sites that are present within the 5'UTR of the *hag* mRNA (10, 18). FliW inhibits binding of CsrA to the 5'UTR of the *hag* mRNA, allowing the translation of flagellin (19, 20). FliW, which can also bind to flagellin, is sequestered as cytoplasmic levels of flagellin rise, allowing CsrA again to inhibit translation of the *hag* mRNA. This cycle is thought to enable homeostasis of cytoplasmic flagellin concentrations over a low and narrow threshold (19). A recent study in *Campylobacter jejuni* has shown that CsrA regulation by FliW might be much more conserved among flagellated eubacteria than anticipated so far (21). Additional experiments demonstrated that the *hag* mRNA is not the only target of CsrA, but rather the most abundant in a pool of several motility-related mRNAs that fall under CsrA regulation (21).

To delineate the molecular mechanism by which FliW antagonizes CsrA binding to the 5'UTR of the *hag* mRNA, we determined the crystal structure of FliW alone and in complex with CsrA. Our structural analysis shows that FliW interacts with a C-terminal extension of CsrA (CsrA-C). In contrast to sRNAs that act as competitive inhibitors of mRNA-binding to CsrA, FliW acts in a noncompetitive manner by allosterically modulating CsrA.

Results

Crystal Structure of FliW Reveals a Minimalistic β -Barrel Fold. We wanted to determine the structure of FliW because it shares no similarity with any other proteins on the primary sequence level. Because thermophilic proteins are often better suited for crystallography, we

Significance

CsrA proteins are repressors of translation that target the Shine–Dalgarno sequence of the ribosome-binding site. Small noncoding RNAs competitively sequester CsrA by offering multiple binding sites that mimic the Shine–Dalgarno. Antagonism of CsrA by small noncoding RNAs is a widely conserved mechanism of posttranscriptional regulation. Here we present the first crystal structure of protein FliW, which allosterically regulates CsrA in a highly specific manner. Our phylogenetic analysis reveals that the FliW–CsrA regulon is subject to coevolution and represents the ancestral state in flagellated bacteria.

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 5DMD, 5JAK, and 5DMB for GtFliW in P2(1), GtFliW in P22(1) 2(1), and GtCsrA/FliW, respectively].

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Search for FliW-related structures by the DALI server (22) did not result in obvious domain similarities. FliW exhibits a minimalistic β -barrel-like fold consisting of six β -strands (Fig. 1B). The strands $\beta 3$ to $\beta 5$ form one-half, whereas strands $\beta 6$, $\beta 8$, and

As concluded from SEC, the crystal structure of *GtCsrA*/*FliW* shows a heterotetramer consisting of two *CsrA* and two *FliW* molecules (Fig. 24). *CsrA* forms a symmetric homodimer along the crystallographic twofold axis. The interface of the *CsrA* homodimer is established by five β -strands, with $\beta 1$ being part of the opposing

Data collection and refinement	GtFliW	GtFliW	GtCsrA–FliW
Data collection			
Space group	P2 ₁	P22 ₁ 2 ₁	C2
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	47.54	31.24	108.88
	42.52	77.99	61.68
	71.92	87.05	42.90
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90.00	90.00	90.00
	102.66	90.00	98.08
	90.00	90.00	90.00
Energy (keV)			
Resolution (Å)	46.38–1.45	43.52–1.80	42.48–2.30
	(1.53–1.45)	(1.95–1.80)	(2.42–2.30)
<i>R</i> _{merge}	0.056 (0.555)	0.052 (0.51)	0.056 (0.518)
<i>I</i> / <i>σ</i> <i>I</i>	9.5 (1.9)	18.7 (3.5)	12.0 (1.8)
Completeness (%)	99.4 (96.6)	100.0 (99.8)	99.5 (99.6)
Redundancy	3.3 (3.1)	7.2 (7.5)	3.1 (2.9)
Refinement			
Resolution (Å)	46.38–1.45	43.52–1.80	42.48–2.30
No. reflections	49,625	20,382	11,552
<i>R</i> _{work} / <i>R</i> _{free}	20.4	22.4	22.4
	23.6	24.0	28.8
No. atoms	2,658	1,226	1,767
Protein	2,355	1,131	1,694
Ligand	0	0	0
Water	303	95	73
Rmsd			
Bond lengths (Å)	0.01	0.01	0.01
Bond angles (°)	1.1	1.4	1.81
Ramachandran (%)			
Preferred	97.49	98.00	95.00
Allowed	1.43	2.0	4.52
Outliers	1.08	0.00	0.48

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Fig. 2. Crystal structure of CsrA–FliW. (A) Cartoon representation of the GtCsrA–FliW crystal structure shows that the CsrA homodimer (gray) binds two FliW molecules (green). “N” and “C” indicate N and C termini, respectively. The dashed line indicates the twofold symmetry axis. (B) Electrostatic surface representation of FliW and cartoon representation of a CsrA monomer rainbow-colored from the N to the C terminus. Helices $\alpha 1$ and $\alpha 2$ of CsrA–C bind into an extended groove of FliW (Left) located opposite to the negatively charged area of FliW (Right) (compare also to Fig. 1C). (C) Coomassie-stained SDS/PAGE of an in vitro pull-down assay using GtFliW as bait and GtCsrA, GtCsrA $\Delta\alpha 2$, and GtCsrA $\Delta\alpha 1/\alpha 2$ as prey. (D) Detailed view of the interaction of FliW with $\alpha 1$ (Upper) and $\alpha 2$ (Lower) of CsrA–C. Residues in FliW that were tested in the in vitro pull-down assay are shown in red (compare with E). (E) Coomassie-stained SDS/PAGE of an in vitro pull-down assay using wild-type GtFliW and its variants as bait and GtCsrA as prey.

monomer. This finding is in perfect agreement with many other structures of CsrA homodimers obtained by X-ray crystallography and NMR (6–8, 15). The CsrA–FliW interface is primarily established through the C-terminal extension of CsrA (CsrA–C) (Fig. 2A). Each of the CsrA half sites interacts with one FliW molecule through approximately $\sim 1,405 \text{ \AA}^2$ of buried surface area (Fig. 2B). The binding site of CsrA–C localizes opposite to the negative charged area of FliW (Fig. 2B). CsrA–C consists of two α -helices, $\alpha 1$ (residues: 44–59) and $\alpha 2$ (residues 60–74), that are connected by a short loop. CsrA–C binds into an extended groove at the surface of FliW via an extensive network of polar and hydrophobic interactions (Fig. 2B and Fig. S3). Successive deletion of helices $\alpha 1$ and $\alpha 2$ from CsrA abolishes its interaction with FliW in vitro (Fig. 2C), and variations of FliW residues critical for CsrA binding abolish the in vitro formation of the CsrA/FliW complex (Fig. 2D and E). Taken together, our structural and biochemical analysis shows that FliW interacts with CsrA–C. Thus, the CsrA homodimer is able to recruit two FliW molecules, yielding a wing-shaped heterotetramer.

FliW Distorts CsrA Binding to the 5'UTR. To understand the molecular mechanism by which FliW antagonizes CsrA binding to the Shine–Dalgarno-containing 5'UTR of the *hag* mRNA, we

compared the structure of GtCsrA–FliW with that of mRNA-bound CsrA. We chose the structure of CsrA bound to a Shine–Dalgarno present within the 5'UTRs of *hcnA* mRNA [PDB ID code 2JPP (8)]. This structural comparison is justified on the grounds that the Shine–Dalgarno sequences of the *hcnA* and *hag* mRNAs share a high degree of sequence conservation within their “CANGGANG” CsrA-binding motifs (Fig. 3A). Both structures were superimposed on the CsrA homodimers that share high structural similarity with an rmsd of 0.746 over all C α atoms (Fig. 3B, Right).

Our structural comparison shows that the Shine–Dalgarno and FliW share overlapping interfaces at CsrA (Fig. 3B). Moreover, Shine–Dalgarno and FliW engross similar spatial entities and charge distributions relative to CsrA. The overlapping binding sites at CsrA involve residues 125–129 of FliW and bases C9 and G10 of the Shine–Dalgarno (Fig. 3C). Additionally, the negatively charged side of FliW (i.e., amino acids E70, D72, D74, D75, and E79) resides in close proximity at CsrA, where the phosphate backbone of the Shine–Dalgarno would otherwise localize (Fig. 3D). This negative patch of FliW will lead to electrostatic repulsions with the phosphate backbone of the Shine–Dalgarno and therefore hinder RNA binding.

Fig. 3. FliW and RNA interaction with CsrA. (A) Sequence alignment of the 5'UTR regions of the *hag* and *hcnA* mRNAs from *B. subtilis* (Bs) and *P. fluorescens* (Ps), respectively. A black bar indicates the Shine-Dalgarno (SD) sequence. The red triangle indicates the central base G11. (B) Side-by-side views of the structures of CsrA (red) bound to *hcnA* (orange) (Left), CsrA (gray) bound to FliW (green) (Center) and the superimposition of both structures (Right). The Upper and Lower panels show two orientations of the same that are rotated by 90° relative to each other. (C) Detailed view on the overlapping binding sites of FliW (green) and *hcnA* (orange) at CsrA (gray). (D) Detailed view of how FliW (green) hinders interaction of CsrA (gray) with *hcnA* (orange) in a steric (Left) and electrostatic manner (Right).

Discussion

Different Mechanisms of CsrA Regulation by FliW and sRNA. In *B. subtilis*, CsrA inhibits translation of flagellin by binding to two adjacent sites present within the 5'UTR of *hag* (18). Interaction of FliW with CsrA enables translation of flagellin, which itself can also sequester FliW (19, 20). This pattern of interactions permits FliW to antagonize initiation of flagellin translation (via CsrA) in a flagellin-dependent manner. FliW thus appears to participate in the homeostatic maintenance of flagellin concentrations.

Here we have investigated the structural basis for how FliW antagonizes CsrA, enabling ribosomes to access the Shine-Dalgarno present within the 5'UTR of the *hag* mRNA. Our crystal structure shows that FliW binds to CsrA primarily through its C-terminal extension (CsrA-C), but also through contacts with the CsrA RNA-binding domain. FliW shares overlapping interfaces with the Shine-Dalgarno at CsrA, which makes the simultaneous binding of FliW and Shine-Dalgarno extremely unlikely (Figs. 3 and 4A and B). Moreover, one-half of FliW is highly negatively charged. This side of the protein localizes in close proximity to where the RNA is found in Shine-Dalgarno/CsrA structures. This negatively charged surface of FliW provides strong electrostatic repulsions inhibiting RNA binding. Therefore, FliW allosterically antagonizes CsrA in a noncompetitive manner by excluding the 5'UTR from CsrA (Figs. 3 and 4A). This mechanism is in strong contrast to that of sRNAs also regulating CsrA. Recent structural analysis has elegantly demonstrated that the sRNA RsmZ sequesters CsrA by offering multiple CsrA binding sites that are similar to Shine-Dalgarno sequences (13, 14) (Fig. 4B). As such, sRNAs regulate CsrA by acting as competitive inhibitors of the original Shine-Dalgarno sequences. Our analysis shows that FliW acts, in contrast to sRNAs, by a noncompetitive mechanism through a highly specific binding site offered by CsrA-C.

Therefore, FliW is highly specific to CsrA, but also to the process of flagellar assembly via its interaction with flagellin. Although a precise molecular understanding of the FliW-flagellin interaction is

lacking, a previous study indicated a role of the C terminus of flagellin for its interaction with FliW (23). FliW therefore links the presence of mature, cytoplasmic flagellin molecules with the production of new flagellin via CsrA. Such a mechanism might be useful in the light that flagellin is one of the most abundant proteins in flagellated bacteria (one flagellar filament consists of over 20,000 flagellin copies), and therefore, should be homeostatically controlled (Fig. 4A).

FliW Is an Ancient Regulator That Is Evolutionarily Coupled to CsrA.

Our study shows that the interaction of FliW and CsrA relies on CsrA-C. We therefore wanted to know whether the presence of CsrA-C relates to FliW among flagellated bacteria. To address this question, we compared the conservation of the CsrA-FliW module by performing a systematic search using the National Center for Biotechnology Information (NCBI) BLAST tool and subsequent phylogenetic inference (Fig. 4C and Table S1). Our analysis confirms that FliW and CsrA form an evolutionary conserved unit among flagellated bacteria, as has been suggested previously (19, 24, 25). Manual inspection of CsrA proteins from organisms that also contain FliW shows that these possess a C-terminal elongation (Fig. 4C, Fig. S4, and Table S1). Vice versa, CsrA proteins from flagellated species missing FliW also lack the C-terminal elongation, whereas the RNA-binding domain is highly conserved (Fig. S4). We therefore suspect that CsrA-C and FliW are subject to coevolution. Absence of CsrA-C and FliW in only some species suggests their secondary loss during the course of evolution. Taking these data together, we find that both proteins apparently form an ancient regulatory module of flagellated bacteria.

Regulation of CsrA by FliW or sRNAs. Our study also raises the question how CsrA proteins lacking a FliW binding site are regulated. Phylogenetic analysis shows that CsrA proteins without an extended C terminus are primarily found within the γ -proteobacteria (that also lack FliW) (Fig. 4C and Table S1). In the γ -proteobacteria, CsrA activity is regulated through the

generating 20 distinct trees to select the one with the best-likelihood. Subsequently, 100 bootstrap inferences were carried out and the support values drawn on the best tree (Figs. S8 A and B and S9). Trees were visualized using FigTree (tree.bio.ed.ac.uk/software/figtree/).

Protein Production and Purification. The genes encoding for CsrA and FliW proteins from *G. thermodenitrificans* NG-80 were amplified from genomic DNA by PCR and cloned into pET16b and pET24d vectors (Novagen) via the NcoI and BamHI restriction sites. FliW contained an N-terminal hexa-histidine tag. Proteins were produced in BL21 (DE3) (Novagen). After cell lysis by a Microfluidizer (M110-L, Microfluidics), cell debris was removed by high-speed centrifugation and proteins were purified by Ni-ion affinity- and size-exclusion chromatography, as described recently (43). The SEC buffer consisted of 20 mM Hepes-Na (pH 7.5), 200 mM NaCl, 20 mM KCl, and 20 mM MgCl₂.

Crystallization and Structure Determination. Crystallization was performed by the sitting-drop method at 20 °C in 600-nL drops consisting of equal parts of protein and precipitation solutions. GtFliW crystallized at 20-mg/mL concentration within 1–5 d in 0.1 M MES pH 6.0, 40% (vol/vol) PEG 400, 5% (wt/vol) PEG 3000 and 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.5, and 30% (wt/vol) PEG 8000. GtCsrA–FliW crystallized at 15 mg/mL in a condition containing 0.2 M potassium fluoride, 20% (wt/vol) PEG 3350. Prior data collection, crystals were flash-frozen in liquid nitrogen using a cryo-solution that consisted of mother-liquor supplemented with 20% (vol/vol) glycerol. Data were collected under cryogenic conditions at the European

Synchrotron Radiation Facility (Grenoble, France) at beamlines ID23-2 and ID30A-1.

Data were processed with XDS (44) and ccp4-implemented SCALA (45). Structures were determined by molecular replacement with PHASER (46), manually built in COOT (47), and refined with PHENIX (48). Search models were the structures of a putative contractile protein (FliW/bh3618; PDB ID code 2AJ7), GtFliW (present study), and RsmA (PDB ID code 2JPP). Figures were prepared with Pymol (www.pymol.org).

In Vitro Pull-Down Assays. For in vitro pull-down assays CsrA, FliW, and their variants were coproduced in *E. coli* BL21(DE3). The bait protein contained a hexa-histidine tag at its N terminus, whereas the prey protein did not. Cells were resuspended in a lysis buffer containing 20 mM Hepes-Na, 200 mM NaCl, 20 mM MgCl₂, 20 mM KCl, and 40 mM imidazole, pH 8.0. After cell lysis and removal of cell debris by high-speed centrifugation, the lysate was incubated with Ni-agarose beads for 5 min. After excessive washing of the beads with lysis buffer, bound protein was eluted in a buffer containing 20 mM Hepes-Na, 200 mM NaCl, 20 mM MgCl₂, 20 mM KCl, and 500 mM imidazole, pH 8.0. Eluates were analyzed by Coomassie-stained SDS/PAGE.

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