Structured and Dynamic Disordered Domains Regulate the Activity of a Multifunctional Anti-σ Factor

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ABSTRACT The anti-σ factor NepR plays a central role in regulation of the general stress response (GSR) in alphaproteobacteria. This small protein has two known interaction partners: its cognate extracytoplasmic function (ECF) σ factor and the anti-anti-σ factor, PhyR. Stress-dependent phosphorylation of PhyR initiates a protein partner switch that promotes phospho-PhyR binding to NepR, which frees ECF σ to activate transcription of genes required for cell survival under adverse or fluctuating conditions. We have defined key functional roles for structured and intrinsically disordered domains of Caulobacter crescentus NepR in partner binding and activation of GSR transcription. We further demonstrate that NepR strongly stimulates the rate of PhyR phosphorylation in vitro and that this effect requires the structured and disordered domains of NepR. This result provides evidence for an additional layer of GSR regulation in which NepR directly influences activation of its binding partner, PhyR, as an anti-anti-σ factor. We conclude that structured and intrinsically disordered domains of NepR coordinately control multiple functions in the GSR signaling pathway, including core protein partner switch interactions and pathway activation by phosphorylation.

IMPORTANCE Anti-σ factors are key molecular participants in a range of adaptive responses in bacteria. The anti-σ factor NepR plays a vital role in a multiprotein partner switch that governs general stress response (GSR) transcription in alphaproteobacteria. We have defined conserved and unconserved features of NepR structure that determine its function as an anti-σ factor and uncovered a functional role for intrinsically disordered regions of NepR in partner binding events required for GSR activation. We further demonstrate a novel function for NepR as an enhancer of PhyR phosphorylation; this activity also requires the disordered domains of NepR. Our results provide evidence for a new layer of GSR regulatory control in which NepR directly modulates PhyR phosphorylation and, hence, activation of the GSR.

Cells employ numerous mechanisms to modulate gene expression in response to changes in the physical and chemical state of the environment. In bacteria, this process is commonly mediated by one of two mechanisms of transcriptional control: (i) two-component signal transduction (TCS) and (ii) alternative σ factor (σ) regulation. The alphaproteobacteria respond to multiple environmental stressors via an atypical, hybrid TCS-σ signaling pathway that controls activity of an extracytoplasmic function (ECF) σ factor, EcfG (1–3). σEcfG activity is regulated at the post-translational level by a partner switch mechanism involving its anti-σ factor, NepR, and the anti-anti-σ factor, PhyR (1). Briefly, phosphorylation of the C-terminal receiver domain of PhyR promotes NepR binding to the σEcfG-like domain of PhyR (PhyR-SL) (4, 5); this frees σEcfG to associate with RNA polymerase (RNAP) and activate transcription (Fig. 1). PhyR, NepR, and σEcfG are broadly conserved in the class Alphaproteobacteria and have been demonstrated to regulate transcription and cell survival in the face of various environmental stressors (1, 6–16).

The primary EcfG family σ factor of Caulobacter crescentus, annotated σC, is a demonstrated regulator of transcription and cell survival under adverse growth conditions (11, 16, 17). The C. crescentus histidine kinase (HK) PhyK phosphorylates PhyR upon stress encounter (16), which promotes its association with NepR and releases σC to activate transcription (5, 16) (Fig. 1A). C. crescentus NepR contains two highly conserved central helices (α1 and α2) connected by a short, 4-residue linker (5). Poorly conserved regions of primary structure (see Fig. S1A in the supplemental material) that we term flanking regions 1 (FR1) and 2 (FR2) border α1 and α2, respectively (2) (Fig. 1C). High-resolution structures of PhyR-SL in complex with NepR determined by X-ray crystallography (5) and nuclear magnetic resonance (NMR) spectroscopy (4) support a model in which NepR α1–α2 is the main structural element that interacts with PhyR-SL (Fig. 1B). These experimental structural data also provide evidence that FR1 and FR2 termini are dynamic and disordered (see Fig. S1B). Though the dynamic termini have highly divergent primary structures, there is evidence that FR1 is required for proper NepR function as an anti-σEcfG factor in Sinorhizobium meliloti (7). However, the
response (GSR) regulatory system in C. crescentus

In vivo anti-PhyR

This protein can also act to stimulate phosphorylation of its anti-PhyR, thereby increasing its affinity for NepR and releasing

function requires both its conserved central helical domain and

unconserved, disordered amino-terminal domain.

Thus, in the context of a fully intact general stress response (GSR) regulatory system in C. crescentus cells, NepR function requires both its conserved central helical domain and unconserved, disordered amino-terminal domain.

Our studies of NepR structure and function further reveal that this protein can also act to stimulate phosphorylation of its anti-PhyR-binding partner, PhyR. Specifically, NepR strongly enhances the rate of PhyR phosphorylation and increases steady-state levels of PhyR-P in vitro; this stimulatory activity requires full-length NepR protein, including the FR1 and FR2 regions. Our data thus provide evidence for an additional layer of GSR control in which low-affinity interaction of full-length NepR with un-phosphorylated PhyR functions to enhance phosphorylation of the PhyR receiver domain and subsequent activation of GSR transcription. Our data support a model for NepR as a multifunctional anti-PhyR factor composed of two functionally distinct regions: (i) a central helical domain that forms an ordered three-dimensional structure and directly interacts with ECF σ and the σ-like domain of PhyR and (ii) an intrinsically disordered amino terminus that determines stable binding to full-length PhyR and ECF σ and that is required for stimulation of PhyR receiver domain phosphorylation.

RESULTS

Unconserved and conserved domains govern NepR binding to full-length PhyR. Ribosome profiling of the 5’ region of nepR revealed three possible start codons (18), suggesting that nepR translation could initiate from multiple sites to yield different isoforms. We show that translation of nepR is highest when all three start codons are present (see Fig. S2 in the supplemental material). To test whether these different putative isoforms of NepR may differentially interact with PhyR and σ5, we used a bacterial two-hybrid (BTH) protein interaction system (19). Three alleles of nepR starting at each of the putative translation start sites (codon 1-NepRFL [full length], codon 8-NepRSC2 [start codon 2], and codon 14-NepRSC1 [start codon 3]) were fused to the T18 fragment and transformed into an Escherichia coli reporter strain expressing full-length PhyR (phyR), PhyR-SL (phyR-SL), or σ5 (sigT) fused to T25. Serial truncation of the first 13 residues of NepR does not have a statistically significant effect on binding to PhyR, PhyR-SL, or σ5 (Fig. 2). Thus, all three versions of NepR are functional in a BTH assay, insofar as they interact with full-length PhyR, PhyR-SL, and σ5.

The observed interactions between full-length PhyR and alleles of NepR by BTH assay suggest that PhyR is phosphorylated in the E. coli cytoplasm, as strong PhyR-NepR binding is dependent on phosphorylation of the PhyR receiver domain (1, 5, 16). To test this hypothesis, we generated T25-PhyR with a mutation in the conserved aspartyl phosphorylation site (D192) of the PhyR receiver domain and transformed it into an E. coli hybrid (BTH) protein interaction system (19). Three alleles of nepR starting at each of the putative translation start sites (codon 1-NepRFL [full length], codon 8-NepRSC2 [start codon 2], and codon 14-NepRSC1 [start codon 3]) were fused to the T18 fragment and transformed into an E. coli reporter strain expressing full-length PhyR (phyR), PhyR-SL (phyR-SL), or σ5 (sigT) fused to T25. Serial truncation of the first 13 residues of NepR does not have a statistically significant effect on binding to PhyR, PhyR-SL, or σ5 (Fig. 2). Thus, all three versions of NepR are functional in a BTH assay, insofar as they interact with full-length PhyR, PhyR-SL, and σ5.

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observed in this strain (see Fig. S3A in the supplemental material), supporting the hypothesis that PhyR is phosphorylated in the *E. coli* reporter strain BTH101. Phosphorylation may be occurring through spurious interaction with an *E. coli* histidine kinase or by a low-molecular-weight phosphoryl donor, such as acetyl phosphate (AcP) (20).

The NepR N and C termini (FR1 and FR2, respectively) are dynamic (4) and disordered (4, 5) in experimental structures of NepR bound to PhyR-SL and display low sequence conservation across species (see Fig. S1A in the supplemental material) (5). Indeed, neural network algorithms (21, 22) trained on a known set of ordered and disordered structures in the Protein Data Bank (PDB) predict that FR1 and FR2 have properties of intrinsically disordered polypeptides (see Fig. S1B). To extend our structure-function analysis of these unconserved, disordered regions of NepR, we generated three additional truncated alleles: *nepR*<sub>H9004</sub>FR1 (expressing residues Q31 to E68), *nepR*<sub>H9004</sub>FR2 (expressing residues M1 to E62), and *nepR*<sub>SV</sub> (SV, short version; expressing residues Q31 to E62). These *nepR* alleles were fused to T18 and transformed into reporter strains expressing full-length PhyR, PhyR-SL, or α<sup>7</sup> fused to T25. Deletion of the disordered N and C termini of NepR does not affect binding to the isolated PhyR-SL domain or to α<sup>7</sup> via BTH assay (Fig. 2). However, deletion of the first 30 residues (FR1) of the NepR amino terminus results in a significant (*P < 0.01*) reduction in PhyR binding (Fig. 2). *NepR<sub>SV</sub>* (in which both FR1 and FR2 are deleted) has further-reduced binding to PhyR (*P < 0.01*). To further control these BTH interaction studies, we conducted the “reverse” experiment, in which each allele under investigation was reciprocally fused to either T25 or T18. No differences in interaction were observed when alleles were expressed from the reciprocal plasmid (see Fig. S3B). As an additional control, we confirmed that the proximity of the T18 subunit to NepR<sub>SV</sub> does not interfere with T25-PhyR interaction by adding a 30-amino-acid (aa) linker between T18 and NepR<sub>SV</sub>. We observed an equivalently weak interaction between NepR<sub>SV</sub> and PhyR in this strain (see Fig. S3B).

Finally, we tested the function of the 4-residue linker sequence between NepR α1 and α2 in NepR substrate binding (Fig. 1C). This region of NepR forms extensive polar contacts with the α-like (SL) domain of *C. crescentus* PhyR (5), suggesting that it plays an important role in PhyR-NepR interaction. A NepR allele with the linker sequence mutated to polyalanine (T18c-NepR<sub>poly-A</sub>) does...
not interact with PhyR, PhyR-SL, or $\sigma^T$ in our BTH assay, confirming the importance of this short linker sequence in the interaction of NepR with its substrates (Fig. 2). Western blot controls confirm that reduced PhyR binding to the NepR$_{sv}$ and NepR$_{poly-A}$ alleles is not a function of variable concentrations of these fusion proteins in the cell (Fig. 2).

**Disordered termini are required for NepR anti-$\sigma^T$ function in vivo.** We next sought to assess the functional implications of our two-hybrid interaction data (Fig. 2) using GSR-dependent transcription in *C. crescentus* cells as a functional readout. We first attempted to generate a strain of *C. crescentus* in which the chromosomal copy of nepR was deleted. We were unable to delete nepR alone but were able to simultaneously delete nepR and sigT. Similar results have been described in *Sphingomonas* species (14) and in *Sinorhizobium meliloti*, where nepR could not be deleted unless its cognate ecfG was first deleted (23). We successfully transformed the *C. crescentus* ΔnepR $\sigma^T$-Galactosidase activity measured in a WT and ΔnepRsigT strain (Fig. 3B). Leaky expression of NepR$_{FL}$ from P$_{sv}$ is also evident in this strain: we observed repression of nepR expression by addition of 0.5 mM vanillate expressed from a vanillate-inducible promoter (P$_{sv}$-nepR$_{FL}$) and terminally truncated (nepR$_{SV}$) nepR alleles from a vanillate-inducible promoter (P$_{sv}$-nepR$_{SV}$) or P$_{sv}$-nepR$_{SV}$). Boundaries of expressed nepR alleles are as follows: nepR$_{FL}$, M1 to E68; nepR$_{SV}$, Q31 to E62. $\beta$-Galactosidase activities were assayed in the presence (+) and absence (−) of nepR induction (0.5 mM vanillate) and in the presence (+) and absence (−) of osmotic upshock stress (150 mM sucrose). Transcription was compared to an empty vector (EV) control strain. Stability and function of HA-tagged nepR alleles were further evaluated by dot blotting and $\beta$-galactosidase transcriptional assays described in Fig. S4 in the supplemental material. All assays were performed in triplicate; error bars represent standard deviations.

**FIG 3** Functional analysis of full-length and truncated NepR alleles as regulators of GSR transcription in *C. crescentus*. (A) Measured $\beta$-galactosidase activity from the $\sigma^T$-dependent P$_{xyl}$-$\Delta$AT reporter plasmid. $\beta$-Galactosidase activities were measured in WT and ΔnepR ΔsigT backgrounds containing a plasmid expressing sigT from a xylose-inducible promoter (P$_{xyl}$-sigT), in the presence (+) or absence (−) of sigT inducer (0.2% xylose) and the presence (+) or absence (−) of osmotic upshock stress (150 mM sucrose). Empty vector (EV) controls (P$_{xyl}$ and P$_{van}$) are also included. (B) $\sigma^T$-dependent transcription measured in a ΔnepR ΔsigT strain expressing sigT from P$_{sv}$-sigT. Transcription was assayed as a function of full-length (nepR$_{FL}$) and terminally truncated (nepR$_{SV}$) nepR alleles expressed from a vanillate-inducible promoter (P$_{sv}$-nepR$_{FL}$ or P$_{sv}$-nepR$_{SV}$). Boundaries of expressed nepR alleles are as follows: nepR$_{FL}$, M1 to E68; nepR$_{SV}$, Q31 to E62. $\beta$-Galactosidase activities were assayed in the presence (+) and absence (−) of nepR induction (0.5 mM vanillate) and in the presence (+) and absence (−) of osmotic upshock stress (150 mM sucrose). Transcription was compared to an empty vector (EV) control strain. Stability and function of HA-tagged nepR alleles were further evaluated by dot blotting and $\beta$-galactosidase transcriptional assays described in Fig. S4 in the supplemental material. All assays were performed in triplicate; error bars represent standard deviations.
TABLE 1

| PhyR       | MBP-NepRFL | MBP-NepRSV |
|------------|------------|------------|
|            | $K_a$ (1/M · s) $\times 10^a$ | $K_d$ (1/s) $\times 10^{-1}$ | $K_{\mu}$ nM |
| His-Phys-R-FL | 5.08 ± 1.78 | 1.1 ± 0.16 | 191 ± 7 |
| His-Phys-R-P  | 1.34 ± 0.15 | 0.97 ± 0.31 | 744 ± 289 |
|             | 4.65 ± 0.98 | 2.08 ± 0.26 | 455 ± 54 |

*ND, not determined.

in a heterologous system (Fig. 2). The implications of this finding are discussed below.

The dynamic, disordered NepR termini are required for binding to PhyR-P in vitro. The unconserved termini of NepR are required for strong binding to full-length PhyR in a BTH assay (Fig. 2) and for proper $\sigma^T$-dependent regulation of transcription in C. crescentus (Fig. 3B). However, BTH assays demonstrate that the termini of NepR are largely dispensable for binding to either the $\sigma$-like domain of PhyR (Phys-R-SL) or $\sigma^T$ (Fig. 2). As BTH assays may lack the sensitivity to quantify small changes in protein interactions, we used surface plasmon resonance (SPR) to more accurately quantify NepR interaction with PhyR. We measured the association and dissociation rate constants ($k_{a}$ and $k_{d}$) of NepRFL and NepRSV binding to PhyR and PhyR-SL. NepR proteins were prepared as fusions to maltose-binding protein (MBP), while PhyR proteins were prepared as His tag fusions.

The equilibrium affinity of NepRFL for Phy-R-FL calculated from the measured rate constants was 191 ± 7 nM (Table 1; see also Fig. S5A in the supplemental material), which is in agreement with previously reported measurements (5). Removal of the termini from NepR decreases its equilibrium affinity for Phys-R-SL to 455 ± 54 nM (Table 1; see Fig. S5B). Thus, removal of the NepR termini clearly compromises binding to Phys-R-SL, even though we observed NepRSV-Phys-R-SL interaction in our BTH assay (Fig. 2). The measured equilibrium affinity of full-length NepR (NepRFL) for phospho-Phys-R (Phys R-P) is 744 ± 289 nM (Table 1; see Fig. 5C). We were unable to detect binding between NepRSV and full-length Phys-R-P by SPR (see Fig. S5D); no binding was detected between PhysR or Phys-R-SL and an MBP control sample (see Fig. S5F). The SPR data are consistent with our BTH interaction data (Fig. 2) and provide additional experimental support for a model in which the unconserved NepR termini play a key functional role in binding to full-length Phys-R-P. We were unable to quantify $\sigma^T$ binding to NepR, due to our inability to purify isolated, soluble $\sigma^T$. Like ecfG family $\sigma$ factors from other systems (1, 7, 9, 14), C. crescentus $\sigma^T$ is largely insoluble when expressed in isolation in a heterologous system; our attempts to solubilize and refold $\sigma^T$ inclusion bodies were unsuccessful.

NepRtermini stabilize the anti-$\sigma$-ECF $\sigma$ complex. Coexpression of His-$\sigma^T$ with untagged NepRFL or NepRSV enabled purification of soluble $\sigma^T$/NepRFL and $\sigma^T$/NepRSV complexes by affinity chromatography (Fig. 4A). We further purified these soluble complexes by size exclusion chromatography (see Fig. S6A in the supplemental material). The difference in size of the two proteins in these complexes and the diffuse resolution and gel staining of the 3.6-kDa (32-amino-acid [aa]) NepRFL peptide made it difficult to draw conclusions about complex stoichiometry. As such, we coexpressed and copurified His-tagged $\sigma^T$ bound to MBP-tagged NepR alleles; Coomassie blue staining of the two resolved fusion proteins in these complexes provides evidence that $\sigma^T$/NepRFL and $\sigma^T$/NepRSV purify at a 1:1 ratio (Fig. 4B; see also Fig. S6B).

Although both NepR alleles interact with $\sigma^T$ in these copurification assays, size exclusion chromatography-purified $\sigma^T$/NepRFL complex has a lower helical content and a lower melting temperature ($T_m = 51.3 \pm 0.5^\circ C$) than $\sigma^T$/NepRSV ($T_m = 53.9 \pm 0.6^\circ C$) as assessed by a circular dichroism (CD) thermal denaturation assay (Fig. 4C and D). To directly transcribe, ECF $\sigma$ factors undergo a conformational transition from a closed state to an open state, in which $\sigma$ can bind RNA polymerase and contact the −35 and −10 boxes in the promoter region (24). Reduced folded stability of the $\sigma^T$/NepRSV complex is consistent with a model in which full-length NepR stabilizes the closed, inactive conformation of $\sigma^T$ to a greater extent than does NepRSV (Fig. 3B).

We acknowledge that the difference in folded stability between these two $\sigma$-anti-$\sigma$ complexes is relatively small; a more complete explanation of the inability of NepRSV to fully stabilize $\sigma^T$ in its closed, inactive form will likely require a better understanding of concentrations and affinities of competing $\sigma$ factors and solvent conditions in the cell during stress (25).

NepR-dependent enhancement of PhyR phosphorylation requires the unconserved NepR termini. Studies of the response regulators (RRs) DrrB and CheY have demonstrated that substrate binding or the presence of additional domains adjacent to the receiver domain can impact RR phosphorylation (26, 27), and it has been reported previously that NepR can affect PhyR phosphorylation (28). We sought to quantify the effect of NepR binding on phosphorylation of the anti-anti-$\sigma$ factor PhyR. In vitro phosphorylation of PhyR with its cognate histidine kinase, PhyK, has proven unsuccessful due to difficulty in purifying an active form of this kinase (16). As an alternative approach, we incubated PhyR with the high-energy phosphodonor acetyl phosphate (AcP), which is known to phosphorylate many bacterial RRs and has been successfully used as a proxy for phosphorylation of RRs by their cognate kinase (29). PhyR is phosphorylated at a low level in the presence of $[^{32}P]AcP$. Addition of equimolar (10 $\mu$M) MBP-NepRFL strongly enhances the phosphorylation rate and steady-state PhyR-P level in this assay, suggesting that NepR can function to control PhyR phosphorylation (Fig. 5A).

Addition of equimolar (10 $\mu$M) MBP-NepRSV only weakly enhanced production of PhyR-P, demonstrating that the unconserved, disordered termini (FR1 and FR2) are required for full NepR-dependent activation of PhyR phosphorylation. Mutation of the conserved site of PhyR aspartyl phosphorylation to alanine (PhyRD192A) prevented PhyR phosphorylation in either the presence or the absence of NepR (Fig. 5A).

We considered two biochemical models for the observed increase in steady-state PhyR-P levels in vitro: (i) NepR stabilizes the phosphoryl group on PhyR-P or (ii) NepR binding enhances PhyR phosphorylation by AcP. To test these two models, we first measured the half-life of phosphoryl loss from PhyR-P alone, and
in the presence of equimolar MBP, MBP-NepRFL, and MBP-
NepRSV. The half-life of PhyR\textsuperscript{~P} is \textasciitilde47 h in the absence of NepR
and \textasciitilde49 h in the presence of NepR (Table 2), indicating that NepR
does not significantly enhance the stability of PhyR\textsuperscript{~P}. To test
whether NepR stimulates PhyR phosphorylation, we incubated
PhyR with AcP (in the presence and absence of NepRFL and
NepRSV) and measured the increase of PhyR\textsuperscript{~P} as a function of
time. Considering PhyR phosphorylation as a first-order process,
we observe an approximately 50-fold increase in the apparent rate
of PhyR phosphorylation in the presence of NepRFL compared to
PhyR alone (Fig. 5B). NepRSV has only a weak stimulatory effect
on PhyR phosphorylation; the level of measured PhyR\textsuperscript{~P} after a
750-min incubation is 30\% of that for NepRFL (Fig. 5B). We con-
clude that the stimulatory effect of NepR on the rate of PhyR
phosphorylation requires one or both of the unconserved NepR
termini. It remains to be determined if NepR promotes PhyK-
dependent phosphorylation of PhyR \textit{in vivo}.

**DISCUSSION**

The anti-\sigma factor NepR plays a key role in regulation of the general
stress response in alphaproteobacteria (1, 2). Structures of NepR
bound to the \sigma-like domain of PhyR (PhyR-SL) (4, 5) revealed
two conserved central helices (\sigma\textsubscript{1} and \sigma\textsubscript{2}) composed of approximately
30 residues that interact with PhyR-SL (Fig. 1B). A four-
residue linker between \sigma\textsubscript{1} and \sigma\textsubscript{2} plays a critical functional role in
NepR binding to \sigma\textsubscript{7}, PhyR, and PhyR-SL. The N and C termini of
NepR (FR1 and FR2, respectively) are poorly conserved (2), dy-
namic, and disordered and do not evidently interact with PhyR-SL
in these structures (4, 5). Nonetheless, \textit{nepR} orthologs across the
class \textit{Alphaproteobacteria} invariably contain unconserved, disor-
dered extensions that flank the conserved central helical domain.
This raised the question of what role, if any, these flanking regions
have in NepR function as an anti-\sigma factor.

NepR FR1 and FR2 are not required for binding to \sigma\textsubscript{7}
or PhyR-SL but are necessary to bind full-length phospho-PhyR
(PhyR\textsuperscript{~P}) (Fig. 2; Table 1; see also Fig. S5 in the supplemental
material). Biophysical analysis of purified \sigma\textsubscript{7}/NepR complexes
provides evidence that FR1 and FR2 increase the folded stability of
the \sigma\textsubscript{7}/NepR complex (Fig. 4), supporting a model in which the
dynamic and disordered NepR termini function to stabilize the
“closed” inhibited form of \sigma\textsubscript{7}. Indeed, our \textit{in vivo} studies of the
model, transient NepR interaction with unphosphorylated PhyR may "prime" PhyR for phosphorylation and subsequent high-affinity binding to NepR. Alternatively, it is possible that the PhyR-NepR interaction in vivo is chaperoned by another protein(s) (e.g., the PhyR kinase, PhyK). It remains to be determined if NepR promotes kinase-dependent phosphorylation of PhyR in the C. crescentus cell. Nonetheless, our results are consistent with a recent observation that the presence of NepR improves phosphorylation of Sphingomonas melonis PhyR by cognate kinases (28). Thus, NepR-dependent enhancement of PhyR phosphorylation may be a general feature of GSR regulation in alphaproteobacteria.

Our data provide an interesting example of a two-component receiver domain that is allosterically regulated by interaction with a nonkinase binding partner. Thus, there are biochemical regulatory parallels between the NepR-PhyR general stress system of alphaproteobacteria and the E. coli chemotaxis system, in which the CheY receiver phosphorylation rate is enhanced by CheZ or FliM binding (26, 27). Though the structural mechanism of NepR as an activator may be a general feature of GSR regulation in alphaproteobacterial GSR, including repression of $\sigma^T$ transcriptional activity and anti-anti-$\sigma$ (i.e., PhyR) activation by phosphorylation (Fig. 6).

**MATERIALS AND METHODS**

**nepR translation start assay.** To measure translation from each of the three putative nepR start codons, we constructed translational fusions of each potential start site to lacZ in the reporter plasmid pPR9TT (cut with AvrII-PstI). Primers used to amplify these different alleles are listed in Table S1 in the supplemental material. All plasmids with the corresponding inserts were sequenced confirmed. Purified reporter plasmids were transformed into electrocompetent Caulobacter crescentus CB15. Single colonies of these reporter strains were used to inoculate 3 ml of peptone-yeast extract (PYE) medium supplemented with 2 $\mu$g/ml chloramphenicol (Chlor). Cultures were incubated overnight at 30°C and shaken at 220 rpm. Overnight cultures were diluted back in fresh PYE medium (with 2 $\mu$g/ml Chlor) at an optical density at 660 nm (OD$_{660}$) of ~0.05. At an OD$_{660}$ of ~0.2 (30°C/220 rpm), $\beta$-galactosidase activity was measured in triplicate as previously described (30). For strain information, see Table S2.

**TABLE 2** Calculated phospho-PhyR (PhyR~P) half-life in the presence of buffer, MBP, MBP-NepR$_{FL}$ or MBP-NepR$_{SV}$

| Protein | PhyR~P half-life (h) |
|---------|---------------------|
| PhyR~P + buffer | 45.7 ± 6.8 |
| PhyR~P + MBP | 44.6 ± 8.5 |
| PhyR~P + MBP-NepR$_{FL}$ | 49.5 ± 2.2 |
| PhyR~P + MBP-NepR$_{SV}$ | 47.8 ± 3.8 |

*a Values presented are the averages of results from three independent experiments ± standard deviations.

**C. crescentus** regulatory system provide evidence that the intrinsically disordered terminal domains are necessary for NepR function as an anti-$\alpha^T$ factor in the cell (Fig. 3). In brief, the central $\alpha_1-\alpha_2$ helical domain of NepR is not sufficient to function as a regulated anti-$\alpha$ factor on its own even though it is competent to bind both $\alpha$ ($\alpha^T$) and anti-anti-$\alpha$ (PhyR-SL) substrates. The unconserved, dynamic, and disordered NepR amino terminus plays an indispensable role in binding of the central helical domain to its substrates in the context of the fully intact regulatory system. The structural basis by which intrinsically disordered regions enable NepR function as a regulated anti-$\alpha$ factor is an open area of investigation. Certainly, we cannot exclude the possibility that FR1 and FR2 are ordered and structured when interacting with full-length PhyR~P or $\alpha^T$. Indeed, NMR studies of the orthologous system in Sphingomonas species demonstrate that NepR undergoes a disorder-order transition when binding PhyR-SL (4), although the structural states of FR1 and FR2 when bound to full-length PhyR or $\alpha^T$ remain undefined.

Our study has also detailed a biochemical function of NepR as a protein that enhances the rate of PhyR phosphorylation and steady-state phospho-PhyR levels in vitro (Fig. 5). Our observation that NepR promotes PhyR phosphorylation (Fig. 5) appears to conflict with an apparent lack of stable association between unphosphorylated PhyR and NepR (Table 1). It may be that weak or transient PhyR-NepR interaction, below the detection limit of our methods, promotes phosphorylation of PhyR. In such a
Bacterial two-hybrid protein interaction assay. To assay interaction between NepR fragments [FL, SV, SV + linker, SC2, SC3, ΔFR1, ΔFR2, and poly-A] and PhyR (wild type [WT], D192A, or SL) or σ73 inside bacterial cells, we used a bacterial two-hybrid system (19). The different nepR alleles were cloned into the pUT18c vector (digested with BamHI-EcoRI), which generated C-terminal fusions to the T18 fragment of adenylate cyclase. PhyR, phyR-SV, and sigT were cloned into the pKT25 vector (digested with XbaI-KpnI). As a control the “reverse experiment” was also performed, and the different nepR alleles (FL and SV) were cloned into the pKT25 vector (digested with XbaI-KpnI); phyR, phyR-SL, and sigT were cloned into pUT18c (digested with BamHI-EcoRI and KpnI-EcoRI, respectively). The nepRsv + linker was synthesized as a GeneBlock (IDT). Primers used for PCRs are listed in Table S1 in the supplemental material; all plasmids were sequence confirmed. pUT18c and pKT25 combinations were transformed into electrocompetent E. coli reporter strain BTH101 and plated on LB agar supplemented with ampicillin (Amp; 100 μg/ml), kanamycin (Kan; 50 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml). Control strains transformed with pKT25-Zip, pKT25-phyR/phyR-SL(sigT) and pUT18c, pKT25-phyR/phyR-SL(nepr) and sigT were cloned into the pKT25 vector (digested with XbaI-KpnI); phyR, phyR-SL, and sigT were cloned into pUT18c (digested with BamHI-EcoRI and KpnI-EcoRI, respectively). The nepRsv + linker was synthesized as a GeneBlock (IDT). Primers used for PCRs are listed in Table S1 in the supplemental material; all plasmids were sequence confirmed. pUT18c and pKT25 combinations were transformed into electrocompetent E. coli reporter strain BTH101 and plated on LB agar supplemented with ampicillin (Amp; 100 μg/ml), kanamycin (Kan; 50 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml). Control strains transformed with pKT25-Zip, pKT25-phyR/phyR-SL(sigT) and pUT18c, pKT25-phyR/phyR-SL(nepr) and sigT were cloned into pUT18c (digested with BamHI-EcoRI and KpnI-EcoRI, respectively). The nepRsv + linker was synthesized as a GeneBlock (IDT). Primers used for PCRs are listed in Table S1 in the supplemental material; all plasmids were sequence confirmed.

Western blot analysis. Western blotting assays were carried out using antisera against the T18 fragment fused to various NepR alleles. Briefly, overnight cultures of the BTH101 strains transformed with different versions of pUT18c and pKT25 plasmids were used to inoculate 350 ml of LB medium supplemented with Amp (100 μg/ml), Kan (50 μg/ml), and IPTG (1 mM); these cultures were grown overnight (30°C, 220 rpm) and used to inoculate 3 ml of fresh LB (Amp-Kan-IPTG) at an OD600 of ~0.05. Once the OD600 of these diluted cultures reached 0.5, cells were harvested by centrifugation at 8,000 rpm for 20 min at 4°C and resuspended in 10 ml of 25 mM Tris-HCl (pH 7.6), 125 mM NaCl buffer supplemented with phenylmethylsulfonyl fluoride (PMSF; 1 μl/ml), 1 mM EDTA, and 0.1% (vol/vol) Triton X-100. Cells were disrupted by three passages through a French pressure cell and clarified by centrifugation at 14,000 rpm for 20 min at 4°C. Soluble fractions were transferred to new tubes, and total protein concentration was estimated using a commercial bicinchoninic acid (BCA) protein assay kit (Pierce). Appropriate lysate dilutions were heated in 3X SDS loading buffer at 95°C for 5 min, and 20 μl of sample was loaded onto a 15% SDS-PAGE gel and run for 2 h at 200 V. Samples were transferred to a Millipore 0.45-μm polyvinylidene difluoride (PVDF) membrane (Millipore) using a Trans-Blot turbo transfer system (Bio-Rad). After transfer, the membrane was washed in Tris-buffered saline–Tween 20 (TBST) supplemented with 5% milk and blocked overnight in fresh milk solution. The membrane was then incubated with 10 ml of TBST, 5% milk with a 1:1,000 dilution of anti-T18 antibody (3D1; KeraFast) for 1 h at room temperature. After a 30-min wash with TBST, the membrane was incubated for 1 h in 10 ml TBST, 5% milk with a 1:5,000 dilution of HRP-conjugated anti-mouse–horseradish peroxidase (HRP; Thermo Scientific) secondary antibody. After a final 30-min wash in TBST, the Western blot was developed with SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific). To visualize the bands and quantify the total amount of protein loaded in the gel, we used the Bio-Rad ChemiDoc MP imaging system.

PhyR Western blotting assays were performed using the sample preparation and transfer protocols detailed above. For PhyR recognition, anti-PhyR antibodies derived against Brucella abortus PhyR (15) were used at a dilution of 1:5,000 in 10 ml TBST, 5% milk. For visualization, a 1:5,000 dilution of HRP-conjugated mouse secondary antibody was used.

For dot blot analysis of hemagglutinin (HA)-tagged NepRFL and NepRsv in Caulobacter, HA nucleotide sequence was inserted in frame at the C or N terminus of the nepR alleles by PCR sewing and cloned into pMT806 (digested with Ndel-Xhol). After sequence confirmation, all plasmids were transformed into the electrocompetent C. crescentus CB15 ΔnepR ΔsigT/pMT464-sigT strain (see strain and primer information in Tables S1 and S2 in the supplemental material). Overnight cultures of CB15 ΔnepR ΔsigT/pMT464-sigT/pMT806, CB15 ΔnepR ΔsigT/pMT464-sigT/pMT806-ΔnepRFL HA and pMT806-ΔnepRFL-HA, and CB15 ΔnepR ΔsigT/pMT464-sigT/pMT806-ΔnepRFL-ΔnepRsv HA and pMT806-ΔnepRFL-ΔnepRsv-HA were grown to an OD600 of ~0.1 in 5 ml of PYE (25 μg/ml Kan plus 1 μg/ml Chlor) and induced with vanillate (0.5 mM) and xylose (0.15%) for 4 h. Cells were spun down and suspended in 3X SDS loading buffer to an OD600 of 100. Samples were boiled and then spotted onto a 0.2-μm PVDF (Millipore) membrane. After transfer, the membrane was washed in Tris-buffered saline–Tween 20 (TBST) supplemented with 5% milk and blocked overnight in fresh milk solution. The membrane was then incubated with 10 ml of TBST, 5% milk with a 1:2,000 dilution of HA.
antibody (Thermo Scientific) for 1 h at room temperature. After a 30-min wash with TBST, the membrane was incubated for 1 h in 10 ml TBST, 5% milk with a 1:5,000 dilution of HRP-conjugated anti-mouse secondary antibody. After a final 30-min wash in TBST, the Western blot was developed with SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific). To visualize the spots and quantify them, we used the Bio-Rad ChemiDoc MP imaging system.

Construction of C. crescentus mutant strains. To delete the nepR-sigT operon from the C. crescentus chromosome, a sacC suicide plasmid, pNPTSl18, carrying 500-nucleotide regions flanking the 5′ and 3′ ends of the nepR-sigT operon was built. Primers (carrying EcoRI-Sall restriction sites) used to construct the deletion allele by overlapping PCR are listed in Table S1 in the supplemental material. The nepR-sigT knockout plasmid was transformed into the electroprototrophic C. crescentus CB13 wild-type strain, and cells containing the integrated knockout plasmid were selected on PEG agar supplemented with 5 μg/ml Kan. Sucrose counterselection for crossover recombination to yield the nepR-sigT deletion strain (ΔnepR ΔsigT) was performed as previously described (31, 32).

Plasmids pRKLac290-PsigU (encoding Tet), pMT464-sigT (encoding Kan), pMT806-nepRFL, and pMT806-nepRSV (encoding Cam) were transformed into ΔnepR ΔsigT strain by electroporation. pMT464-sigT was used to induce expression of sigT from a xylose-inducible promoter (Pxy); pMT806 (restriction sites Ndel-XhoI) (33) was used to generate constructs from which NepRF and NepRSV could be expressed from a vanillate-inducible promoter (Pvan). Primers used to build these constructs are listed in Table S1 in the supplemental material. pRKLac290-PsigU and pMT464-sigT were purified from preexisting strains FC634 and FC2251, respectively (11). All plasmids were sequence verified. To control for plasmid effects, empty pMT464 and pMT806 plasmids were transformed when needed into wild-type C. crescentus CB15 (strain number FC19) and the ΔnepR ΔsigT strain.

Stress response transcription assays. It is known that transcription of sigU is upregulated by the general stress factor σ74 upon hypersmotic or oxidative stress challenge (17). The plasmid pRKLac290-PsigU (encoding Tet) (11), which contains the sigU promoter transcriptionally fused to lacZ, was conjugated into the different C. crescentus backgrounds (in some cases carrying pMT464 and pMT806 Pvan and Pxy constructs) to assay σ74-dependent transcription. All C. crescentus strains were grown in PYE (2 μg/ml Chlor, 5 μg/ml Kan, and 1 μg/ml Tet) and diluted to a starting OD_{660} of 0.05 (30°C, 220 rpm). Cells were hypersmotically stressed by adding 150 mM sucrose to the culture medium at the beginning of the experiment. Induced expression of phyR and nepR alleles from the xyl and van promoters was carried out by adding 0.15% xylose or 0.5 mM vanillate to the culture medium. β-Galactosidase activities were measured at an OD_{660} of 0.25 in triplicate as previously described (34).

Recombinant protein expression strain construction. Previously published E. coli Rosetta (DE3)pLysS strains were used for the heterologous overexpression of Catabobacter His-PhyR, His-PhyR-SL, MBP-NepRF, and MBP-NepRSV (5); these strains are listed in Table S2 in the supplemental material.

Primers were used to amplify and build the other expression strains are listed in Table S1 in the supplemental material. For expression of MBP-NepRSV, the nepR-SV PCR product was cloned into pMaltc2g (Amp+) digested with EcoRI-HindIII. For expression of His-PhyR (or PhyR-SL), pET25-PhyR (or PhyR-SL) was used as a PCR template and the corresponding insert was cloned into pETDuet-1 (Amp+). The His tag was carried by σ74, the corresponding insert was cloned at the first position of pETDuet-1, using EcoRI-NotI as restriction sites. The different NepR alleles (NepRF, NepRSV, MBP-NepRF, and MBP-NepRSV) were then inserted at the second position using the Ndel-Kpnl restriction sites. To amplify by PCR the MBP-tagged NepR alleles, we used the pMalc2g-NepR FL and SV as the templates. After sequence confirmation, all the different expression plas-
Circular dichroism thermal denaturation measurements. The thermal denaturation profile of the different σ²/NepR complexes was assayed using a Jasco J-1500 circular dichroism spectrometer. After dialysis in Tris-NaCl buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl), 300 μl of protein purified by nickel affinity chromatography and size exclusion chromatography was loaded in a 1.0-mm quartz cuvette. Protein spectra were assayed at a 20 μM concentration. To ensure that all samples were correctly folded, we first acquired a full, buffer-subtracted CD spectrum (260 to 180 nm) for each sample at 26°C. For the thermal denaturation assay, temperature was gradually increased from 26°C to 74°C (2°C/minute ramp). As the temperature was raised, the CD intensity at 222 nm decreased. The corresponding melting curve (i.e., loss of signal at 222nm) was normalized, plotted, and fitted. Melt measurements on all samples were performed four times using independent protein preparations.

In vitro phosphorylation assays. His-PhyR, His-PhyR<sub>1262A</sub>, MBP-NepR<sub>V</sub>, MBP-NepR<sub>SV</sub>, and MBP were purified as described above. In vitro phosphorylation of PhyR using radiolabeled acetyl phosphate ([<sup>32</sup>P]AcP) was performed as previously described (35, 36). Briefly, for a 1-h reaction mixture, 300 μl of AcP buffer (25 mM Tris-HCl [pH 8.0], 60 mM potassium acetate, 10 mM MgCl₂) was added to equal-volume SDS loading buffer at the indicated time points and stored at 20°C until gels were run. All gels were exposed for equivalent periods of time on the same phosphor screen and imaged on a Bio-Rad FX imager. For each condition, the percentage of phosphorylated PhyR was calculated using ImageJ; the PhyR<P>−/−/MBP-NepR<sub>V</sub> final time point was considered 100%. For calculating PhyR<P>−/−/PhyR<sub>D</sub> phosphorylation, unincorporated [<sup>32</sup>P]AcP was first removed by running the sample over a Zeba 7,000-molecular-weight-cutoff (MWCO) desalting column that was equilibrated in dialysis buffer. All samples were separated on Bio-Rad Any kD TGX gels before exposure to a phosphor screen (Molecular Dynamics) and subsequent visualization on a Bio-Rad FX imager. Gel images were analyzed using ImageJ (37). All experiments were performed in triplicate.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl?doi=10.1128/mBio.00910-15/-/DCSupplemental.

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