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Regulation of CsrB/C sRNA decay by EIIA^{Glc} of the phosphoenolpyruvate: carbohydrate phosphotransferase system

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Summary

Csr is a conserved global regulatory system, which uses the sequence specific RNA binding protein CsrA to activate or repress gene expression by binding to mRNA and altering translation, stability and/or transcript elongation. In *Escherichia coli*, CsrA activity is regulated by two sRNAs, CsrB and CsrC, which bind to multiple CsrA dimers, thereby sequestering this protein away from its mRNA targets. Turnover of CsrB/C sRNAs is tightly regulated by a GGDEF-EAL domain protein, CsrD, which targets them for cleavage by RNase E. Here, we show that EIIA^{Glc} of the glucose-specific PTS system is also required for the normal decay of these sRNAs and that it acts by binding to the EAL domain of CsrD. Only the unphosphorylated form of EIIA^{Glc} bound to CsrD *in vitro* and was capable of activating CsrB/C turnover *in vivo*. Genetic studies confirmed that this mechanism couples CsrB/C sRNA decay to the availability of a preferred carbon source. These findings reveal a new physiological influence on the workings of the Csr system, a novel function for the EAL domain, and an important new way in which EIIA^{Glc} shapes global regulatory circuitry in response to nutritional status.

Keywords

sRNA decay; Csr system; PTS; GGDEF-EAL protein; global regulation; CsrD

Introduction

The Csr (carbon storage regulator) or Rsm (repressor of stationary phase metabolites) system is present in diverse eubacteria, where it globally regulates metabolism, biofilm

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formation, motility, virulence, quorum sensing, and stress response systems (Romeo, 1998, Babitzke & Romeo, 2007, Romeo *et al.,* 2013, Vakulskas *et al.,* 2015). The RNA binding protein CsrA/RsmA of the Csr system regulates gene expression by interacting with sequences in mRNA, thus altering translation, mRNA stability, and/or transcript elongation. CsrA governs genes responsible for bacterial lifestyle transitions, repressing processes that are triggered upon entry into the stationary phase of growth and conferring stress resistance, while activating processes such as glycolysis, which support vigorous growth. In *Escherichia coli*, CsrA activity is mainly controlled by the noncoding sRNAs, CsrB and CsrC, which contain multiple CsrA binding sites, allowing them to antagonize CsrA activity by sequestering it away from lower affinity mRNA targets (Liu *et al.,* 1997, Weilbacher *et al.,* 2003). Fluctuations in CsrB/C levels play a central role in regulating Csr system and the bacterial lifestyle.

Multiple factors ensure appropriate expression of *csrB/C*. Transcription is activated by the BarA-UvrY two-component signal transduction system (TCS) in response to carboxylic acids (Suzuki *et al.,* 2002, Chavez *et al.,* 2010, Martínez *et al.,* 2014). In a complex negative feedback loop, CsrA regulates *csrB/C* transcription by activating both the expression of the response regulator UvrY and its phosphorylation by the sensor-kinase BarA (Suzuki *et al.,* 2006, Camacho *et al.,* 2015, Suzuki *et al.,* 2002). Amino acid starvation and other stresses activate *csrB/C* transcription via the stringent response components ppGpp and DksA (Edwards *et al.,* 2011) and two DEAD-box RNA helicases, DeaD and SrmB, activate *csrB/C* transcription by distinct mechanisms (Vakulskas *et al.,* 2014).

In contrast to synthesis, the decay of CsrB/C RNAs is not well understood. A specificity factor, CsrD, is necessary for degradation of CsrB/C by the housekeeping nucleases RNase E and PNPase (Suzuki *et al.,* 2006). CsrD does not appear to be a nuclease, but renders CsrB/C susceptible to degradation by RNase E, thus affecting the expression of CsrA-regulated genes in a predictable fashion. CsrD contains GGDEF and EAL domains, which are often responsible for synthesis and degradation of the secondary messenger cyclic dimeric (3′→5′) GMP (c-di-GMP). However, biochemical and genetic studies indicated that CsrD displays no c-di-GMP synthetic or hydrolytic activity and that CsrD activity is not regulated by c-di-GMP *in vivo*. At present, the molecular mechanism of CsrD effects on CsrB/C degradation is unclear. CsrD bound nonspecifically to CsrB/C *in vitro* (Suzuki *et al.,* 2006). Accordingly, one hypothesis is that CsrD evolved from the GGDEF-EAL domain family, becoming an RNA binding protein. How CsrD activity is regulated is another open question. CsrA weakly represses *csrD* expression in *E. coli* and *Salmonella* Typhimurium (Suzuki *et al.,* 2006, Jonas *et al.,* 2010), but does not seem to affect CsrB turnover in *E. coli* (Gudapaty *et al.,* 2001), and no other factors are known to affect CsrD activity.

Glucose is the preferred carbon and energy source for *E. coli* and is taken up primarily by the glucose-specific phosphoenolpyruvate-dependent sugar-phosphotransferase system, PTS (Deutscher *et al.,* 2006, Deutscher *et al.,* 2014, Lengeler & Jahreis, 2009). This system consists of two cytoplasmic proteins, enzyme I (EI) and histidine phosphocarrier protein (HPr) that are used for transporting many sugars, and two glucose-specific proteins, enzyme IIA^{Glc} ($EIIA^{Glc}$) and the membrane-bound enzyme $IIBC^{Glc}$ ($EIIBC^{Glc}$). Glucose uptake is coupled to its phosphorylation. The phosphoryl group is donated by PEP and transferred to

glucose via a phosphorylation cascade formed by EI, HPr, EIIA^{Glc}, and EIIBC^{Glc} proteins. Thus, the phosphorylation state of the PTS proteins depends both on extracellular carbon availability and the metabolic state of the cell.

The glucose-PTS proteins also mediate regulatory functions (Gabor *et al.*, 2011, Deutscher *et al.*, 2014). EIIA^{Glc} is a central regulator of carbon metabolism. Unphosphorylated EIIA^{Glc} mediates inducer exclusion by binding to and inhibiting transporters of non-PTS sugars (Deutscher *et al.*, 2014, Deutscher *et al.*, 2006). It also inhibits metabolism of alternative carbon sources, e.g. by binding to glycerol kinase (Postma *et al.*, 1984). In contrast, phosphorylated EIIA^{Glc} (EIIA^{Glc}-P) binds to and stimulates the activity of adenylate cyclase, which produces cAMP. This compound acts as a secondary messenger that binds to the cAMP receptor protein (Crp), forming a transcription factor (cAMP-Crp) that exerts global effects on the proteome, ensuring efficient resource utilization (Krin *et al.*, 2002, Park *et al.*, 2006, Bao & Duong, 2013). With the discovery of novel EIIA^{Glc} binding partners in various species, EIIA^{Glc} has been found to participate in chemotaxis (Neumann *et al.*, 2012), respiration/fermentation (Koo *et al.*, 2004), biofilm formation (Pickering *et al.*, 2012) and virulence (Kim *et al.*, 2010, Mazé *et al.*, 2014). The EIIBC^{Glc} protein also carries out a variety of regulatory functions (Lux *et al.*, 1995, Nam *et al.*, 2001, Tanaka *et al.*, 2000, Lee *et al.*, 2000).

In a screen for EIIA^{Glc} binding partners in *Vibrio cholerae*, a CsrD homologue, MshH was identified (Pickering *et al.*, 2012). While no function was assigned to this interaction, it was hypothesized that EIIA^{Glc} might affect the decay of Csr sRNAs. Here, we present the results of a detailed investigation of the role of EIIA^{Glc} in CsrB/C decay in *E. coli*. Unphosphorylated EIIA^{Glc} binds specifically to the EAL domain of CsrD and stimulates CsrB/C turnover. We propose that this mechanism helps to increase the concentration of free CsrA when it is needed to support growth, and simultaneously poises the Csr system for rapid response to changing environmental conditions.

Results

EIIA^{Glc} activates CsrB/C decay via CsrD

To start to investigate the possible connection between EIIA^{Glc} and the Csr system in *E. coli*, we decided to determine if EIIA^{Glc} participates in the degradation of CsrB/C in this species. To do so, we first determined the stability of CsrB/C in the presence or absence of EIIA^{Glc} (*Δcrr*) after the addition of rifampicin to the exponentially growing cultures. Deletion of *crr* decreased CsrB and CsrC decay rates by about 5-fold and 3-fold, respectively (Figure 1A–D). Ectopic expression of *crr* complemented the *crr* defect, confirming that EIIA^{Glc} somehow regulates CsrB/C decay (Figure 1A–D). As discussed below, a site-directed *crr* mutant allele, encoding an EIIA^{Glc} protein that cannot be phosphorylated, H91A, also complemented the *crr* deletion (Figure 1A–D).

As shown in Figure 1E, while EIIA^{Glc} greatly stimulated CsrB/C decay, it modestly reduced the levels of CsrB/C in the cell. A similar observation was also made previously in a *csrD* mutant strain, and was shown to be the result of the Csr regulatory circuitry (Suzuki *et al.*, 2006; Figure 1F). CsrA indirectly activates transcription of CsrB/C via the BarA-UvrY TCS

(Suzuki *et al*., 2002). Thus, when CsrB/C decay is inhibited, these sRNAs accumulate and sequester CsrA, causing a decrease in their transcription and an attenuated effect on their levels.

We next performed an epistasis experiment to determine whether the effect of $EIIA^{Glc}$ on CsrB/C decay was dependent on CsrD. A *Δcrr ΔcsrD* double deletion strain was severely defective in CsrB/C decay (Figure 2A and B), as reported previously for the *ΔcsrD* mutant (Suzuki *et al*., 2006). While ectopic expression of *crr* in the *Δcrr ΔcsrD* strain failed to restore CsrB/C decay, ectopic overexpression of *csrD* enhanced CsrB/C decay rates to wild-type levels (Figure 2A and B). This finding suggested that CsrD functions downstream of $EIIA^{Glc}$ in CsrB/C turnover.

A possible explanation for the epistasis results is that $EIIA^{Glc}$ affects CsrD levels in the cell by altering its stability or synthesis. However, deletion of *crr* had no effect on the levels of a chromosomally encoded and biologically functional CsrD-FLAG protein (Figure 2C and Figure S1).

In vitro binding of $EIIA^{Glc}$ to CsrD requires the EAL domain

Because $EIIA^{Glc}$ regulates the activities of several proteins via direct binding, it was reasonable to speculate that $EIIA^{Glc}$ affects CsrB/C decay via direct binding to CsrD in *E. coli*. To test this idea, we examined the binding of $EIIA^{Glc}$ and CsrD in an *in vitro* binding assay or pull-down assay using His-tagged $EIIA^{Glc}$ and a soluble recombinant CsrD protein ($CsrD^{\Delta TM}$) in which the N-terminal transmembrane domains of CsrD were replaced with a maltose binding protein (MBP) tag (Figure 3A). Previous studies showed that the transmembrane domains of CsrD were dispensable for its activity when the protein was ectopically expressed (Suzuki *et al*., 2006). In this assay, CsrD was mixed with Ni-NTA resin with or without His-tagged $EIIA^{Glc}$. As shown in Figure 3B, the $CsrD^{\Delta TM}$ variant was retained by the Ni-NTA resin when $EIIA^{Glc}$ was bound to it, but remained in the unbound fraction when $EIIA^{Glc}$ was absent, indicating that $EIIA^{Glc}$ bound directly to CsrD.

To determine which domain of CsrD protein is involved in the interaction with $EIIA^{Glc}$, we tested similar MBP fusions of CsrD lacking the EAL ($CsrD^{\Delta EAL}$), GGDEF ($CsrD^{\Delta GGDEF}$), or HAMP-like domain ($CsrD^{\Delta HAMP}$ and $CsrD\Delta^{Coil}$), using the pull-down assays. While the other CsrD variants retained the ability to bind to $EIIA^{Glc}$, $CsrD^{\Delta EAL}$ lost all detectable binding, suggesting that the EAL domain is involved in this interaction (Figure 3B and 3C). Moreover, the EAL domain alone bound to $EIIA^{Glc}$ in this assay (Figure 3D). These results indicated that $EIIA^{Glc}$ binds specifically to the EAL domain of CsrD.

To examine the binding reaction of $EIIA^{Glc}$ with CsrD in more detail, the size and composition of the $EIIA^{Glc}$-EAL complex was analyzed by gel-filtration chromatography. The free EAL and $EIIA^{Glc}$ eluted at positions corresponding to sizes of their monomeric forms (EAL, 72kDa; $EIIA^{Glc}$, 20 kDa) (Figure 3E and F). When $EIIA^{Glc}$ and EAL were mixed to allow binding, and fractionated on Superdex 200 (HiLoad™ 16/60, GE Healthcare), a new peak was observed at a position corresponding to a size of 98kDa, approximately that of a heterodimer of $EIIA^{Glc}$-EAL (92kDa). To determine the ratio of $EIIA^{Glc}$ and EAL in the complex, column fractions corresponding to the presumptive

EIIA^{Glc}-EAL complex and the free EIIA^{Glc} were analyzed by SDS-PAGE with Commassie blue staining and quantification of the stained proteins (Figure 3F). This experiment revealed the molar ratio of EAL bound to EIIA^{Glc} in peak fractions (11 and 12) to be 1:1 suggesting that EIIA^{Glc} binds to the EAL domain of CsrD in a one to one ratio.

In the pull down assay, the relative amount of CsrD$^{\Delta TM}$ or CsrD$^{\Delta GGDEF}$ bound by EIIA^{Glc} was much greater than that of proteins that lacked the intact HAMP-like domain, CsrD$^{\Delta HAMP}$ or CsrD$^{\Delta Coil}$ (Figure 3B and C). The HAMP domain typically promotes dimerization or protein-protein interactions and plays important roles in signal transduction (Hulko *et al.*, 2006). We used gel filtration assays to determine the *in vitro* oligomeric states of CsrD variants containing or lacking the HAMP-like domain. All CsrD variants containing the HAMP-like domain, CsrD$^{\Delta TM}$, CsrD$^{\Delta GGDEF}$ and CsrD$^{\Delta EAL}$, eluted at volumes consistent with their tetrameric forms (Figure S2 and Table S4). In contrast, the CsrD variants with a disrupted HAMP-like domain, CsrD$^{\Delta HAMP}$ and CsrD$^{\Delta Coil}$, eluted as apparent monomers (Figure S2 and Table S4). The precise way in which tetramerization affected EIIA^{Glc} binding by the CsrD variants was not further investigated.

EIIA^{Glc} regulates CsrB/C decay in a phosphorylation-dependent manner

EIIA^{Glc} typically modulates the activity of its binding partners in a phosphorylation-dependent manner. Accordingly, we tested the effect of the phosphorylation state of EIIA^{Glc} on CsrB/C decay. We first investigated the impact of the unphosphorylated EIIA^{Glc} on CsrB/C turnover using a site-directed mutant protein that could not be phosphorylated (EIIA^{Glc} H91A). Plasmid complementation of the *Δcrr* strain with EIIA^{Glc} H91A restored CsrB/C decay rates to slightly higher than in the wild-type strain (Figure 1A–D), demonstrating that phosphorylation of EIIA^{Glc} was dispensable for activation of CsrB/C turnover.

Next, we performed pull-down assays to determine the effect of EIIA^{Glc} phosphorylation on binding to CsrD. In these experiments, CsrD containing an N-terminal MBP tag was bound to amylose resin and then mixed with EIIA^{Glc} in reactions that were designed to produce either the phosphorylated or unphosphorylated form of this protein. In one reaction, *E. coli* Hpr, EI and PEP were mixed to provide phosphorylated EIIA^{Glc}. In the other reaction, pyruvate was added instead of PEP to maintain EIIA^{Glc} in the unphosphorylated form. Strikingly, while most of the unphosphorylated EIIA^{Glc} bound to CsrD, no binding was observed between the phosphorylated EIIA^{Glc} and CsrD (Figure 4). These results were in agreement with the observation that EIIA^{Glc} did not require phosphorylation for activation of CsrB/C turnover *in vivo* (Figure 1A–D), and indicated that the binding of unphosphorylated EIIA^{Glc} to CsrD activates CsrB/C sRNA decay.

cAMP-Crp modestly represses CsrB turnover

While the unphosphorylated EIIA^{Glc} bound to CsrD *in vitro* and was able to activate CsrB/C decay *in vivo*, we wondered if the phosphorylated form of EIIA^{Glc} might affect CsrB/C turnover via its important role in cAMP-Crp production (Krin *et al*, 2002, Park *et al*, 2006). Deletion of *cyaA* or *crp* modestly increased the CsrB decay rate by 2-fold (Figure 5A), while exhibiting weak or negligible effects on CsrC decay (Figure 5B). The increased decay rate of

CsrB in the *cyaA* mutant was restored by exogenous cAMP (10 mM), confirming that cAMP-Crp somehow inhibits CsrB decay. Deletion of *cyaA* or *crp* in the *Δcrr* background had twofold effects on CsrB decay rates that were similar to those in the wild-type background, and deletion of *crr* had similar fivefold effects on CsrB decay in both the wild type strain and its isogenic *crp* and *cyaA* mutants (Figure 5A). These findings confirmed that the major effect of $EIIA^{Glc}$ on CsrB decay is mediated independently of cAMP-Crp. The modest effect of cAMP-Crp in the *Δcrr* background was likely due to basal adenylate cyclase activity in the *crr* mutant (Lévy *et al.*, 1990, Feucht & Saier, 1980, Reddy & Kamireddi, 1998).

CsrB/C decay is regulated in response to carbon availability via the phosphorylation state of $EIIA^{Glc}$

The phosphorylation state of $EIIA^{Glc}$ is determined by carbon sources that are taken up and metabolized (Hogema *et al.*, 1998, Deutscher *et al*., 2014). Preferred carbon sources such as glucose lead to net dephosphorylation of PTS proteins, including $EIIA^{Glc}$, whereas unfavorable carbon sources or carbon starvation conditions cause the accumulation of phosphorylated $EIIA^{Glc}$. Because the unphosphorylated $EIIA^{Glc}$ bound to CsrD *in vitro* and promoted CsrB/C decay *in vivo* (Figures 1, 4), we expected that CsrB/C decay rates should be elevated in the presence of glucose. Consequently, we first examined CsrB/C decay in minimal medium supplemented with 0.2% glucose, glycerol or succinate. Both the phosphorylation state of $EIIA^{Glc}$ and CsrB/C decay rates responded predictably to these carbon sources; more rapid decay was observed in glucose compared to glycerol or succinate (Figure 6).

To verify that the phosphorylation state of $EIIA^{Glc}$ determines the decay rates of CsrB/C in response to different carbon sources, we examined CsrB/C turnover in a strain that expresses the mutant $EIIA^{Glc}$ protein, H91A, which cannot be phosphorylated. Because the strain expressing H91A has a significant growth defect in minimal medium (data not shown) the WT and H91A strains were first grown in LB broth to exponential phase and then washed and inoculated into minimal medium lacking a carbon compound or containing 0.2% glucose or succinate. The phosphorylation state of $EIIA^{Glc}$ was determined from growth in LB (Figure 7A) and 10 min after inoculation into minimal media (Figure 7B). $EIIA^{Glc}$ phosphorylation in LB was ~40% and increased to ~90% in media with succinate or lacking a carbon source, while it decreased to 4% at 10 min after glucose exposure. Decay rates of CsrB/C were determined 10 min after inoculation (Figure 8). The decay rates of CsrB and CsrC in the wild-type strain (WT) were ~3.5 and 2.5-fold greater, respectively, in medium with glucose vs. no carbon source. A more modest, but reproducible difference (~2 fold) was observed for CsrB/C decay rates in glucose compared to succinate. These data support the observations described above, showing that CsrB/C decay rates vary in response to different carbon conditions, although the difference in decay between succinate and carbon-deficient media does not seem to be explained by $EIIA^{Glc}$ phosphorylation alone (Figure 8), as both conditions resulted in similar $EIIA^{Glc}$-P levels (Figure 7B). Most importantly, CsrB/C decay in the H91A strain was rapid and virtually identical in all three media, confirming that the phosphorylation state of $EIIA^{Glc}$ determines CsrB/C decay in response to carbon substrate availability. The levels of CsrB/C RNAs (Figure 8E) were consistent with these decay rates,

but as observed previously (Figure 1), the effects of turnover may be attenuated via the feedback loop of the Csr circuitry (Figure 1F; Suzuki *et al.*, 2002; 2006).

EIIA^{Glc} and MshH promote Csr sRNA decay in Vibrio cholerae

EIIA^{Glc}, RNase E and CsrD orthologs are widespread in *Enterobacteriaceae*, *Vibrionaceae*, and *Shewanellaceae* species (Suzuki *et al.*, 2006, Vakulskas *et al.*, 2015, Comas *et al.*, 2008), suggesting that a common mechanism may exist for Csr sRNA decay in members of these bacterial families. As a proof of principle, we tested the effects of EIIA^{Glc} and the CsrD homolog, MshH, on decay of the *V. cholerae* sRNAs, CsrB, CsrC and CsrD (Lenz *et al.*, 2005). The *V. cholerae* sRNAs exhibited longer half-lives compared to the *E. coli* sRNAs under our growth conditions (Figure S3). Nevertheless, deletion of *mshH, crr* or both genes greatly decreased CsrB and CsrD turnover. These effects were not apparent for CsrC, which was already extremely stable in the wild-type strain. This experiment demonstrated the potential of EIIA^{Glc} and CsrD to activate the decay of Csr sRNAs in this important member of the *Vibrionaceae* family.

Discussion

Here, we identified a new regulatory function for EIIA^{Glc}, in which it binds to the sRNA decay protein CsrD and stimulates CsrB/C decay when glucose is present. This mechanism should enhance the concentration of free CsrA, which activates glycolysis and represses gluconeogenesis, secondary metabolism, and stress resistance responses such as biofilm formation (Babitzke & Romeo, 2007, Vakulskas *et al.*, 2015, Romeo *et al.*, 2013). Because CsrA regulates lifestyle transitions in many bacterial species and interacts with hundreds of transcripts in *E. coli* (Babitzke & Romeo, 2007, Patterson-Fortin *et al.*, 2013, Edwards *et al.*, 2011, Vakulskas *et al.*, 2015), we propose that this represents a particularly important role for EIIA^{Glc}. A high rate of turnover can facilitate rapid changes in transcript levels. Therefore, EIIA^{Glc}-CsrD interactions should not only allow CsrB/C decay rates to be reset in response to changing glucose availability, but may poise the Csr system for rapid responses to other cues or conditions when glucose is present. The *V. cholerae* CsrD ortholog protein MshH and EIIA^{Glc} also activated the decay of CsrB and CsrD sRNAs (Figure S3). We suspect that the mechanism described for *E. coli* CsrB/C turnover operates in many species of *Enterobacteriaceae*, *Vibrionaceae* and *Shewanellaceae*.

The conclusion that only the unphosphorylated form of EIIA^{Glc} is able to promote CsrB/C decay through binding interactions with CsrD was based on a combination of biochemical and genetic evidence. CsrD bound only to the unphosphorylated form of EIIA^{Glc} *in vitro* (Figure 4). Furthermore, a non-phosphorylatable protein, EIIA^{Glc}-H91A, sustained CsrB/C decay rates that were similar to or even greater than the wild-type protein (Figure 1A–D). The presence of glucose also caused net dephosphorylation of EIIA^{Glc} and supported rapid decay of CsrB/C sRNAs relative to carbon starvation conditions or alternative carbon sources (Figure 6–8). Importantly, EIIA^{Glc}-H91A supported high decay rates under all of these conditions, confirming that the effects of carbon availability on CsrB/C decay are mediated thorough altered phosphorylation of EIIA^{Glc} (Figure 8). A previous study with MshH of *V. cholerae* concluded that both the phosphorylated and unphosphorylated forms of

EIIA^{Glc} bind to the CsrD homolog. This conclusion was based on the observation that in a two-hybrid assay, MshH interacted with mutant proteins designed to mimic the unphosphorylated (EIIA^{Glc}-H91A) or the phosphorylated (EIIA^{Glc}-H91D) forms of EIIA^{Glc} (Pickering *et al.*, 2012). However, we caution that EIIA^{Glc}-H91D does not appear to mimic EIIA^{Glc}-P. Another putative EIIA^{Glc}-P mimic, EIIA^{Glc}-H91E, was unable to activate adenylate cyclase (Reddy & Kamireddi, 1998). Similarly, effects of EIIA^{Ntr}-P were not mimicked by replacing its phosphorylatable His residue with either Asp or Glu (Lüttmann *et al.*, 2009). Finally, we constructed EIIA^{Glc}-H91D in *E. coli* and found that it behaved similarly to the unphosphorylated EIIA^{Glc} rather than EIIA^{Glc}-P in CsrB/C decay (Figure S4).

The phosphorylated form of EIIA^{Glc} activates cAMP synthesis by binding to adenylate cyclase. Because cAMP and Crp modestly repressed CsrB decay (Figure 5), we propose that EIIA^{Glc}-P indirectly and modestly represses CsrB turnover, reinforcing the positive effect of unphosphorylated EIIA^{Glc} on CsrB decay. Because a potential Crp binding site was predicted in the untranslated leader region of *csrD* (data not shown), we tested the possibility that Crp inhibits CsrB decay by controlling *csrD* expression. Weakly positive to negligible effects of Crp were observed on CsrD levels (Figure S5), which might contribute to the effect of Crp on CsrB decay.

Given that EIIA^{Glc} acts via CsrD without altering its levels in the cell and that overexpression of *csrD* restored CsrB/C decay in a strain deleted for the EIIA^{Glc} gene, *crr* (Figure 2), we propose that EIIA^{Glc} functions as an allosteric activator of CsrD, perhaps similar to the role of EIIA^{Glc}-P in activating adenylate cyclase (Saier, 1989, Park *et al.*, 2006). Structural studies of EIIA^{Glc} show that it possesses a concave face that allows it to interact with globular target proteins (Hurley *et al.*, 1993, Chen *et al.*, 2013, Wang *et al.*, 2000, Cai *et al.*, 2003). Other EIIAs seem unable to duplicate this function (Deutscher *et al.*, 2006). We deleted the genes for five other EIIAs (*fruB*, *mtlA*, *chbA*, *manX* and *ptsN*) and found that none of them regulated CsrB/C decay (data not shown).

This study also expands our understanding of the functionality of the EAL domain. This is not the first report of a catalytically inactive EAL domain performing a regulatory role via protein-protein interactions (Römling *et al.*, 2013). The enzymatically inactive EAL domain protein YdiV of *E. coli* binds to the transcription activator $FlhD_4C_2$ and prevents it from binding to target DNA (Li *et al.*, 2012). Similarly, the EAL domain of the *E. coli* photoreceptor YcgF (BluF) binds to the MerR-like repressor YcgE (BluE) in the presence of blue light and prevents it from binding to DNA (Tschowri *et al.*, 2009). Complex regulation exists for the EAL domain of FimX, which binds to c-di-GMP as well as the PilZ protein, and is required for biogenesis of the Type IV pilus (Guzzo *et al.*, 2009). In all of these cases, the EAL domain-containing protein acts as a sensor that uses its EAL domain to transmit information to another protein. In contrast, the EAL domain of CsrD acts as a receiver, to detect signaling information from a sensory protein. We did not examine other EAL domain proteins for binding interactions with EIIA^{Glc}. However, none of the binding partners in *E. coli* or the potential ones in *V. cholerae* contains an EAL domain except for CsrD/MshH (Deutscher et al., 2014, Pickering et al., 2012). The modular structure of CsrD suggests that the effect of EIIA^{Glc} might be transmitted through other CsrD domains, such as the GGDEF

domain, which is also necessary for CsrD activity (Suzuki *et al.*, 2006), although this possibility has not been explored.

Our previous data suggested that CsrD might function as a CsrB/C binding protein, converting the sRNAs into substrates for RNase E degradation (Suzuki *et al.*, 2006). However, $EIIA^{Glc}$ did not increase the binding affinity or specificity of CsrD for CsrB in electrophoretic mobility shift assays (Figure S6). Presently, we cannot distinguish whether (i) additional unknown factors or conditions are required to reconstitute the effect of $EIIA^{Glc}$ on CsrD RNA binding, (ii) $EIIA^{Glc}$ stimulates CsrD activity at a step in the decay pathway other than CsrB/C binding, or (iii) perhaps RNA binding by CsrD is an *in vitro* artifact and not part of its biological function.

While the circuitry surrounding the Csr system is extensive, its role in carbon and energy pathways is particularly wide-ranging and important (Edwards *et al.*, 2011, Patterson-Fortin *et al.*, 2013, Yang *et al.*, 1996, Romeo, 1996, Romeo *et al.*, 2013, Sabnis *et al.*, 1995, Pernestig *et al.*, 2003). Previous studies have shown that carboxylic acid-containing end products of carbon metabolism, such as acetate and formate, stimulate CsrB transcription via the BarA-UvrY TCS (Chavez *et al.*, 2010). Thus, the synthesis pathway and the newly discovered turnover pathway for CsrB/C should mediate reinforcing positive effects on the levels of these sRNAs when preferred carbon resources have been expended and end products have accumulated. The resulting decrease in CsrA activity under this condition should promote the transition from glycolytic metabolism and active growth to gluconeogenesis, glycogen biosynthesis and the formation of a stress resistant phenotype. We caution that other regulators influence the expression of CsrB/C RNAs, e.g. ppGpp, DksA, DeaD and SrmB helicases, and impact the workings of this circuitry. An understanding of the combinatorial effects of all of these factors, and perhaps unknown ones, will require additional investigation.

Experimental procedures

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1. Bacterial strains were routinely grown in Luria-Bertani (LB) broth unless otherwise indicated. For synthetic minimal medium, minimal medium A (Hogema *et al.*, 1998) and M9 minimal medium supplemented with indicated carbohydrates were used. When necessary, the following antibiotics were added to the growth media: ampicillin (100 μg ml^{-1}), tetracycline (15 μg ml^{-1}), kanamycin (50 μg ml^{-1}), and chloramphenicol (25 μg ml^{-1}). *E. coli* and *V. cholerae* strains were grown at 37°C and 27°C, respectively. Stationary phase cultures were routinely used to inoculate LB broth or minimal media unless otherwise indicated. For strains carrying *cyaA* deletion or *crp* disruption, exponentially growing cultures were used to inoculate LB broth supplemented with or without 10 mM cAMP to minimize the growth defect.

Construction of plasmids and mutant strains

The plasmids and related primers and restriction sites used in this study are listed in Table S2 and S3. The plasmid pCRR, used for complementation of a *crr* deletion strain, carries the

crr gene under a mutant *lacUV5* promoter, in which −8 A of the −10 hexamer consensus was replaced with −8 T for decreased promoter activity (Moyle *et al.*, 1991). To generate pCRR, the *crr* gene was amplified from chromosomal DNA of *E.coli* MG1655 and ligated into vector pBR322. Plasmid pCRRH91A expressed the mutant *crr* gene, producing the protein $EIIA^{Glc}$H91A. This plasmid was constructed similarly to pCRR except that the His91Ala (CAC to GCC) substitution was introduced by the megaprimer PCR procedure (Ke & Madison, 1997). Plasmid pBYH4 used for complementation of the *csrD* deletion strain was described previously (Suzuki *et al*., 2006). To construct plasmid pETCRR for expression of C-terminal His-tagged $EIIA^{Glc}$, *crr* was amplified and cloned into plasmid pET24a. Plasmids overexpressing CsrD variants were generated by amplifying the coding regions corresponding to $CsrD^{\Delta TM}$ (residues 156–646), $CsrD^{\Delta HAMP}$ (residues 192–646), $CsrD^{\Delta Coil}$ (residues 156–199 and 220–656), $CsrD^{\Delta GGDEF}$ (residues 156–223 and 393–646), $CsrD^{\Delta EAL}$ (residues 156–385) and EAL (residues 393–646) by standard PCR or overlapping PCR (Urban *et al.*, 1997), and cloning the resulting products into vector pmal-c5x, yielding N-terminally MBP-tagged CsrD variants.

E. coli gene deletions were created by the standard P1 *vir* transduction procedure or the lambda Red system as described (Datsenko & Wanner, 2000). Chromosomal C-terminal FLAG-tagged fusions were generated using the phage lambda Red system as described (Datsenko & Wanner, 2000, Uzzau *et al.*, 2001). The mutant strain H91A carrying a chromosomal point mutation of *crr*, producing $EIIA^{Glc}$ H91A protein, was constructed by using overlapping PCR mutagenesis and the pKOV gene replacement protocol as described (Urban *et al*., 1997, Link *et al.*, 1997).

V. cholerae Δ*crr* and Δ*mshH* mutants were created in the C6706str2 wild-type background (Thelin & Taylor, 1996) by double homologous recombination as previously described (Haugo & Watnick, 2002, Pickering et al., 2012).

Expression and Purification of $EIIA^{Glc}$ and CsrD variants

$EIIA^{Glc}$ was overproduced in *E. coli* strain BL21(DE3) grown in 1L of M9 minimal medium supplemented with 0.8% glucose (w/v). Three hours after the induction with 1mM IPTG at OD_{600} ~ 0.6, cells were harvested and lysed using a French Press. After centrifugation (20,000 × g, 30 min, 4 °C), the soluble fraction of the lysate was applied to a HisTrap column (1ml, GE Healthcare) and eluted with a gradient of imidazole (20–500 mM). Eluted proteins were further purified by gel filtration chromatography (Superdex 75 10/300, GE Healthcare), dialyzed against dialysis buffer (20 mM Tris-HCl, pH7.5, 100 mM NaCl, 1 mM DTT, 10% glycerol) and stored for subsequent experiments.

Overproduction of CsrD variants was from *E. coli* BL21(DE3) strains containing the corresponding plasmids, which were grown in LB medium supplemented with 0.2% glucose (w/v). Cells were induced with 0.3 mM IPTG at OD_{600} ~ 0.6 and lysed as mentioned above. The soluble fraction of the lysate was applied to an MBPTrap column (1ml, GE Healthcare) and eluted with a gradient of 0–10 mM maltose. To obtain homogeneous proteins, eluted proteins were further purified by gel filtration chromatography (Superdex 200 10/300, GE Healthcare) and dialyzed against dialysis buffer.

Northern blotting

Northern blot analysis was performed as previously described, with minor modifications (Vakulskas *et al*, 2014). Bacterial culture were immediately stabilized by the addition of RNAprotect™ Bacteria Reagent (Qiagen) or 0.125 volumes of stop solution (10% phenol/90% ethanol). Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total cellular RNA (1–2 μg) was separated on denaturing 5% acrylamide/7 M urea gels and transferred to a positively charged nylon membrane (Roche Diagnostics) by electroblotting. The membrane was cross-linked using UV light and hybridized overnight at 68°C (CsrB/C of *E. coli*) or 70°C (CsrB/C/D of *V. cholerae*) using a DIG-labeled antisense RNA probe (Table S3), which was prepared with the DIG Northern Starter kit (Roche Diagnostics). Transcripts were detected using the DIG Northern Starter kit (Roche Diagnostics) according to the manufacturer's instructions. Blots were imaged using the ChemiDoc XRS+ system (Bio-Rad) and RNAs were quantified using Quantity One image analysis software (Bio-Rad). Prior to hybridization, the rRNAs (16S and 23S) were stained by methylene blue, which served as loading controls for signal correction.

Western blotting

Western blot analysis was performed using standard laboratory protocols as described (Vakulskas *et al*, 2014). Briefly, total cellular proteins were separated by SDS-PAGE and transferred to 0.2 μm polyvinylidene difluoride membranes (Bio-Rad) by electroblotting. Blots for FLAG epitope-tagged proteins used the anti-FLAG M2 monoclonal antibody (Sigma) at 1:2,000 dilution. Blots for RpoB used anti-RpoB monoclonal antibodies (Neoclone) at 1:50,000 dilution. Western blots were detected using horseradish peroxidase-linked secondary antibodies and the SuperSignal West Femto Chemiluminescent Substrate (Pierce) as recommended by the manufacturer. Proteins were quantified using Quantity One image analysis software (Bio-Rad).

Protein Pull-down assays

Interaction between CsrD variants and $EIIA^{Glc}$ was assessed using purified His-tagged $EIIA^{Glc}$ protein to pull down MBP-tagged CsrD variants. In a 60 μl reaction, 8 μM $EIIA^{Glc}$ and 4 μM CsrD variants were incubated with 15 μl Ni-NTA resin (Qiagen) in binding buffer (50 mM MES pH 6.5, 1 mM DTT, 20 mM Imidazole) at 4°C for 1hour. Unbound proteins remaining in the supernatant solution were collected after brief centrifugation of the resin. The resin was washed three times with 1 ml washing buffer (50 mM MES pH 6.5, 1mM DTT, 60 mM Imidazole), and the bound proteins were eluted with 45 μl of elution buffer (25 mM Tris-HCl pH 8.0, 500 mM NaCl, and 500 mM Imidazole).

To determine the phosphorylation dependence of the interaction between $EIIA^{Glc}$ and CsrD, the MBP-tagged $CsrD^{\Delta TM}$ was used to pull down His-tagged $EIIA^{Glc}$. His-tagged $EIIA^{Glc}$ (8 μM) was incubated in reactions containing EI (1 μM), Hpr (8 μM) and either 5 mM PEP or 5 mM pyruvate in 60 μl buffer (20mM Tris-HCl pH8.0, 2 mM $MgCl_2$, 1 mM DTT) at room temperature for 10 min. These reactions were designed to produce the phosphorylated or unphosphorylated form of $EIIA^{Glc}$, respectively. Reactions were dialyzed against the binding buffer (50mM MES pH6.5, 1mM DTT) with Slide-A-Lyzer dialysis cassette (Thermo) for 1 hour and subsequently incubated at 4°C for 1 hr with $CsrD^{\Delta TM}$ (95 μg) pre-

bound to amylose resin. Unbound proteins were collected after brief centrifugation of the resin and bound proteins were eluted with 60 μl of elution buffer (20 mM Tris-HCl pH7.5, 200 mM NaCl, 10 mM maltose, 1 mM DTT) after extensive rinsing of the resin with binding buffer.

Unbound and bound proteins in the pull-down reactions were detected by SDS-PAGE (10% or 15%, as required) and Coomassie blue staining. Proteins were quantified using Quantity One image analysis software (Bio-Rad).

Determination of the EIIA^{Glc} phosphorylation state

The phosphorylation state of EIIA^{Glc} was determined as previously described (Hogema *et al.*, 1998), with modifications. Briefly, 0.2 ml of bacterial culture (OD_{600} ~ 0.5) was treated with the addition of 20 μl of 10 M NaOH followed by 1 ml of ethanol and 180 μl of 3 M sodium acetate, pH 5.2. After chilling at −80°C for 2 hr, precipitates were collected by centrifugation, rinsed with 70% ethanol, and suspended in 100 μl of sample buffer (0.16 M Tris HCl, pH 7.5, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol). To achieve a good separation of the two forms of EIIA^{Glc}, samples were fractionated on SDS-PAGE gels containing 50 μM of Phos-tag reagent as previously described (Vakulskas *et al.*, 2014). Subsequently, gels were washed with Western transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol, and 0.1% SDS) containing 1 mM EDTA for 10 min, followed by a second wash with transfer buffer for 20 min. Western blot analysis was then performed as described above.

Gel filtration analysis of EIIA^{Glc} in complex with the EAL domain or other CsrD variants

For analysis of the EIIA^{Glc}-EAL complex, a 0.5 ml reaction containing 33 μM EAL, 78 μM EIIA^{Glc} or both proteins were incubated at 4 °C for 30 min and subjected to gel filtration analysis using an AKTA-FPLC system (Superdex 200, HiLoad™ 16/60, 120ml, GE Healthcare), and subsequently eluted at 4°C with a flow rate of 1 ml/min in buffer containing 20 mM Tris HCl pH 7.5, 100 mM NaCl, 1mM DTT. For CsrD variants, 0.5 ml samples containing purified CsrD$^{\Delta TM}$, CsrD$^{\Delta EAL}$, CsrD$^{\Delta HAMP}$, CsrD$^{\Delta Coil}$, or CsrD$^{\Delta GGDEF}$ was separated on the same system. Fractions (3 ml) were collected and analyzed by SDS-PAGE and Coomassie blue staining. For EIIA^{Glc}-EAL analysis, the column was pre-calibrated using 5 gel filtration molecular weight markers (1, sweet potato β-amylase, 200 kDa; 2, yeast alcohol dehydrogenase, 150 kDa; 3, bovine serum albumin, 66 kDa; 4, carbonic anhydrase from bovine erythrocytes, 29 kDa; 5, horse heart cytochrome C, 12.4 kDa), and blue dextran 2000. For CsrD variants analysis, the column was calibrated using 5 molecular weight markers (1, equine spleen apoferritin, 443 kDa; 2, sweet potato β-Amylase, 200 kDa; 3, alcohol yeast dehydrogenase,150 kDa; 4, bovine serum albumin, 66 kDa; 5, carbonic anhydrase from bovine erythrocytes, 29 kDa), and blue dextran 2000. The relative molecular masses of proteins or protein complexes were calculated by logarithmic interpolation from the standards.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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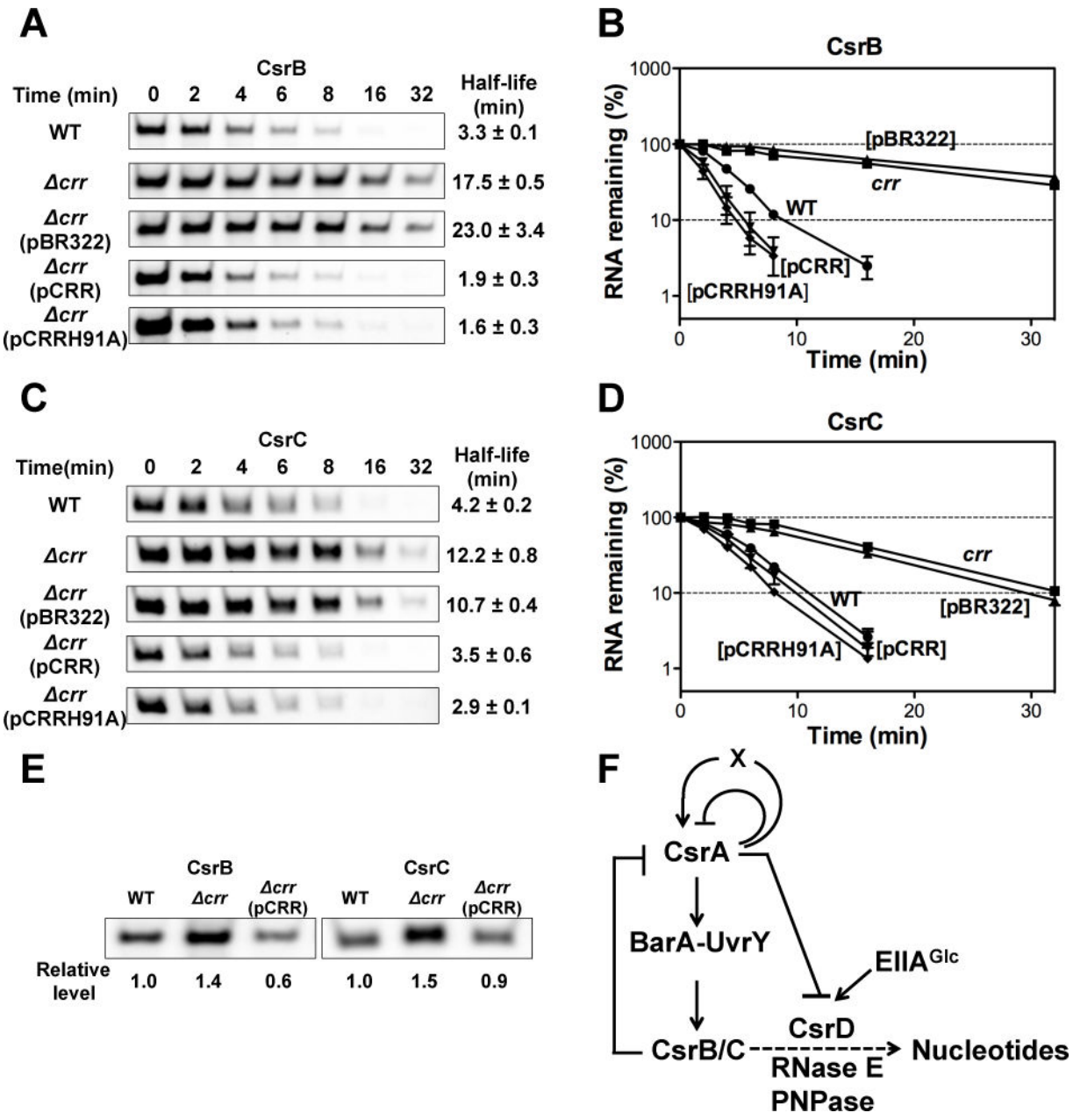

Figure 1.

EIIA^{Glc} affects CsrB/C decay rates and levels in *E. coli.*

A and C. Decay rates of CsrB/C were determined by Northern blotting of RNA extracted from exponential phase cultures (OD_{600} ~0.5) at various times following the addition of rifampicin. Culture identities: *E. coli* MG1655 (WT) or its *Δcrr* mutant with or without plasmid pBR322, pCRR or pCRRH91A (nonphosphorylatable EIIA^{Glc}). The RNA half-lives were determined from the linear portions of their decay curves, shown in B and D. Standard deviations of values from two independent experiments are shown.

E. CsrB/C steady state levels determined by Northern blotting of RNA from exponentially growing cultures (OD_{600} ~0.5), as above.

F. A model for the Csr regulatory circuitry that includes $EIIA^{Glc}$ activation of CsrD-mediated CsrB/C decay. A broken line indicates an undefined mechanism(s).

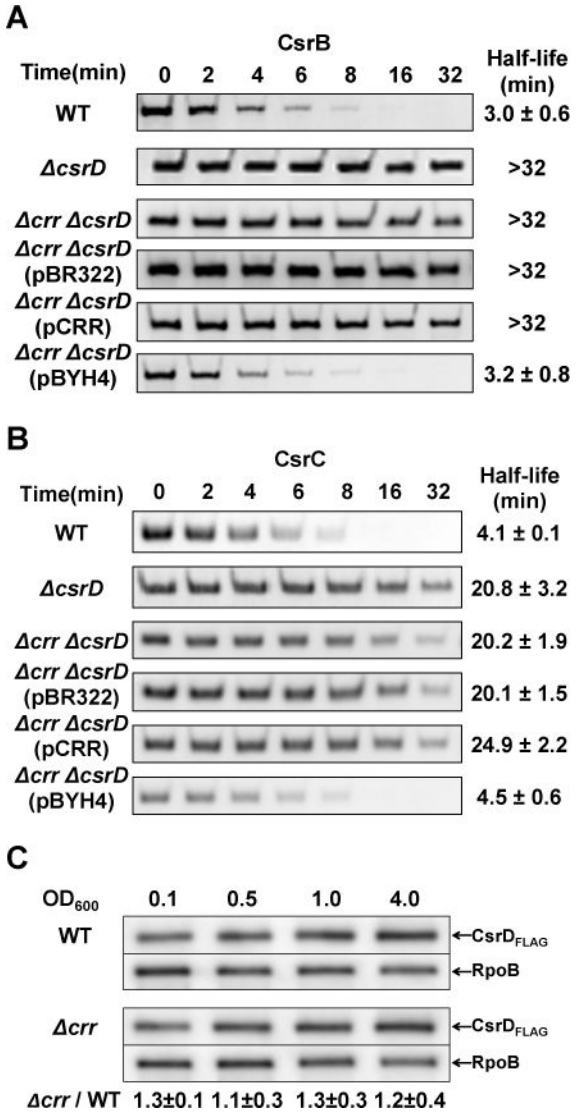

Figure 2.

EIIA^{Glc} (*crr*) activates CsrB/C decay via CsrD, but does not enhance cellular CsrD levels.

A and B. Decay rates of CsrB (A) and CsrC (B) were determined by Northern blotting of RNA from *E. coli* MG1655 (WT), *ΔcsrD*, *Δcrr ΔcsrD* strains with or without plasmid pBR322, pCRR or pBYH4 (expressing CsrD), as described in Figure 1.

C. Western blots depicting the effect of *crr* deletion on the level of CsrD protein. RpoB was used as loading control. CsrD protein levels in *Δcrr* relative to those in the wild-type strain (WT) are shown at the bottom. Standard derivations from triplicate experiments are indicated.

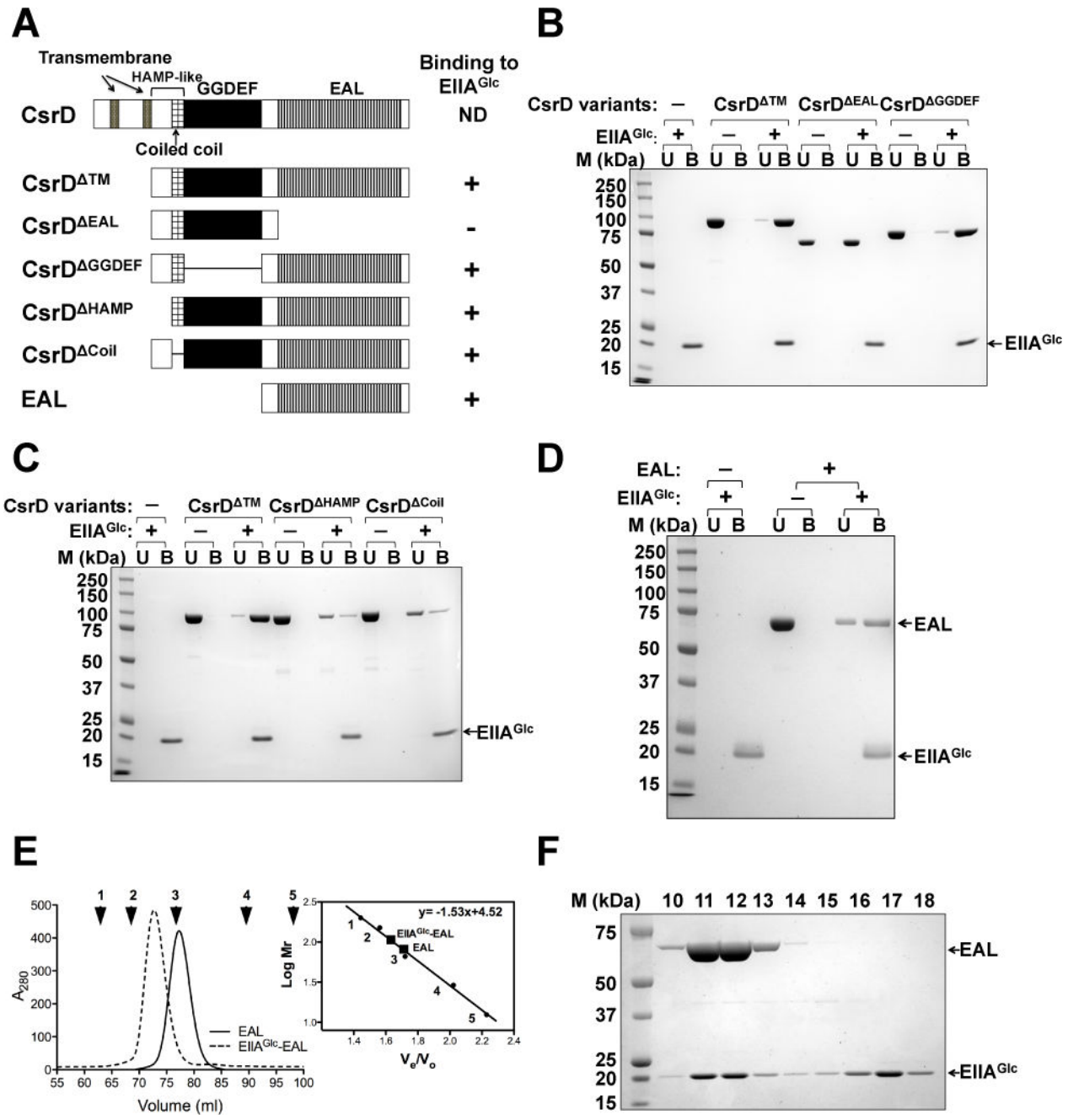

Figure 3.

EIIA^{Glc} interacts specifically with the EAL domain of CsrD.

A. CsrD variants used to identify the domain that binds to EIIA^{Glc}.

B, C and D. *In vitro* assays for binding of EIIA^{Glc} to CsrD variants depicted in panel A. Each reaction contained 8 μM of CsrD variants and 16 μM of EIIA^{Glc}. U and B: unbound and bound proteins. Control reactions were performed in the absence of EIIA^{Glc} to confirm that CsrD varients do not bind nonspecifically to Ni-NTA resin.

E. Gel filtration assay of EIIA^{Glc}, EAL, and EIIA^{Glc}-EAL mixture. Proteins were fractionated on a Superdex 200 column (HiLoad™ 16/60, 120 ml). The solid line

corresponds to EAL domain alone; the dashed line corresponds to the $EIIA^{Glc}$-EAL mixture. The chromatogram for $EIIA^{Glc}$ was not shown because this protein displays little absorbance at 280 nm. Arrows indicate elution volumes of molecular weight markers used to calibrate the column (Experimental procedures).

F. SDS-PAGE and Coomassie blue staining of proteins from gel filtration chromatography fractions of the $EIIA^{Glc}$-EAL mixture shown in panel E. Fractions (3 ml) were collected starting at 40 ml.

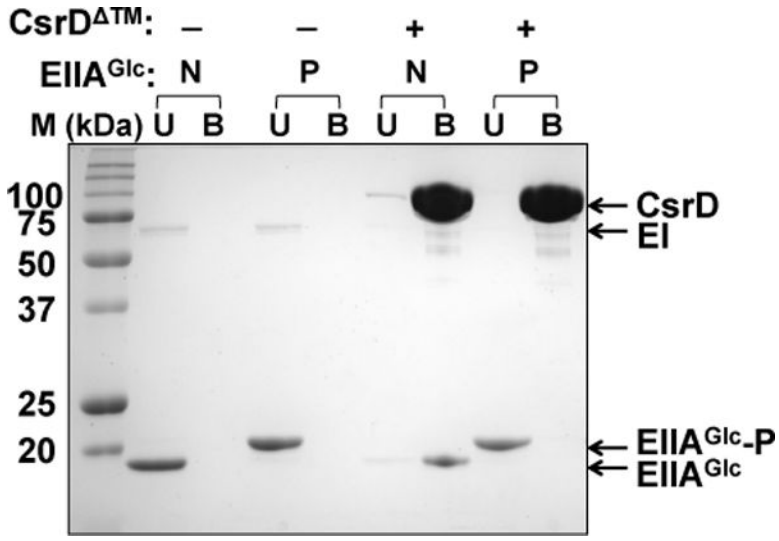

Figure 4.

CsrD binds only to unphosphorylated EIIA^{Glc} in pull-down assays.

MBP-tagged CsrD protein was bound to amylose resin and then mixed with EIIA^{Glc} in the nonphosphorylated (N) or phosphorylated state (P) to permit binding reactions to occur. The reactions were processed as described in the Experimental procedures. Control reactions were performed without CsrD to test for nonspecific binding of the two forms of EIIA^{Glc} to amylose resin. Note that the two forms of EIIA^{Glc} were resolved from each other on 15% SDS-PAGE gel. U and B refer to proteins that were unbound vs. bound by the amylose resin.

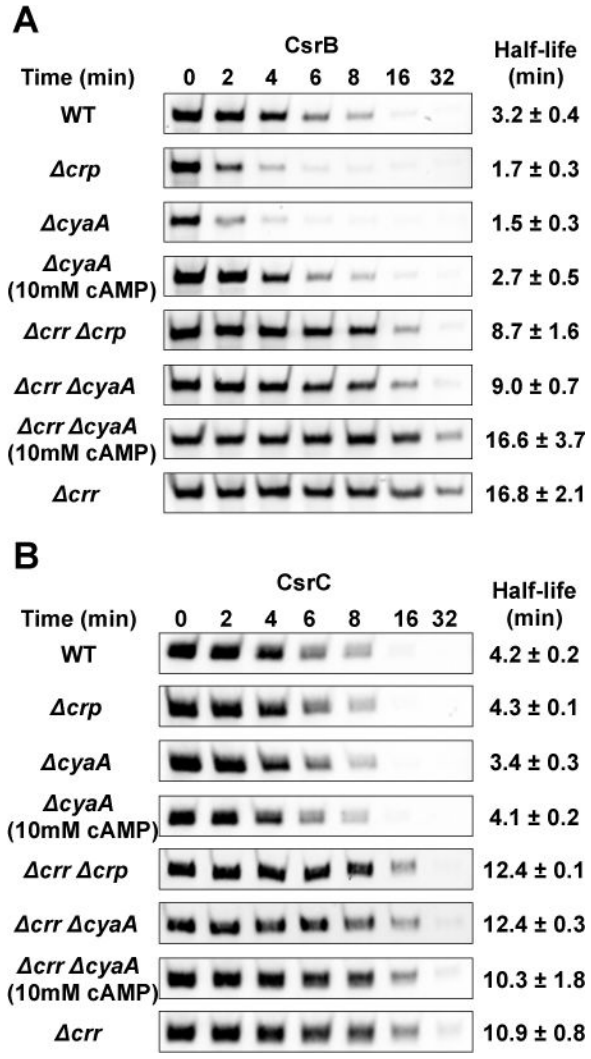

Figure 5.

Effects of cAMP-Crp on CsrB/C decay.

A and B. Decay rates of CsrB/C were determined by Northern blotting of RNA from *E. coli* MG1655 (WT) and isogenic deletion mutants: *cyaA*, *crp*, *cyaA*, *crr crp*, *crr cyaA*, *crr* with or without added 10 mM cAMP, as described in Figure 1.

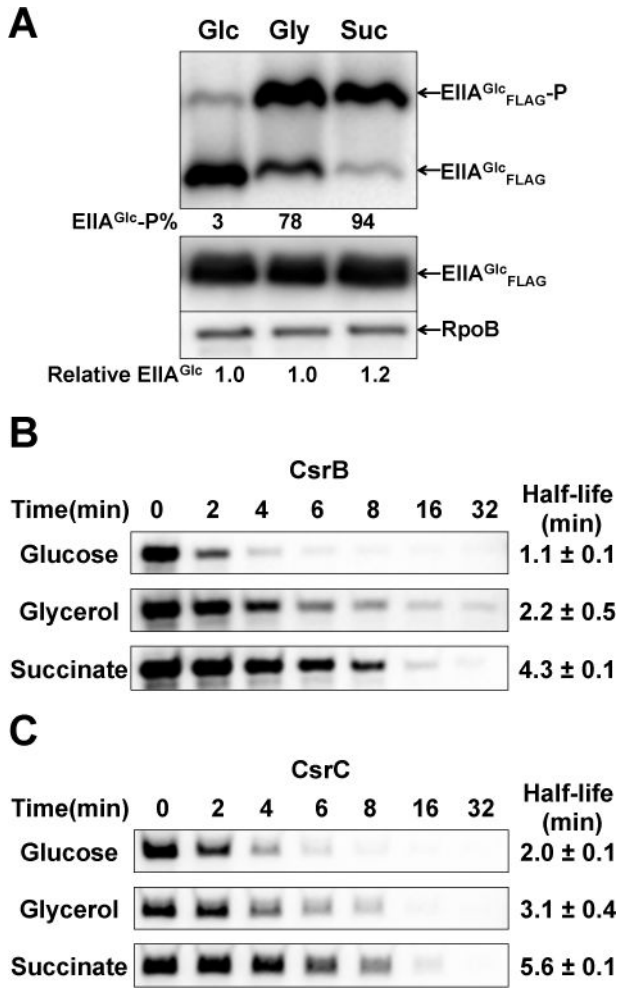

Figure 6.

Effects of carbon sources on EIIA^{Glc} phosphorylation and CsrB/C decay in minimal media. A. Western blot depicting the phosphorylation state of EIIA^{Glc} of *E. coli* MG1655 growing in minimal medium A supplemented with 0.2% glucose, glycerol or succinate. Exponential phase extracts were fractionated in gels with Phos-tag™ reagent and analyzed by Western blot. Relative level of total EIIA^{Glc} was analyzed by western blot without using Phos-tag™ reagent. The percentage of phosphorylated (EIIA^{Glc}-P) and unphosphorylated EIIA^{Glc} (EIIA^{Glc}) and relative total EIIA^{Glc} protein levels are given.

B and C. Northern blot depicting CsrB/C decay rates in *E. coli* MG1655 exponentially growing in minimal medium A supplemented with 0.2% glucose, glycerol or succinate. The RNA half-lives were determined as shown in Figure 1.

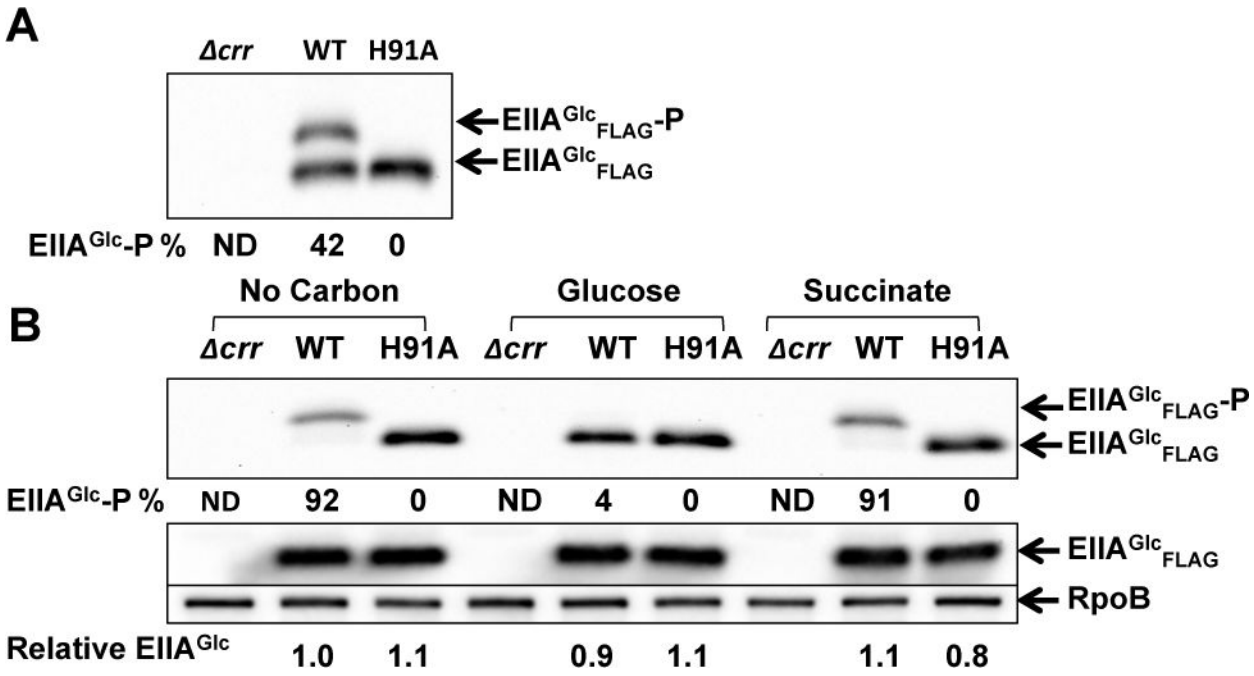

Figure 7.

The phosphorylation state of EIIA^{Glc} before and 10 min after shift from LB broth into minimal media.

A and B. Western blot depicting the phosphorylation state and relative level of EIIA^{Glc} in *E. coli* MG1655 (WT), *Δcrr* and H91A (mutant strain that carrying a chromosomal EIIA^{Glc}H91A point mutation). Extracts were prepared from cultures grown in LB broth (A) and at 10 min after reinoculation into minimal medium without carbon, with 0.2% glucose or succinate (B). The percentage of phosphorylated EIIA^{Glc} relative to total EIIA^{Glc} was determined as described in Figure 6.

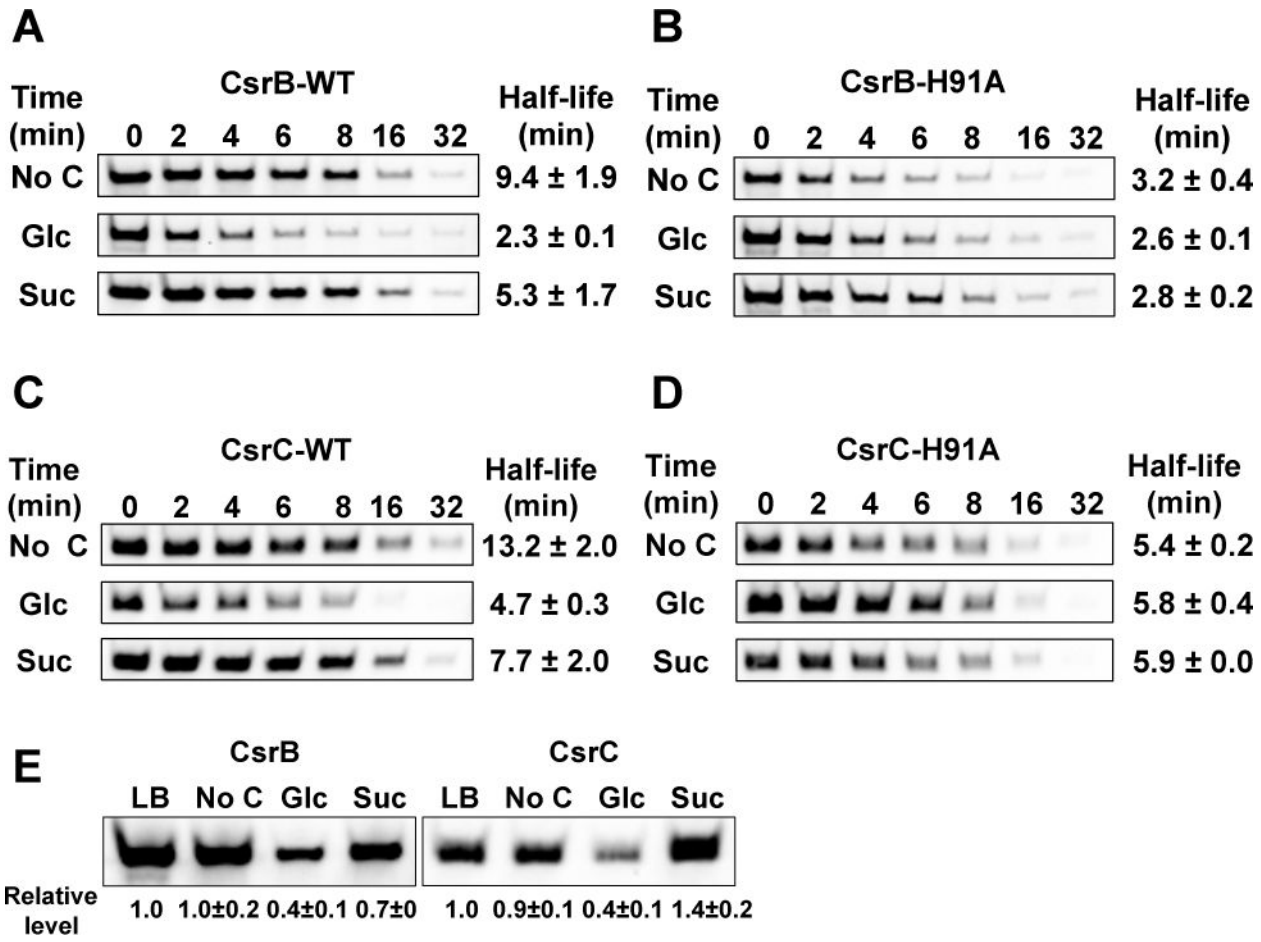

Figure 8.

CsrB/C decay rates and levels after shift from LB to minimal media.

A, B, C and D. Decay rates of CsrB (A and B) and CsrC (C and D) determined by Northern blotting of RNA from *E. coli* MG1655 (WT) and mutant strain H91A (unphosphorylatable $EIIA^{Glc}$). Strains were grown in LB broth to exponential phase, washed and reinoculated into M9 minimal medium without carbon or with 0.2% glucose or succinate. Rifampicin was added 10 min after inoculation into minimal media and RNA half-lives determined as in Figure 1.

E. CsrB/C steady state levels determined by Northern blotting of RNA from *E. coli* MG1655 (WT) in LB broth at exponential growth phase and 10 min after inoculation into minimal media.