Activating mutations in quorum-sensing regulator Rgg2 and its conformational flexibility in the absence of an intermolecular disulfide bond

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Edited by Chris Whitfield

Rap/Rgg/NprR/PlcR/PrgX (RRNPP) quorum-sensing systems use extracellular peptide pheromones that are detected by cytoplasmic receptors to regulate gene expression in firmicute bacteria. Rgg-type receptors are allosterically regulated through direct pheromone binding to control transcriptional activity; however, the receptor activation mechanism remains poorly understood. Previous work has identified a disulfide bond between Cys-45 residues within the homodimer interface of Rgg2 from Streptococcus dysgalactiae (Rgg2sd). Here, we compared two Rgg2sd(C45S) X-ray crystal structures with that of wild-type Rgg2sd and found that in the absence of the intermolecular disulfide, the Rgg2sd dimer interface is destabilized and Rgg2sd can adopt multiple conformations. One conformation closely resembled the “disulfide-locked” Rgg2sd secondary and tertiary structures, but another displayed more extensive rigid-body shifts as well as dramatic secondary structure changes. In parallel experiments, a genetic screen was used to identify mutations in rgg2 of Streptococcus pyogenes (rgg2sp) that conferred pheromone-independent transcriptional activation of an Rgg2-stimulated promoter. Eight mutations yielding constitutive Rgg2 activity, designated Rgg2sp*, were identified, and five of them clustered in or near an Rgg2 region that underwent conformational changes in one of the Rgg2sd(C45S) crystal structures. The Rgg2sp* mutations increased Rgg2sp sensitivity to pheromone and pheromone variants while displaying decreased sensitivity to the Rgg2 antagonist cyclosporine A. We propose that Rgg2sp* mutations invoke shifts in free-energy bias to favor the active state of the protein. Finally, we present evidence for an electrostatic interaction between an N-terminal Asp of the pheromone and Arg-153 within the proposed pheromone-binding pocket of Rgg2sp.

Extracellular chemical communication (quorum sensing, QS) in the low-G + C Gram-positive bacteria (Firmicutes) is best understood to utilize peptide messengers (pheromones) as signals to coordinate gene expression among populations of cells. Pheromone receptors fall into two functional groups, those that detect peptides outside the cell by integral membrane sensors (e.g. ComD, AgrB, BAP, and others), and those located in the cytoplasm that rely on transport of pheromones into the cell (1, 2). The latter are members of the RRNPP family of receptors, which employ the concave pocket of a C-terminal helical repeat domain to bind the peptide ligands (3–7). Most Rap/Rgg/NprR/PlcR/PrgX (RRNPP) proteins, like NprR, PlcR, PrgX, and Rgg, contain an N-terminal DNA-binding domain (DBD), and they regulate transcription conditionally as a function of pheromone binding. Rap proteins are also modulated by peptide ligands, but control gene regulation through interactions with protein mediators of transcription (8–10). As RRNPP proteins serve as communication hubs within bacterial cells, an understanding of their switch-like properties in the process of gene regulation has been a focus of several studies (3, 6, 7, 11–13). Despite a similar overall structure among RRNPP proteins, allosteric modulation of activity by ligands is reported to have disparate mechanisms of function, where conformational and oligomeric differences have been described.

The obligate human pathogen Streptococcus pyogenes (Group A Streptococcus (GAS)) is genetically programmed to utilize several different QS systems (14), including four differ-
Figure 1. Rgg2/Rgg3 quorum-sensing system of S. pyogenes. Short hydrophobic peptide genes (shp2 and shp3) encode precursor peptides that are processed and secreted to generate mature SHP pheromones. Pheromones are actively transported to the cytoplasm by the oligopeptide permease transporter, Opp. Opps are allosteric regulators of the Rgg receptor proteins. In the absence of SHP, transcription of shp promoters is repressed by Rgg3. The presence of SHP accomplishes two events: it causes Rgg3 to release DNA, allowing Rgg2 to bind at the promoter, and SHP stimulates Rgg2 to become a transcriptional activator. The conformational changes in Rgg2 and Rgg3 incurred by SHP pheromones are unknown.

Results

X-ray crystal structures of Rgg2sd(C45S)

X-ray crystal structures of apo Rgg2sd(C45S) were determined at 2.39 and 2.20 Å resolution in two crystal forms, named Rgg2(C45S)x1 and Rgg2(C45S)x2, respectively (Fig. 2 and supplemental Fig. S1 and Table S1). As described below and under “Experimental procedures”, the Rgg2sd(C45S) structures were compared with the previously determined apo wild-type Rgg2sd structure (PDB code 4YV6). To identify any gross conformational changes in Rgg2sd(C45S) relative to wild-type Rgg2sd, the entire Rgg2sd(C45S)x1 or Rgg2sd(C45S)x2 dimer (both protomers A and B, simultaneously) were aligned to apo
wild-type Rgg2sd (root-mean-square deviation for pairwise comparisons of structurally aligned Cα atoms = 0.66 and 0.90 Å, respectively) (**supplemental Fig. S1A**). These alignments revealed that Rgg2sd(C45S)x1 closely matches the structure of wild-type Rgg2sd whereas Rgg2sd(C45S)x2 has undergone significant conformational changes. Specifically, residues 170–173, which were part of a loop (residues 170–177) connecting wild-type Rgg2sd protomer B repeat 3 helix B (R3B) and repeat 4 helix A (R4A), have adopted α-helical structure, extending the Rgg2sd(C45S)x2 R3B approximately one helical turn. We propose that the Rgg2sd(C45S)x2 rigid body (tertiary structure) and secondary structure changes are enabled by structural flexibility otherwise absent upon formation of the Cys-45–Cys-45 intermolecular disulfide bond that connects the wild-type Rgg2 DBDs. It is important to note that because Rgg2sd(C45S) in the Rgg2sd(C45S)x1 crystal form adopts tertiary and secondary structure conformations similar to those of the wild-type Rgg2sd structure, it is not in fact the presence of the serine residue (the substitution for Cys-45) that enables the conformational change, but rather the lack of the intermolecular disulfide bond. We conclude that the Rgg2sd(C45S)x2 crystal form traps one of many conformations that Rgg2sd can adopt in the absence of the intermolecular disulfide bond.

**Constitutively active mutations of Rgg2sd map to the dynamic loop of Rgg2sd(C45S)x2**

As the Rgg2sd(C45S) mutant protein displayed a capacity to adopt a conformation under crystallization conditions not seen for the wild-type protein, we tested the possibility that this residue change would also confer differences in Rgg2 transcriptional activity. A test of this substitution had been conducted for Rgg2sd (20) but not for Rgg2sp. Thus, we constructed nonsense mutations at residue 45 of Rgg2sp to generate C45S and C45A and transferred these variants to _S. pyogenes_. The reporter strain used, RVW119 (Δ_rgg2 shp2 shp3_), contains a chromosomal deletion of rgg2, null mutations in coding sequences of _shp2_ and _shp3_ (and is therefore unable to produce SHP pheromones), and a chromosomally integrated _shp3-lux_ reporter. Luminescence from cultures expressing the C45S, C45A, and wild-type Rgg2 variants displayed similar responses to a range of SHP concentrations (Fig. 3A); thus, in this assay, the apparent conformational stability provided by the intermolecular disulfide bond _in vitro_ did not affect transcriptional activity.

Instead, to begin to map regions of the Rgg2 structure that correlate with the protein’s ability to modulate transcription, we adopted a genetic selection strategy to identify mutants of Rgg2capable of promoting transcription in the absence of an inducing SHP pheromone. We speculated that amino acid changes providing Rgg2 enhanced transcriptional activity may suggest regions of Rgg2 that undergo conformational changes during protein activation. The _aphA3_ gene, encoding resistance to kanamycin, was placed under the control of the _shp2_ promoter to create plasmid pRVW31 (Δ_aphA3) as a scheme to select for resistant colonies in the absence of

**Figure 2. Structural comparison of Rgg2sd(C45S)x2 and wild-type Rgg2sd (PDB code 4Y46).** Both the entire structure of Rgg2sd(C45S)x2 protomer A and the repeat domain of protomer B are shifted 4° around the hinge axis (red cylinder) when aligned to the protomer B DBDs of Rgg2sd(C45S)x2 and wild-type Rgg2sd (PDB code 4Y46). A and B, left, these panels are identical except that α-helices are depicted as detailed schematics or labeled cylinders, respectively. A, right, expanded view of the area enclosed by the black dashed line in the left panel. B, right, side view obtained by rotating the structures in 90° in the direction indicated by the arrow. In all panels, Rgg2sd(C45S)x2 protomer A residues 67–70 are colored magenta, Rgg2sd(C45S)x2 and Rgg2sd(wt) protomer B residues 170–177 are colored yellow and brown, respectively. Wild-type Rgg2sd residue Cys-45 and Rgg2sd(C45S)x2 residue Ser-45 are labeled accordingly and depicted in ball-and-stick format.
SHP. Optimization of kanamycin concentrations needed to select for Rgg2 activation was conducted and led to the finding that 100 μg/ml was sufficient (see under “Experimental procedures”).

Plasmid pRVW31 was then integrated into the chromosome of a GAS NZ131 Δrgg2 Δrgg3 derivative that contains the deletions Δrgg2 Δrgg3 to generate strain RVW89. As expected, without rgg2 this strain was unable to grow on THY plates containing 100 μg/ml kanamycin. pJC217, a plasmid capable of replication in GAS and containing rgg2 with its endogenous promoter, was treated with variable concentrations of ethyl methanesulfonate (EMS), a chemical agent that alkylates guanine bases, as a means to generate point mutations in the plasmid. EMS-treated pJC217 was transferred to RVW89 by electroporation, and kanamycin-resistant mutants were selected on THY agar. THY medium inhibits SHP signaling because it supplies a high concentration of nonspecific nutrient peptides that compete with SHP pheromones for transport into the cytoplasm through the oligopeptide transporter Opp, rendering GAS non-responsive to SHP (17). Mutant selection was conducted on THY to enhance the likelihood that kanamycin-resistant clones would not arise because of unintended induced SHP signaling, but rather only from mutations in rgg2 that cause SHP-independent activation.

Plasmids were recovered from 47 resistant clones, and the rgg2 gene was sequenced. Of these, 31 were found to have mutations in the coding sequence of rgg2 and 19 were of known unique

Figure 3. A, Rgg2Sp(C45S) and Rgg2Sp(C45A) mutants display only minor changes in transcriptional activation in response to sSHP peptides. The maximum luminescence, normalized to cell density (RLUs), attained by NZ131 Δrgg2 sshp2GGGsshp3GGG Pshp3-luxABCDE cells (strain RVW119), complemented with indicated Rgg2 variants on a multicopy plasmid, are reported here as a function of sSHP2 concentration, ranging from 0 to 1,000 nm. B, location of Rgg2* mutations depicted as spheres on Rgg2Sd(C45S) x2 protomers A (green) and B (blue). Rgg2Sd(C45S) x2 regions of conformational flexibility in protomer A (residues 67–70) and protomer B (residues 170–177) are colored magenta and yellow, respectively. C, mutated Rgg2* proteins support varying levels of constitutive Pshp3-lux activity. RLUs attained by RVW119 cells complemented with indicated Rgg2 variants on a multicopy plasmid are shown. Cells were treated with either 100 nm sSHP2 or equivalent concentration of reverse sequence peptide (revSHP). D, Rgg2* mutants demonstrate significant alterations in peptide sensitivity. RLUs attained by RVW119 cells complemented with indicated Rgg2 variants on a multicopy plasmid are reported here as a function of sSHP2 concentration, ranging from 0 to 1,000 nm. Wild-type Rgg2 treated with equivalent concentrations of revSHP peptide is included as a control (black line). All data are representative of three biological replicates. Error bars show standard deviation. E, frequencies of rgg2* mutations. Total hits indicate the total number of times a given mutation was found. Unique hits indicate the total number of times the mutation was identified in a separately mutagenized pool.
Rgg2* mutant positions correlate with structural shift

provenance as they had arisen in separately mutagenized pools of plasmid (Fig. 3E). As these mutations tentatively conferred gain of function properties, we designate them rgg2*. Interestingly, a majority of the mutations (L163F, M167I, S174L, Q176K, and V184M) were located near the loop (residues 170–177) identified as dynamic region in Rgg2sd(C45S)2 (described above). The correlation of these mutations to a region where dynamic movements were observed suggested to us that a conformational movement of the loop is an important step for Rgg2 transcriptional activation.

Rgg2sp* mutants are sensitized to peptides in cultures

To ensure that constitutive activation of the Rgg2/3 quorum-sensing pathway observed in our selections was due to observed changes in the amino acid sequence of Rgg2sp, and not secondary mutations elsewhere on the plasmid, point mutants were regenerated in pLC217 by site-directed mutagenesis (except for Rgg2sp* V184M, see under “Experimental procedures”). The resulting plasmids, or a plasmid containing unaltered rgg2sp, were transferred into RWV119 (∆rrg2 sphp2EGG sphp3EGG), which contains a chromosomally integrated Pshp3lux reporter. This rgg3+ strain was chosen as an ideal strain to test the ability of Rgg2sp* mutants to function in the presence of Rgg3, the known negative regulatory counterpart to Rgg2. It was also imperative to conduct experimentation in a shp-null genetic background to uncouple exogenous pheromone responses from endogenous pheromone production (which is strongly influenced by positive-feedback regulation), and it was previously demonstrated that multicopy expression of wild-type rgg2 in a shp-replete background was sufficient to induce QS activity (17). Initially, the luciferase reporter activity of each strain was assessed under non-inducing conditions (i.e. without sHP), but to control for general effects that addition of a similar peptide may have on cells, we added to the cultures a peptide comprising the reversed-sequence of SHP (revSHP), known not to stimulate the Rgg2/3 pathway (17). All Rgg2sp* variants displayed a basal level of bioluminescence that was greater than that generated by wild-type Rgg2sp (Fig. 3C). Despite there being only modest changes (∼2–3-fold increases) for many of the mutants (D60N, W140L, S174L, Q176K, V184M, and G232R), activity levels were statistically significant for each except D60N (p < 0.05, Student’s t test). Two mutants, L163F and M167I, displayed substantially higher levels of luminescence than wild type in the absence of sHP3. When treated with 100 μM sHP3, nearly all Rgg2sp* mutants responded by inducing transcription of luciferase to levels comparable with or higher than wild type, except W140L and G232R, which were induced less than 1.5-fold (p < 0.05, Student’s t test). Overall, the panel of Rgg2sp* mutants provide multiple examples of Rgg2sp activation that will be used to elucidate mechanisms of regulation. For further analysis, we chose four mutants (D60N, L163F, S174L, and G232R) displaying distinct activities and localization within Rgg2sp. Considering that the variability in response to a single concentration of sHP3 was disparate among Rgg2sp* mutants, we generated dose-response curves with a titration of sHP3 for each selected mutant (Fig. 3D). These results indicated that L163F and S174L were 10–30 times more sensitive to sHP3 than wild type, or in other words, less SHP3 was required to reach equivalent luminescence levels observed in wild type. This was in contrast to D60N, which displayed a similar pattern of response to pheromone as the wild type. Previously, a collection of peptides designed to test the primary features of SHP were assessed for their ability to activate Rgg2sp (17). To summarize previous findings, the negative charge of aspartic acid at the N terminus was an important feature of SHP3, because changing this position to asparagine abrogated function, whereas a substituted glutamic acid remained active. Two other pheromone variants were synthesized to shorten the length by one residue from either end of the peptide, and each displayed poor or no activity at 50 nM (17). We titrated each peptide variant in cultures of Rgg2sp* mutants to test the possibility that selectivity for peptides might be altered (Fig. 4, A–E). Remarkably, D60N, L163F, and S174L each displayed increased responses to the peptide variants. L163F showed the greatest degree of sensitivity to all tested peptides, although the narrow dynamic range of luminescence activation made it difficult to precisely determine the concentration at which half-maximal induction (EC50) occurred. S174L also displayed a substantial increase in sensitivity to the peptide variants as compared with wild-type Rgg2sp, and it even responded to revSHP, although only at the highest concentration tested, 1 μM. G232R displayed limited responsiveness across the range of peptides and concentrations tested, and there was no appreciable increase over basal levels in RLUs, even at the highest concentrations of peptide tested. These results indicated that Rgg2sp* D60N, L163F, and S174L mutants were more amenable to activation, even by ligands that are not optimal inducers.

CsA is known to inhibit Rgg2sp by competing with SHPs for binding to the receptor and blocking transcriptional activation (15). We tested Rgg2sp* mutants D60N, L163F, S174L, and G232R for the ability of CsA to inhibit activation of luciferase expression. Dose-response curves of the four rgg2sp* alleles in strain RWV119 (∆rrg2 shp2EGG shp3EGG) stimulated with 50 nM sHP2 were generated with titrations of CsA that ranged between 0.16 and 10 μM (Fig. 3F). Rgg2sp* mutants D60N, L163F, and S174L displayed partial sensitivity to CsA but required higher concentrations than seen for wild type to be inhibited, and complete inhibition of transcription was not possible under the conditions employed. In concordance with the observation that G232R does not respond to sHP2, CsA had no appreciable inhibitory effect on this mutant. To determine whether CsA inhibition worked only by competing with SHP pheromone for the binding pocket of Rgg2 or by also altering the conformation of our Rgg2sp* to resemble wild type more closely, we assessed transcriptional activity in the absence of sHP2 of Rgg2sp* L163F, our most constitutively active mutant, following treatment with CsA. Using the same approach as described above, but in the absence of sHP2 pheromone, CsA reduced constitutive Rgg2sp* L163F transcriptional activity in luciferase assays at higher concentrations (>2.5 μM) (Fig. 4G). At 10 μM, CsA reduced L163F activity to approximately that of wild-type Rgg2sp in the absence of peptide pheromone. These results suggest that not only does CsA compete with the SHP pheromones at the binding pocket, but as our previous struc-
tural studies suggest, it locks Rgg2 in an inactive (pheromone-free) conformation.

**Rgg2**

To better understand the altered QS activity of our selected Rgg2 variants, Rgg2 wild type, D60N, L163F, S174L, and G232R were purified via affinity chromatography. As it had previously been shown not to interfere with in vitro assays, an N-terminal MBP tag used for purification purposes was retained during all ensuing experiments to prevent protein aggregation (18). We first sought to confirm that MBP-Rgg2 was functional in vivo and did not possess any unexpected Rgg2-like activity, secondary to the expression of the MBP tag. We expressed the MBP-Rgg2 fusion under the native **P<sub>rgg2</sub>** promoter in multicopy on plasmid pMBP-Rgg2, and observed peptide responsiveness in RVW119 (∆**rgg2** s**h2**<sub>GGG</sub> **shp3**<sub>GGG</sub> **P<sub>shp3</sub>-lux**ABCDE (strain RVW119) cells complemented with indicated Rgg2 variants on a multicopy plasmid, are reported here for increasing concentrations of indicated peptides, ranging from 0 to 1000 nM. F, Rgg2<sup>*</sup> mutants demonstrate reduced sensitivity to the competitive inhibitor of Rgg–SHP interaction, CsA. The maximum light level, normalized to cell density (RLUs), attained by RVW119 cells complemented with indicated Rgg2 variants on a multicopy plasmid were treated with 50 nM sSHP2 and concentrations of CsA ranging from 10 to 0.16 μM. Data were normalized based on maximum readings obtained in the absence of CsA. G, in the absence of sSHP2 peptide, Rgg2<sup>*</sup> constitutive activity can be reduced by CsA. Data were based on maximum RLU readings obtained at **A**<sub>600</sub> reading closest to 0.15. All data shown here are representative of at least three biological replicates. Error bars show standard deviation.

**Figure 4.** A–E, wild-type Rgg2 and select Rgg2<sup>*</sup> variants show different levels of induction when treated with peptide variants. The maximum light levels, normalized to cell density (RLUs) attained by GAS NZ131 ∆**rgg2** s**h2**<sub>GGG</sub> **shp3**<sub>GGG</sub> **P<sub>shp3</sub>-lux**ABCDE (strain RVW119) cells complemented with indicated Rgg2 variants on a multicopy plasmid, are reported here for increasing concentrations of indicated peptides, ranging from 0 to 1000 nM. F, Rgg2<sup>*</sup> mutants demonstrate reduced sensitivity to the competitive inhibitor of Rgg–SHP interaction, CsA. The maximum light level, normalized to cell density (RLUs), attained by RVW119 cells complemented with indicated Rgg2 variants on a multicopy plasmid were treated with 50 nM sSHP2 and concentrations of CsA ranging from 10 to 0.16 μM. Data were normalized based on maximum readings obtained in the absence of CsA. G, in the absence of sSHP2 peptide, Rgg2<sup>*</sup> constitutive activity can be reduced by CsA. Data were based on maximum RLU readings obtained at **A**<sub>600</sub> reading closest to 0.15. All data shown here are representative of at least three biological replicates. Error bars show standard deviation.
results did not support this hypothesis. MPB-Rgg2\textsubscript{sp}* D60N and S174L demonstrated a similar affinity for FITC-sSHP2, as seen in the overlapping polarization curves (Fig. 5A). Intriguingly, MPB-Rgg2\textsubscript{sp}* L163F and G232R failed to display any polarization. Because purification yields of MPB-Rgg2\textsubscript{sp}* L163F and G232R had been notably lower than other variants, we were concerned that these mutations may cause protein instability. Although thermal stability determined by micro-scale thermophoresis did not indicate any significant differences in unfolding-transition midpoint temperatures (T\textsubscript{m}) or in aggregation onset temperatures (T\textsubscript{agg}) (supplemental Fig. S3), circular dichroism (CD) spectra of all five purified MPB-Rgg/Rgg2\textsubscript{sp}* proteins indicated that L163F and G232R were somewhat altered, suggesting conformational differences existed (supplemental Fig. S4). Though perhaps overly cautious, we continued studies only with D60N and S174L variants.

To explore the possibility that Rgg2\textsuperscript{*} mutants may have decreased affinity for CsA when compared with wild-type Rgg2, we conducted competitive fluorescence anisotropy experiments to determine the apparent K\textsubscript{d} value for CsA in wild-type Rgg2 versus the S174L mutant (Fig. 5B). Although both wild-type Rgg2\textsubscript{sp} and Rgg2\textsubscript{sp}* S174L demonstrated equivalent affinities for sSHP2 in direct binding experiments, in light of the decreased sensitivity for CsA inhibition \textit{in vivo} by the Rgg2\textsubscript{sp}* mutants, we wondered whether changes to the conformation of Rgg2\textsubscript{sp}* mutants might alter affinity for CsA. When compared with previously published results (21), the calculated K\textsubscript{d} value for wild-type Rgg2\textsubscript{sp} was equivalent (0.53 \textmu M, 95% confidence interval, 0.25–1.08 \textmu M); however, CsA’s affinity for Rgg2\textsubscript{sp}* S174L was increased to 2.15 \textmu M (95% confidence interval, 1.46–3.16 \textmu M). These results suggest that the conformational changes incurred by the S174L mutation decreased the affinity of CsA for Rgg2\textsubscript{sp}*. As neither MPB-Rgg2\textsubscript{sp}* D60N nor S174L showed altered SHP-binding capacity \textit{in vitro} when compared with wild-type MPB-Rgg2\textsubscript{sp}, we considered the possibility that the point mutations might enhance the DNA-binding affinity of the protein, although prior studies have not indicated that increased access to the promoter would result in constitutive transcriptional activity (18). Nevertheless, to test this possibility, we conducted electrophoretic mobility shift assays (EMSA) using fluorescently labeled double-stranded DNA containing the minimum Rgg2/3-binding sequence (18). As shown previously, titration of wild-type MBP-Rgg2 between 0 and 400 nM led to a complete shift of the probe, indicating \textit{in vitro} MBP-Rgg2-DNA binding, even in the absence of SHP peptide (Fig. 5C).

Figure 5. A, MBP-Rgg2\textsubscript{sp}, D60N, and S174L bind peptide with equivalent affinity. Direct fluorescence polarization was conducted utilizing FITC-labeled sSHP2. Wild-type Rgg2\textsubscript{sp}, D60N, and S174L all displayed equivalent affinity for labeled peptide resulting in overlaying binding curves. MBP-Rgg2\textsuperscript{*} L163F and G232R failed to display any significant degree of polarization. Data are representative of three technical replicates; error bars show standard deviation. B, CsA has lower affinity for Rgg2\textsubscript{sp}* S174L. Competitive fluorescence polarization was conducted utilizing FITC-labeled sSHP2. Indicated MBP-Rgg2\textsubscript{sp} species were incubated with FITC-labeled sSHP2 for 10 min prior to the addition of increasing concentrations of CsA. The reaction proceeded for 20 min before assessment. Data are representative of two technical replicates. C–E, MBP-Rgg2\textsuperscript{sp} D60N and S174L DNA binding are limited in the absence of SHP peptide. Electromobility shift assays were conducted using 20 nM fluorescently labeled DNA and with 0, 50, 100, 200, or 400 nM MPB-Rgg2 proteins of the indicated species. 400 nM wild-type MBP-Rgg2 is included in all gels, indicated by (+). No protein control is indicated by --.
However, the DNA migration shift was substantially decreased or not apparent at all for D60N or S174L at concentrations up to 400 nM Rgg2Sp*, and even at 1,600 nM Rgg2Sp*, the shift remained incomplete (Fig. 5, D and E, and data not shown). These paradoxical findings, which indicated a decreased ability to bind DNA, led us to suspect that the mutations were affecting either proteins’ conformational stability (i.e., shifting their conformational equilibrium) or their propensity to bind DNA in vitro. We hypothesized that if this were true, then binding peptide ligands could stabilize the receptors; therefore, the EMSA was repeated in the presence of either the sSHP2 active peptide or the reverse-peptide (revSHP) at 2 μM concentrations. The addition of the sSHP2 peptide increased the DNA-binding affinity of both MBP-Rgg2Sp D60N and S174L. Notably, MBP-Rgg2Sp S174L, when sSHP2 was provided, demonstrated a shift that was similar to wild-type MBP-Rgg2Sp across all concentrations of protein. As hypothesized, the addition of revSHP or the vehicle control (DMSO) failed to improve the Rgg2Sp position 153 (20). These findings are consistent with other works relating to pheromone-binding pockets of RRNPP-type regulators, many of which rely upon polar and electrostatic interactions to coordinate their peptide ligands (22). We therefore tested the formal hypothesis that Rgg2Sp Arg-153 forms a salt bridge with the aspartic acid of the SHP peptides by substituting residues in Rgg2Sp and in SHP peptides. First, we generated R153A and R153E mutants in Rgg2Sp, changing the large, positively charged arginine residue to a small, neutral alanine, and to the oppositely charged glutamate, respectively. Transcriptional activity was then assessed in a Δrgg2 shp2GGE shp3GGE pShp3-lux background. When Rgg2Sp(R153A) was treated with wild-type synthetic pheromone (sSHP3(WT)), an ~15-fold reduction in maximum transcriptional activity compared with wild-type Rgg2Sp was observed (Fig. 6A); this result matched well with the previously reported equivalent mutation in Rgg2Sp (20). Stimulation of Rgg2Sp(R153A) with sSHP3(WT) saw a further decrease in transcriptional response, reduced by 100-fold compared with the response seen with wild-type Rgg2Sp (Fig. 6A). Next, we predicted that if sSHP3 variants with altered C-terminal residues (sSHP3(D16N) and sSHP3(D16R)) were used to stimulate the Rgg2Sp alleles, patterns of transcriptional activation would correlate with the expected electrostatic interactions between Rgg2Sp residue 153 and SHP ligands. In other words, we anticipated that if residue 153 provides an interaction with SHP3, then attractive electrostatic interactions would induce an enhanced transcriptional response, and repulsive interactions would have decreased responses. Luciferase activity of each Rgg2Sp allele was measured following stimulation with the sSHP3 variants and compared with the responses stimulated by sSHP3(WT) (Fig. 6B). For wild-type Rgg2Sp, activity induced by sSHP3(D16N) and sSHP3(D16R) was comparatively 4- and 10-fold less, respectively, than seen with sSHP3(WT). Rgg2Sp(R153A) displayed equivalent responses to both sSHP3(WT) and sSHP3(D16N), whereas sSHP3(D16R) treatment was 2.9-fold reduced. Finally, Rgg2Sp(R153E) showed a 6- and 3-fold increase in activity over sSHP3(WT) when treated with sSHP3(D16N) and sSHP3(D16R), respectively. Thus, electrostatic interaction between Rgg2Sp position 153 and the N terminus of the mature
Rgg2* mutant positions correlate with structural shift

SHP provides an important contribution to peptide recognition and allosteric regulation.

Discussion

A model for Rgg2 activation draws upon prior work investigating the Rgg2* and Rgg3* regulatory system in Group A Streptococcus (Fig. 1). A peculiar feature of this system is the dual use of both a transcriptional activator (Rgg2) and a repressor (Rgg3) to bind the same DNA sequences, control the same promoters, and respond similarly to two similar pheromones (SHP2 and SHP3) (17–19). Previous studies showed that recombinant Rgg2* forms homodimers in solution, as well as when in complex with the inhibitory molecule CsA (20). Because Rgg2* affinity for DNA is not influenced by pheromone binding (18), we propose that SHP binding triggers an Rgg2* conformational change enabling a productive interaction with RNA polymerase. The proteins show a high degree of similarity and synteny with one another (52% identical, 72% similar). Of the eight residue changes accounting for Rgg2* mutants, seven are conserved in Rgg3 (with the exception of Gln-176). How these proteins, which in all likelihood have a similar three-dimensional structure, control gene expression by opposite mechanisms remains an outstanding question, driving our ongoing studies to understand the fundamental allosteric properties of RRNPP proteins. Given what is known about other members of the RRNPP family of regulators, it is likely that peptide binding causes an allosteric conformational change in Rgg2* at sites relatively distant from the pheromone-binding pocket. However, we stress that in the absence of evidence showing SHP-mediated Rgg2* conformational changes or Rgg2* interactions with RNA polymerase, these conclusions are inferred but not proven. The disproportionally high frequency of Rgg2* mutations occurring between residues 163 and 184, and the proximity of these mutations to both the region of conformational flexibility (residues 170–177) and the pheromone-binding pocket (Fig. 2) (20), led us to hypothesize that the 170–177 region mediates conformational changes that transmit pheromone-binding signal input to polymerase-activating signal output.

How might Rgg2* mutations function to drive constitutive receptor activation? In the RRNPP family member PlcR, binding of the ligand PapR results in a conformation change in a helix that triggers a large reorientation of the PlcR DNA-binding domains, allowing for DNA binding and transcriptional activation (12). But unlike PlcR, Rgg2 binds promoter DNA even in the absence of bound pheromone. In fact, increased occupancy of the promoter by Rgg2 is not sufficient to induce transcription since prior work showed that Rgg2 requires pheromones for activation, even in the absence of the Rgg3 repressor (19). Therefore, even if Rgg2* mutants are more able than wild type to outcompete Rgg3 for promoter sites, it would not explain their increased transcriptional activity (17, 19). Thus, Rgg2* mutations must incorporate, at the very least, an enhanced ability to recruit RNA polymerase and/or stimulate initiation of transcription. Surprisingly, Rgg2* D60N and S174L mutants displayed a decreased affinity for DNA, at least in vitro. Despite a reduced ability to bind DNA, these mutants were selected by having enhanced basal transcriptional activity, conferring enhanced kanamycin resistance in the absence of pheromone in our selection system and, independently, constitutive luminescence activity in transcriptional reporters. Interestingly, addition of sSHP to recombinant Rgg2* D60N and S174L in vitro restored DNA-binding ability to levels seen for wild-type Rgg2*. It is possible that the D60N and S174L mutants affect Rgg2* by driving their conformational equilibrium away from a DNA-binding proficient conformation but toward one more amenable to pheromone- and/or polymerase-triggered activation and DNA binding. This concept is expanded on below. Regardless of the mechanism, the negative effects imposed by mutations on DNA binding must be compensated by other positive attributes that result in a net increased ability to induce transcription, likely through positive interactions with RNA polymerase.

Rgg2* response to SHP pheromone was disparate among the Rgg2* mutants. Two of the Rgg2* mutants, L163F and G232R, were refractory to pheromone binding. Although L163F was more responsive to peptides and reached higher activation levels, G232R remained primarily in a low, constitutive state. In contrast, S174L displayed an enhanced sensitivity to sSHP2 peptide (i.e., the EC_{50} for sSHP2 were lower than that seen for wild-type Rgg2). Rgg2(L163F), Rgg2(S174L), and Rgg2(D60N) even responded to SHP derivatives that were previously shown to be poor inducers of wild-type Rgg2* (Fig. 4) (17). However, surprisingly, affinity of the Rgg2* mutants for peptides was not enhanced in vitro, as determined in fluorescence anisotropy studies (Fig. 5). How can the paradox of mutants displaying enhanced sensitivity to pheromones be reconciled with the observation that they present equivalent affinities for the ligands? As mentioned above, we propose that the Rgg2* mutations have an underlying effect of shifting the free energy of Rgg2* toward a state amenable to SHP- or polymerase-dependent activation. Generation of Rgg2* mutations is reminiscent of LuxN mutants displaying altered sensitivities to autoinducer molecules (pheromones) of the AI-1 quorum-sensing system of Vibrio harveyi (23). In these studies, mutations in LuxN conferred measurable shifts in the biases of the receptor’s basal kinase and phosphatase enzymatic activities (23). In the case of Rgg2, the protein does not have kinase/phosphatase activity like LuxN, but instead it functions as a transcriptional activator. LuxN* mutants (having a free-energy shift toward the phosphatase-ON state) displayed enhanced sensitivity toward the pheromone, accounting for lower EC_{50} values, just as we describe for Rgg2* mutations. Interestingly, three of the four LuxN* mutations were located outside of the putative AI-1-binding site of LuxN, and when accounting for the free-energy shift of the enzyme, their affinities for the pheromone were unchanged.

We feel it is important to point out that although an antagonist compound of LuxN also retained equivalent IC_{50} values for the LuxN* alleles in the cited report (23), in the case of Rgg2* mutants, we observed diminished IC_{50} values for CsA as compared with its effect on wild-type Rgg2* (Fig. 5). The binding affinity for CsA was also modestly reduced, at least for the tested mutant S174L (Fig. 5B). It remains unclear why affinities for pheromone were unaltered in the Rgg2* mutants but were reduced for CsA. It is possible that CsA’s size (nearly twice the
size of SHP) or relative rigidity (CsA is cyclical although SHPs are linear) may hinder interactions with the binding pocket if subtle changes in pocket shape exist in Rgg2* mutants that do not interfere with SHP binding. Most importantly, it was interesting to find that CsA could inhibit the basal activity of Rgg2Sp* L163F in the absence of pheromone, suggesting that CsA stabilizes Rgg2 in an inactive conformation. This could have important implications for developing Rgg2 inhibitors that function in the presence of constitutively active Rgg2 mutations. If CsA or its derivatives are pursued as antivirulence lead compounds against Rgg2/3-containing streptococci, selection of Rgg2* alleles should be taken into consideration. Debate has emerged whether targeting quorum-sensing pathways by small-molecule antagonists offers a viable alternative to antibiotic therapeutics (25–29). Receptor mutants (i.e. Rgg2Sp*) identified in this report obviate the potential of natural mutations that give rise to altered protein activities that resist inhibitor action. However, whether such mutants would confer extended fitness to the organism will depend on the role the regulatory system delivers in an organism’s life cycle and the advantages that social regulation of behavior provides.

To summarize our findings, we have generated a model that integrates structural and genetic information pertaining to Rgg2 (Fig. 7). The results herein describe regions of Rgg2 conformational flexibility, which may indicate movements that initiate or result in activation of the protein. Because culturing conditions did not indicate transcriptional activation by the Rgg2Sp(C45S) mutation, we employed a genetic screen that identified active Rgg2Sp* mutants. Several of these mutants’ locations in Rgg2Sp correspond to the same regions seen to move in the Rgg2Sd(C45S)x2 crystal structure. We suggest these spatial correlations provide supportive evidence to the concept that the observed intramolecular movements are related to protein activation. Rgg2Sp* mutants display altered ranges of activity in response to pheromone, but not altered affinity toward SHP, indicating a shift in their free-energy bias.

Ideally, Rgg proteins and the RRNPP family could stand as new therapeutic targets whose allosteric misregulation would effectively modulate bacterial behavior. The ability to predict how an RRNPP regulator responds to a pheromone or to a modulatory compound would be a powerful tool for antivirulence or biotechnology strategies that would benefit greatly by manipulating bacterial gene expression through use of chemical signals.

**Experimental procedures**

**Bacterial strains and culture conditions**

Strains used in this study are described in supplementary Table S1. *S. pyogenes* was grown in Todd Hewitt medium (BD Biosciences), supplemented with 0.2% (w/v) yeast extract (Amresco). Solid media contained 1.4% agar. All luciferase experiments were conducted in a chemically defined medium containing 1% glucose (w/v) (17, 30). Antibiotics were used at the following concentrations: chloramphenicol, 3 µg/ml; erythromycin, 0.5 µg/ml; kanamycin, 100 µg/ml; spectinomycin, 100 µg/ml. Cloning was conducted in *Escherichia coli* strain BH10C. *E. coli* XL10-Gold cells (Agilent Technologies) were used for the generation of Rgg2 point mutations. BL21(DE3) cells were used for protein expression. *E. coli* cells were cultivated in Luria-Bertani medium (BD Biosciences), supplemented with the following antibiotics as appropriate: ampicillin, 100 µg/ml; chloramphenicol, 10 µg/ml; erythromycin, 500 µg/ml; kanamycin, 100 µg/ml; spectinomycin, 100 µg/ml. Peptide pheromones were purchased from Neo Scientific (Woburn, MA). Lyophilized peptides were dissolved in DMSO to a stock concentration of 2 mm based on the preparation’s purity and lyophilized weight.

**Genetic selection design**

Plasmid pRVW38 (*P*<sub>_shp2_</sub>aphA3) was generated from pSAR56 (*P*<sub>_shp2_</sub>-luxAB) (17) by digesting pSAR56 with NdeI and EcoRI. *aphA3* was amplified from pOsKaR using primers RW110 and RW111 (31). The plasmid was assembled using the NEBuilder HiFi assembly kit (New England Bioscience) prior to
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transformation into electrocompetent E. coli strain BH10C. The plasmid was verified by sequencing. To validate the selection system, pRVW31 was integrated into the genome of wild-type GAS NZ131 and cultured in the presence or absence of a synthetic SHP peptide (sSHP3), and bacteria were challenged with a range of concentrations of kanamycin. As predicted, cells treated with sSHP3 were able to divide and grow at normal rates to high density at concentrations of kanamycin up to 1,000 μg/ml, whereas cells treated only with the solvent used to apply pheromones (DMSO vehicle) failed to grow in concentrations at or above 100 μg/ml kanamycin. pRVW31 was subsequently transferred to RVW89 (NZ131 Δrgg2 Δrgg3 P_sha2-aphA3) for use in the selection. As the selection was conducted on THY medium, a medium where Rgg2/3 QS is non-functional, this strain was effectively rendered SHP-non-responsive as well. The plasmid pJC217, encoding rgg2 under its own promoter, was treated with EMS at various concentrations (ranging from 1 μl of neat EMS/μg of plasmid to 1 μl of a 1:10 dilution of EMS/μg of plasmid). Following sodium thiosulfate treatment to neutralize the EMS, 100 ng of plasmid was introduced into RVW89 via electroporation and plated on THY agar with kanamycin 100 μg/ml and spectinomycin 100 μg/ml. Isolated mutants were outgrown in THY broth under selection, and genomic DNA was obtained. Primers specific to pJC217, RV290. The plasmid was assembled with NEBuilder HiFi master mix (New England Bioscience) and verified by colony PCR. All primers are described in Table S3.

mutant rgg2 strain generation

Mutations of interest, with the exception of Rgg2 V184M, were generated de novo in pJC217 using a QuickChange Lightning site-directed mutagenesis kit (Agilent) and verified by sequencing. Rgg2 V184M was unable to be obtained via site-directed mutagenesis. Rather, the rgg2 coding sequence from an EMS-treated sample was amplified by primers RW214 and RW215 (supplemental Table S3) and cloned into pLZ12 digested with EcoRI and BglII using Gibson Assembly with NEBuilder HiFi master mix (New England Bioscience). Plasmids bearing mutant rgg2 were transformed into a P_sha2 luciferase reporter strain lacking rgg2, shp2, or shp3, RVW119 (Δrgg2 shp2 GGG shp3 GGG P_sha3 luxABCDE). Rgg2* purification

Nucleotide changes to the rgg2-coding sequence were made to the maltose-binding protein–rgg2 expression plasmid pCA104 (MBP-Rgg2), described previously (18) using Quick-Change Lightning site-directed mutagenesis (Agilent) as described previously. Following outgrowth, mutations were verified by Sanger sequencing. Plasmids encoding wild-type Rgg2 or Rgg2* mutations (D60N, L163F, S174L, or G232R) were introduced into BL21(DE3) cells by electroporation and selected on ampicillin-containing LB agar. Protein purification proceeded as described previously (18, 21). In brief, 500-ml cultures of BL21(DE3) cells were grown to early log (A600 = 0.5) and induced with 0.5 mM IPTG for 6 h at 25 °C. Cells were pelleted and lysed via sonication in Buffer A (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol) with 1× EDTA-free protease inhibitor (Thermo Fisher Scientific) and 1 mg/ml lysozyme (Sigma). Supernatants were clarified by centrifugation and applied to an amylose column (GE Healthcare). The column was washed 12 times with Buffer A, and Rgg2 was eluted in 3 column volumes of Buffer B (Buffer A + 20% glycerol at −80 °C until use).

Additional cloning

pMOS-Rgg2 was generated by amplifying the MBP-Rgg2 fusion from pCA104 using primers RW277 and RW288 (18). The pLZ12-sp backbone containing the Rgg2 fragment was generated by inverse PCR from pJC217 using primers RW287 and RW290. The plasmid was assembled with NEBuilder HiFi master mix (New England Bioscience) and verified by colony PCR. All primers are described in supplemental Table S2.

Luminescence assays

Luminescence assays were conducted as described previously (32). In brief, overnight cultures of GAS were diluted 1:100 into chemically defined medium containing appropriate antibiotics. Cells were grown at 37 °C until reaching an A600 of ~0.1. 100 μl of bacteria were transferred to each well of 96-well plates, and indicated SHP peptides or inhibitors were added at appropriate concentrations. Plates were incubated at 37 °C in a BioTek Synergy 2 plate reader (Winooski, VT); growth was assessed by absorbance at 600 nm, and luminescence was recorded as counts/s by the luminometer. Relative light units (RLUs) were calculated by dividing luminescence readings by A600 values.

Rgg2* production for crystallization

S. dysgalactiae rgg2(C45S) was cloned in pTB146 as described previously (20). His-Sumo-Rgg2(C45S) was overexpressed in E. coli strain BL21(DE3) by first growing the cells at 37 °C in LB medium containing 100 μg/ml ampicillin to A600 = 0.5 and then inducing expression with 0.5 mM IPTG for 16 h at 16 °C. The cells were collected by centrifugation and lysed in Buffer C (20 mM Tris-HCl (pH 8.0), 400 mM NaCl, 10% glycerol) supplemented with 20 μg/ml DNase. Lysate supernatant was applied to His-60 nickel resin (Clontech) equilibrated in Buffer C. His-Sumo-Rgg2(C45S) was eluted by washing the column with increasing amounts of imidazole and analyzed for purity using SDS-PAGE. Eluted protein was combined with 1.25 mg of the SUMO protease Ulp1 and dialyzed against 2 liters of Buffer D (20 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 10 mM β-mercaptoethanol, and 0.1% Triton X-100) overnight at 25 °C. The next day, the protein was passed over His-60 nickel resin to separate the cleaved tag from the Rgg2(C45S) protein. The resulting cleaved protein, Rgg2(C45S), was diluted to 100 mM NaCl with Buffer E (20 mM Tris-HCl (pH 8.0), 5 mM sodium phosphate (pH 8.0)), passed through a 0.45-μm filter, and loaded onto an anion-exchange column (Source 15Q; GE Scientific).
Healthcare) equilibrated in Buffer E. Rgg2(C45S) was then eluted in a 50–1,000 mM NaCl gradient of Buffer E. Fractions containing Rgg2(C45S) was pooled, concentrated by ultrafiltration through a 10-kDa filter, and further purified by gel filtration using a Superdex 200 (GE Healthcare) 16/70 column equilibrated in Buffer F (20 mM Tris-HCl (pH 8.0), 150 mM NaCl). Rgg2(C45S) was concentrated using a 10-kDa MWCO centrifugal filter device and stored at −80°C.

Crystallization, X-ray diffraction data collection, and structural alignments

Rgg2sd(C45S)x1 crystals were obtained by the vapor diffusion method at 20°C with 2-μl hanging drops of 4.0 mg/ml Rgg2sd(C45S) mixed 1:1 with mother liquor containing 100 mM Btis tris propane (pH 7.75), 350 mM KSCN, and 18% PEG 3350. Rgg2sd(C45S)x2 crystals were obtained by the vapor diffusion method at 20°C with 0.4-μl sitting drops of 90 μM Rgg2sd(C45S) and 480 μM SHSP2 mixed 1:1 with mother liquor containing 170 mM NH4OAc, 85 mM sodium citrate (pH 5.6), 25.5% PEG 4.000, and 15% glycerol. Prior to X-ray diffraction data collection, the Rgg2sd(C45S)x1 crystal was moved to a solution of the mother liquor supplemented with 10% glycerol and immediately flash-cooled in liquid nitrogen. X-ray diffraction data for Rgg2sd(C45S)x1 were collected using single crystals mounted in nylon loops that were then flash-cooled in liquid nitrogen before data collection in a stream of dry N2 at 100 K. X-ray diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) beamline 14-1 at 1.1808 Å with a MARmosaic 325 CCD detector. X-ray diffraction data for Rgg2sd(C45S)x2 were collected in an identical manner using our in-house Raxis IV++ image plate detector at 1.5408 Å. X-ray data for both crystals were processed using HKL2000 (33). Initial crystallographic phases were determined by molecular replacement using Phaser (34), and the previously determined structure of apo Rgg2sd (PDB code 4YV6) as a search model (20). The final models were generated using iterative cycles of model building in Coot (35) and refinement in phenix.refine (36). Initial refinement included simulated annealing as well as rigid body, individual atomic coordinate, and individual B-factor refinement. Later rounds of refinement employed individual atomic coordinate, individual B-factor, and TLS refinement. TLS groups were selected using the TLSMD server (37). During the final rounds of refinement, the stereochemistry refinement. TLS groups were selected using the TLSMD server

Rgg2* mutant positions correlate with structural shift

Direct fluorescence polarization (FP) assays were conducted as described previously (15). In brief, purified MBP-Rgg2 (or indicated mutant variant Rgg2*) was serially diluted in FP Buffer (Buffer A supplemented with 0.01% Triton and 0.1 mg/ml BSA). A master mix of FITC-sHSP2 was prepared in FP Buffer and aliquoted into a black, ½ area 96-well plate (Corning). Diluted MBP-Rgg2 was added to each well, and allowed to equilibrate for 30 min at 25°C in the dark. The protocol was modified to assess CsA competition as follows: 230 nM MBP-Rgg2 or MBP-Rgg2 S174L was incubated in FP Buffer with 10 nM FITC-sHSP2 for 10 min. CsA was added to the indicated concentration and allowed to equilibrate for an additional 20 min at 25°C in the dark. Plates were assessed on a BioTek Synergy 2 plate reader, and in-system software was used to calculate polarization values.

Electrophoretic mobility shift assays

EMSSAs were conducted as described in Chang et al. (17). A master mix of EMSA Buffer (20 mM HEPES (pH 7.9), 100 mM KC1, 12.5 mM MgCl2, 0.2 mM EDTA (pH 8.0); 0.5 mM dithiothreitol, 50 mg/ml sheared salmon sperm DNA, 0.001 units/μl poly(dI-dC), 100 mg/ml bovine serum albumin, 0.5 mM CaCl2, 12% (v/v) glycerol) with 20 nM double-stranded FITC-Pshp2 probe (CCATTTCCTCCATTTTCAAAAC) was prepared and aliquoted into a 96-well plate. Purified Rgg2/Rgg2* proteins were diluted in EMSA Buffer and added to the probe master mix to the indicated final concentration. sHSP2, revsHSP, or DMSO was also added to reactions at that time. The reaction was allowed to proceed at 25°C for 30 min in the dark. The resulting product was loaded and run on a 5% native-PAGE gel, buffered with 20 mM phosphate. The resulting gels were imaged on a Typhoon Trio imaging system (GE Healthcare).

Circular dichroism

Circular dichroism (CD) spectra were collected on a JASCO J-810 (Easton, MD) system using a 2-cm cuvette. Samples were diluted in CD Buffer (20 mM potassium phosphate (pH 7.6), 100 mM NaF) to a final concentration of 5 μM. Scans were conducted between 260 and 190 nm, using 0.5 nm increments.

Protein thermostability assays

To assess the thermal stability of MBP-Rgg2, purified proteins were subjected to heat denaturation using the Prometheus NT.48 nanoDSF (NanoTemper Technologies). Samples were subjected to a temperature ramp of 1.0°C/min from 20 to 95°C, and fluorescence was constantly monitored. Data were analyzed with the PR.ThermControl software.
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Statistics
All statistical calculations and curve fitting were performed on Graph Pad Prism Version 7 software (La Jolla, CA).

Accession numbers
Atomic coordinates and structure factors for Rgg2sd (C45S)x1 and Rgg2Sd(C45S)x2 have been deposited in the Protein Data Bank, and assigned PDB code 5W4M and PDB code 5W4N, respectively.

Author contributions—Substantial contributions to conception and design were provided by R. V. W., A. K., G. C. C., M. B. N., and M. J. F. Acquisition of data was provided by R. V. W., A. K., G. C. C., and K. M. T. Analysis and interpretation of data were from R. V. W., A. K., G. C. C., M. B. N., and M. J. F. Drafting and revising the article were done by R. V. W., G. C. C., A. K., M. B. N., and M. J. F.

Acknowledgments—We are grateful to the members of the Federle laboratory for their ongoing input on this project, especially for their assistance in the conceptualization of the genetic selection described here. We thank Gerd Prehna for assistance with the circular dichroism experiments. We are grateful to NanoTemper Technologies for providing thermostability analyses.

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