Published in final edited form as:

Mol Microbiol. 2009 October ; 74(1): 44–57. doi:10.1111/j.1365-2958.2009.06849.x.

Symmetric Signaling within Asymmetric Dimers of the *Staphylococcus aureus* Receptor Histidine Kinase AgrC

Elizabeth A. George Cisar^{#1}, Edward Geisinger^{#2}, Tom W. Muir^{1,*}, and Richard P. Novick^{2,*}

¹Laboratory of Synthetic Protein Chemistry, Training Program in Chemical Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

²Molecular Pathogenesis Program and Departments of Microbiology and Medicine, the Kimmel Center for Biology and Medicine of the Skirball Institute, New York University School of Medicine, New York, New York 10016

These authors contributed equally to this work.

Abstract

Virulence in *Staphylococcus aureus* is largely under control of the *accessory gene regulator* (*agr*) quorum sensing system. The AgrC receptor histidine kinase detects its autoinducing peptide (AIP) ligand and generates an intracellular signal resulting in secretion of virulence factors. Although *agr* is a well-studied quorum sensing system, little is known about the mechanism of AgrC activation. By co-immunoprecipitation analysis and intermolecular complementation of receptor mutants, we showed that AgrC forms ligand-independent dimers that undergo *trans*-autophosphorylation upon interaction with AIP. Remarkably, addition of specific AIPs to AgrC mutant dimers with only one functional sensor domain caused symmetric activation of either kinase domain despite the sensor asymmetry. Furthermore, mutant dimers involving one constitutive protomer demonstrated ligand-independent activity, irrespective of which protomer was kinase deficient. These results demonstrate that signaling through either individual AgrC protomer causes symmetric activation of both kinase domains. We suggest that such signaling across the dimer interface may be an important mechanism for dimeric quorum sensing receptors to rapidly elicit a response upon signal detection.

Keywords

quorum sensing; histidine kinase; symmetric signaling; AgrC; signal transduction mechanism

Introduction

Bacteria commonly sense and react to their environment through two-component systems (TCSs) that rapidly convert external stimuli into internal changes in gene expression. These systems are typically composed of a transmembrane sensor module, the receptor histidine protein kinase (HPK), and a cytoplasmic transcription factor, the response regulator (RR). Stimulus perception leads to activation of the receptor, which in turn affects the regulatory activity of the associated RR. Although TCSs respond to a variety of environmental signals, including osmolarity, pH, oxygen, and chemoattractants (Mascher *et al.*, 2006), in most cases the specific activating ligands remain unknown. There is, however, one important

*Correspondence: muir@mail.rockefeller.edu (T.W.M.), novick@saturn.med.nyu.edu.

SUPPLEMENTAL DATA

Supplemental data include five figures and two tables.

class of TCSs with known ligands, namely the quorum sensing autoinduction systems, which regulate their target genes in response to population density. In these systems, the ligand is encoded within the TCS locus, is secreted, and then binds specifically to the receptor, thereby activating the TCS and its own synthesis. Specificity is determined by the highly variable extracellular sensor domain of the receptor, which transmits the ligand-induced activating signal to the highly conserved cytoplasmic histidine kinase (HK) domain. These receptors are typically dimeric and their HK domains consist of two subdomains; one, the DHP subdomain, contains the phosphorylation-site histidine and classically has a helical coiled-coil structure that contains the dimerization interface. The other, the kinase (CA) subdomain, contains the catalytic ATP-binding site, which mediates the *trans*-autophosphorylation of the contralateral histidine (Dutta *et al.*, 1999). Although many studies have elucidated the architectures of HK subdomains and their relationships under basal conditions, the conformational changes associated with the regulation of kinase activity are still generally unclear (Szurmant *et al.*, 2007).

In this paper, we report a series of studies with a prototypical quorum sensing HPK, the AgrC protein of *Staphylococcus aureus*, with a view toward elucidation of the signal transduction mechanism. AgrC is a member of the class 10 HPK family (Grebe & Stock, 1999), each of which has a polytopic transmembrane sensor domain, an arginine or lysine adjacent to the active site histidine and several (but not all) of the other typical HPK motifs defined by Grebe and Stock (Grebe & Stock, 1999). AgrC is encoded by the *agr* locus, which consists of two divergent transcription units, driven by promoters P2 and P3 (Figure 1A). The P2 operon encodes the AgrC–A TCS (of which AgrA is the RR), plus AgrB and D, of which the former participates in processing and secretion of the latter to generate the autoinducing ligand (the AIP), a 7-10 amino acid peptide with a novel thiolactone ring. The AIP activates the *agr* TCS, leading to both P2 autoinduction and generation of the P3 transcript. This transcript, a 514 nt regulatory RNA, RNAIII, is the intracellular effector of the *agr* response (Janzon & Arvidson, 1990, Novick *et al.*, 1993). The *agr* locus, conserved among the staphylococci and with homologs in many other species, has undergone an interesting evolutionary divergence, giving rise to variant specificity groups, of which there are four in *S. aureus* and two or more in several other staphylococcal species (Ji *et al.*, 1997, Jarraud *et al.*, 2000, Dufour *et al.*, 2002). Among these specificity groups, heterologous AIP–AgrC interactions are usually inhibitory, with heterologous AIPs acting as competitive antagonists (Lyon *et al.*, 2002a) and/or inverse agonists (Geisinger *et al.*, 2009).

In previous studies, we and others have performed structure-activity analyses of the AIP (George & Muir, 2007), have localized determinants of receptor specificity (Geisinger *et al.*, 2008, Jensen *et al.*, 2008), and have isolated and mapped a series of constitutive AgrC mutants (Geisinger *et al.*, 2009). In this study, we have demonstrated by co-immunoprecipitation that AgrC exists as a pre-formed dimer. By co-expressing mutant receptors with inactivated histidine or kinase motifs, we have observed ligand-dependent complementation of activity, thereby demonstrating that AgrC activation, like that of other HPKs, occurs through dimerization and *trans*-autophosphorylation. Using this complementary mutant dimer paradigm, we prepared constructs in which the sensory input was exclusively or primarily from one receptor sensor domain. With these constructs, we have demonstrated that input from a single receptor is sufficient for activation. This finding enabled us to test the hypothesis that the protomers in a dimeric receptor are activated sequentially and linearly; that is, an activating signal is transmitted directly to the cytoplasmic domain of the same receptor, activating the kinase, which then phosphorylates the histidine of the other protomer. Our results, however, showed roughly equivalent activation whether the functional receptor was linked to a mutant kinase or a mutant histidine site. Similar mutant dimers, in which one or the other protomer had an additional mutation in the DHP subdomain conferring constitutive activity, exhibited ligand-

independent activity irrespective of which protomer contained the constitutive mutation. These results together suggest that the above linear signaling hypothesis is incorrect and that AgrC signal transduction is inherently symmetric. We suggest that this symmetry is based on bidirectional signaling across the dimer interface between the cytoplasmic domains of the HPK protomers.

Results

Construction and characterization of AgrC phosphorylation site and catalytic domain mutants

The prototypical histidine kinase functions as a dimer and undergoes *trans*-autophosphorylation (Dutta et al., 1999, Stock *et al.*, 2000). In order to test whether AgrC follows this paradigm, we adopted a mutant complementation approach similar to that used to demonstrate functional dimerization and *trans*-autophosphorylation for HPKs in *Escherichia coli* (Yang & Inouye, 1991, Swanson *et al.*, 1993, Ninfa *et al.*, 1993). Our aim was to create and analyze two complementary mutants, one lacking an appropriate phosphorylation site and one with a dysfunctional kinase. When expressed individually, these mutants would be inactive; when co-expressed, if AgrC were to undergo dimerization and *trans*-autophosphorylation, mutant heterodimers would form in which each protomer would complement the other's defect. For this experiment to be convincing, the individual mutants must be inactive so that any activity would be indicative of the presence of mutant heterodimers. Through sequence alignment of the AgrC cytoplasmic domain with that of other HPKs, we identified conserved residues representing the putative phosphorylation site and catalytic motifs (Grebe & Stock, 1999) (Figure 1B, C). The H-box histidine (His239) is the predicted phosphorylation site, and the N-box asparagine (Asn339) and G-box glycine residues (Gly394 and Gly396) are critical for ATP binding in other HPKs (Hirschman *et al.*, 2001, Zhu & Inouye, 2002). Accordingly, we mutated these residues in AgrC-I and tested the activities of the resulting receptor mutants in *S. aureus* β -lactamase reporter cells (Lyon *et al.*, 2002b). These cells contain a chromosomal *agrP3-blaZ* fusion and carry either WT or mutant *agrC*, driven by the *agrP2* promoter. The reporter strains lack the ability to produce AIPs, allowing measurement of AgrC activity in response to treatment with varying concentrations of synthetic AIP. As expected, the H-box mutant (H239Q) was completely inactive (Figure S1). This result is consistent with the prediction that His239 is the site of phosphorylation, as is the fact that His379 of the CA subdomain is the only other histidine in the protein. Surprisingly, mutation of the N-box (N339D) resulted in only partially reduced receptor activation unlike the effect of the corresponding mutation in EnvZ (Zhu & Inouye, 2002). Each individual G-box glycine mutant (G394A and G396A) resulted in weaker activation than that seen with AgrC-I N339D, while the double mutant (G394A, G396A) was completely inactive up to the highest concentration of AIP-I tested (10 μ M). Thus, AgrC-I_{H239Q} and AgrC-I_{G394A, G396A} were selected for subsequent complementation analysis and are hereafter referred to as AgrC-I_{His} and AgrC-I_{Kin}, respectively, to indicate the particular defect of each mutant.

In order to determine the cellular localization and expression levels of AgrC-I_{His} and AgrC-I_{Kin}, we constructed C-terminal GFP-fusions of the two mutants and WT AgrC-I. These GFP fusions enabled direct visualization of receptor localization and abundance via fluorescence microscopy and facilitated measurement of protein levels using western blot analysis (Figure 2). Ligand-dependent kinase function was preserved in the AgrC-IGFP fusion protein, as receptor activation by AIP-I was only slightly decreased compared to the WT receptor (EC_{50} s = 16 and 12 nM, respectively; Figure S2, Table 1). AgrC-I-GFP localized to the cell membrane and was distributed approximately evenly throughout the cell perimeter, as visualized by fluorescence microscopy (Figure 2C-I). The membrane

fluorescence of cells that express WT AgrC-I-GFP and secrete endogenous AIP was much brighter than that of cells lacking the *agrB* and *agrD* genes necessary for AIP biosynthesis (Figure 2D, E), providing visual confirmation of the autocatalytic induction of *agr* expression upon AgrC activation (Novick *et al.*, 1995). Furthermore, the attenuated fluorescence of an AIP producing strain that lacks *agrA* showed that the observed induction of AgrC expression is dependent on the AgrC–A TCS (Figure 2C). The images of cells expressing AgrC-I-GFP_{His} and AgrC-I-GFP_{Kin} fusions (Figure 2F, G) resembled those of uninduced cells carrying WT AgrC-I-GFP (Figure 2C, D), suggesting that the phosphorylation site and catalytic domain mutations do not adversely affect localization or expression. Western blots of total cellular protein prepared from cultures of strains containing the AgrC-I-GFP constructs also showed that expression of the AgrC-I-GFP_{His} and AgrC-I-GFP_{Kin} mutants is comparable to WT AgrC-I in the absence of AIP (Figure 2A). As the precise expression levels of the WT and mutants was not important to the experimental design, the moderate variability in expression between the two mutants was not addressed.

AgrC is a dimer and undergoes *trans*-autophosphorylation

Having established that the individual AgrC-I_{His} and AgrC-I_{Kin} mutants have no detectable kinase activity but are expressed and are appropriately localized, we next co-expressed the two mutants by introducing the compatible plasmids, pEG58 and pEG59, into the reporter strain background described above (see Figure 3A). In cells co-expressing AgrC-I_{His} and AgrC-I_{Kin}, we observed dose-dependent activation by AIP-I, with a slightly increased EC₅₀ value of 40 nM compared to 10 nM for WT AgrC (Figure 3B, Table 1). Similar activation was observed with the GFP-fused variants (Figure S2). The maximal activity of the co-expressed mutants was moderately reduced compared to WT AgrC, consistent with the statistical expectation of the formation of inactive mutant homodimers. In order to rule out plasmid recombination as a source of active AgrC in this hybrid strain, we confirmed plasmid size by agarose gel electrophoresis of whole cell minilysates, and reconfirmed the inactivity of the mutants by backcrossing each plasmid to a naïve reporter background (data not shown).

In order to ensure that the complementary mutations in AgrC-I do not alter ligand recognition, we assessed the ability of a non-cognate AIP to block activation of the co-expressed mutants by AIP-I. The reporter cells co-expressing both mutant AgrCs were treated with a fixed concentration of AIP-I and increasing doses of AIP-III, a potent inhibitor of AgrC-I. In this test, we observed a dose-dependent decrease in activity (Figure 3C, Table 1). A similar result was observed using the inhibitor AIP-II (data not shown). Thus, AgrC-I_{His} and AgrC-I_{Kin} form functional complexes that maintain WT ligand specificity. This observation of intermolecular complementation between an AgrC-I phosphorylation site mutant and a catalytic domain mutant demonstrates that activation of AgrC occurs via *trans*-autophosphorylation within oligomeric complexes. Since activation of the HK domain in this paradigm occurs by a bimolecular reaction, the simplest interpretation of these results is that the functional unit of AgrC is a dimer, which is consistent with the behavior of most or all HPKs in other systems (Stock *et al.*, 2000, Dutta *et al.*, 1999). Although AgrC is the first of the class 10 HPKs to be analyzed in this manner, there is no reason to believe that it and others in the same class would behave dramatically differently from other HPKs. Although our results do not rule out the possibility of higher order oligomers, it is not obvious how a multimeric complex would carry out a bimolecular reaction involving more than two protomer subunits. In other words, any multimeric complex would have to be composed of dimers, and therefore would not be important for interpretation of the results presented here.

AgrC is a pre-formed dimer

We next addressed the question of whether the AgrC dimer/oligomer is pre-formed, as has been shown for VirA of *Agrobacterium tumefaciens* (Pan *et al.*, 1993) and the HPK-associated aspartate receptor, Tar (Milligan & Koshland, 1988), or ligand-induced. For this purpose, we performed co-immunoprecipitation of differentially tagged versions of AgrC in the absence and presence of AIP. To ensure that dimers formed by the tagged AgrCs (AgrC-I-GFP and AgrC-I-HA, respectively) are functional, we first analyzed cells co-expressing the corresponding complementary mutants, AgrC-I-GFP_{His} and AgrC-IHA_{Kin}. This pair exhibited robust dose-dependent activation by AIP-I, with an EC₅₀ of 31 nM (Figure 4A). Western blots of total lysates from cells co-expressing WT AgrC-I-HA and AgrC-I-GFP but not producing AIP revealed the presence of high molecular weight bands that correspond to putative, pre-formed AgrC dimers or oligomers (Figure S3). Next, immobilized GFP antibodies were used to immunoprecipitate AgrC from these lysates, and western blot analysis showed that AgrC-HA is immunoprecipitated only when AgrC-GFP is present and that AgrC-HA interacts with AgrC-GFP in the absence and presence of AIP (Figure 4B). Similar results were obtained when HA antibodies were used to immunoprecipitate the complex (Figure S4). Although the result shown in these western blots clearly establishes that the functional AgrC complexes are pre-formed, it does not clearly distinguish between dimers and higher order oligomers.

In order to rule out the possibility that co-immunoprecipitation of the differentially tagged receptors was due to non-specific interactions with membrane proteins, we examined whether Sortase, an unrelated membrane protein involved in anchoring proteins to the cell wall, was also immunoprecipitated. While it appeared in the total lysate inputs, Sortase was not present in the samples eluted from the beads (Figure S4A). Furthermore, formation of AgrC-GFP / AgrC-HA complexes was not observed when a mixture of lysates from cells expressing only AgrC-GFP or AgrC-HA was analyzed by immunoprecipitation (data not shown), indicating that dimerization occurs only in the cell and not following lysis. These results lend further support to the fundamentally dimeric nature of AgrC and demonstrate that these dimeric complexes form independent of ligand binding.

One intact AgrC sensor domain is sufficient for receptor activation

In the following sections, we have used complementary AgrC mutant dimers to analyze various aspects of AIP–AgrC signaling. We first attempted to evaluate the stoichiometry of the ligand–receptor interaction by means of a truncated sensor domain. We constructed N-terminal truncations of AgrC-I, deleting progressive segments of the transmembrane sensor domain, and made the same truncations of AgrC-I_{Kin}. None of the resulting dimers, with two truncated sensor domains, displayed significant activity in response to AIP-I when expressed alone (Figure S5A). GFP fusions of the truncated mutants revealed that although they localized to the membrane, there was considerable fluorescence throughout the cytoplasm, suggesting that their membrane localization was less complete than that of the WT (data not shown). Nonetheless, co-expression of full-length AgrC-I_{His} with a truncated receptor lacking most of the sensor domain (AgrC-I_{Δ1-135, Kin}) resulted in significant activity (Figure S5B). Note that this truncation removed the second extracellular loop region, which is critical for activation by AIP-I (Geisinger *et al.*, 2008) and presumably necessary for AIP binding. The complementation of this mutant by AgrC-I_{His} was dose-dependent, as shown in Figure S5B, although the maximal activation was only a third of that observed for the full-length complementary pair. These results suggest that if the functional unit is a dimer, there will be only a single sensor domain in the complex between intact and truncated molecules and therefore that ligand binding to a single receptor protomer would be sufficient for activation. This model is consistent with 2:2 stoichiometry for AIP:AgrC binding to the wild-type receptor. Although, as noted above, the functional unit of the HK domain must be

a dimer, the situation would be more complex if the receptor were arranged as a higher order oligomer – for example as a tetramer. In that case, no implication of stoichiometry could be entertained, and the possibility would exist that the ligand-binding pocket could consist of more than one protomer. The implications of this possibility with respect to symmetrical signaling are discussed below.

The weakness of the response in the above pairing could have been a consequence of the relatively weaker membrane localization of the truncated receptor. To address this possibility, we repeated the test with full-length AgrC-I derivatives carrying mutations T104V, S107A, and S116I in the second extracellular loop region (Figure 1B) that reduced sensitivity to AIP-I by about 50-fold (Geisinger et al., 2008). For simplicity, this mutant is hereafter referred to as AgrC-I_{Sensor}. A fluorescence image and western blot of an AgrC-I_{Sensor}-GFP fusion demonstrated that membrane localization and expression levels are very similar to those of the WT (Figure 2A, H, I). We next introduced the histidine or G-box mutations into AgrC-I_{Sensor} and tested these mutants alone and in pairings with AgrC-I_{His}, AgrC-I_{Kin}, or each other, for activation by AIP-I. Co-expression of AgrC-I_{Sensor, His} and AgrC-I_{Sensor, Kin} resulted in activity reduced over 150-fold compared to that observed with the WT sensor domains (Figure 5C, Table 1), represented by an EC₅₀ value of 6.1 μM. However, when AgrC-I_{Sensor, Kin} was combined with AgrC-I_{His} (Figure 5A), activation by AIP-I increased approximately 30-fold, represented by an EC₅₀ value of 200 nM (Figure 5C, Table 1). Unlike the results with the truncated receptors, the complementation in these combinations was characterized by a maximal activation efficacy similar to that observed with the AgrC-I_{His} / AgrC-I_{Kin} pair. Because one of the sensor domains within the AgrC-I_{His} / AgrC-I_{Sensor, Kin} dimer is dysfunctional, these results are consistent with the above observation that only one functional sensor domain is sufficient for activation within an AgrC dimer.

Receptor activation is symmetric

The finding that a single sensor domain is sufficient for activation enabled a test of the intuitive hypothesis that signaling is direct and sequential; that is, the activation signal is transmitted exclusively by each receptor to its own HK domain, which then *trans*-phosphorylates the contralateral histidine. This hypothesis predicts a profound asymmetry in the activation mechanism depending on whether the ligand-bound sensor domain is linked to an active or an inactive kinase. If the linked kinase were functional, activation would be robust; if the linked kinase were inactive, the receptor would be activated very poorly, if at all. To test this prediction, we analyzed the reciprocal mutant combination, AgrC-I_{Sensor, His} and AgrC-I_{Kin} (Figure 5B), and were surprised to find that the prediction was incorrect. The AgrC-I_{Sensor, His} / AgrC-I_{Kin} pair was nearly as active as the reciprocal pairing (AgrC-I_{His} / AgrC-I_{Sensor, Kin}) described above, as reflected in the EC₅₀ values of 310 and 200 nM, respectively (Figure 5, Table 1). Inhibition tests of both pairings demonstrated that the observed activity of the mutant dimers could be antagonized by AIP-III in a dose-dependent fashion (Figure 5D, Table 1). Thus, AgrC activation appears to be symmetric; instead of following the linear pathway predicted above, ligand binding to the sensor domain of either protomer resulted in *trans*-autophosphorylation by the functional kinase domain. A possible explanation for this result is that ligand-induced conformational changes are transferred across the dimer interface, and that these are sufficient to activate the contralateral kinase domain. An alternative possibility is that AgrC activation occurs through a composite AIP binding pocket formed by the sensor domains of both protomers. The possible impact of this on symmetrical signaling is discussed below.

To test the idea of symmetric signaling by AgrC further, we performed an experiment in which the *agrC*s belonging to two different *agr* specificity groups, I & II, were co-

expressed. Note that the sensor domains of AgrC-I & II share only 31% sequence identity, whereas the HK domains are identical, and AIP-I & II are potent cross-group inhibitors. Mutations replacing the active site histidine or inactivating the kinase function of AgrC-II, corresponding to those made in AgrC-I, were constructed and used to test for the formation of heterodimers and their response to AIP-I & II. As with AgrC-I, the AgrC-II mutants (AgrC-II_{His} and AgrC-II_{Kin}) were completely inactive individually (Table 1). Here again, the linear pathway model would predict that the AIP specific for the sensor domain attached to a functional kinase would activate an AgrC-I / -II heterodimer, whereas that specific for the sensor domain attached to a mutant kinase would not. Once more, however, this prediction was incorrect: either of the AIPs activated either of the heterodimers to approximately the same extent (Figure 5E, F, S6A, B). For example, the pairing of AgrC-I_{His} and AgrC-II_{Kin} (Figure 5E) was activated equally well by AIP-I and AIP-II, as represented by EC₅₀ values of 21 and 17 nM, respectively (Table 1). To ensure that the ligand recognition by the unnatural sensor domain pairings was not fundamentally changed, we tested for inhibition by AIP-III, an antagonist of both AgrC-I & II. AIP-III inhibited activation of both AgrC-I / -II mutant heterodimers (Figure 5G, S6C, Table 1). These data lend further support for the above conclusion that a single ligand-sensor domain interaction is sufficient for activation of either kinase domain.

Symmetric signaling by a constitutive AgrC mutant

The results obtained in the previous experiments point towards an activation model in which conformational changes are transduced from either protomer unit to the other within the context of the HK domains – *i.e.*, subsequent to and separable from the initial interaction between ligand and receptor. To test this model, we incorporated a recently isolated constitutive AgrC mutant, AgrC-I_{R238H} (Geisinger et al., 2009) into our complementation analysis. The point mutation in this constitutive variant is located in the predicted dimerization domain, directly preceding the active site histidine. In the absence of ligand, AgrC-I_{R238H} exhibits full activity, reaching a level equivalent to that observed with activation of WT AgrC-I by AIP-I (Figure 6C). As would be expected, the constitutive mutant receptor exists as a pre-formed dimer (Figure 4B, S4B). However, the level of AgrC-I_{R238H} dimerization was less than that of the WT receptor under our experimental conditions, a finding that may be relevant to the mechanism of the constitutively active receptor.

As the dimerization domain of AgrC-I_{R238H} is expected to assume the conformational state associated with normal ligand-dependent receptor activation, we hypothesized that this conformation could be transduced to a sister WT AgrC-I protomer of a dimer, resulting in constitutive *trans*-autophosphorylation. To test this prediction, we introduced the H239Q or G394, G396A mutations to AgrC-I_{R238H} and co-expressed each construct with the complementary AgrC-I mutant. Individually, the AgrC-I_{R238H}, *His* and AgrC-I_{R238H}, *Kin* mutants were inactive (Figure 6C), confirming that ligand-independent activity depends on these critical residues. When AgrC-I_{R238H}, *His* and AgrC-I_{R238H}, *Kin* were co-expressed in the same strain, however, complementation between the mutants was observed, with constitutive activation reaching a level similar to that seen with AIP-I-induced activation of the AgrC-I_{His} / AgrC-I_{Kin} complementary pair (Figure 6C). Next, mutant dimers composed of one constitutive and one normal receptor were analyzed. Combination of AgrC-I_{R238H}, *His*, the mutant containing a constitutively active kinase subdomain, with AgrC-I_{Kin}, as illustrated in Figure 6A, resulted in ligand-independent activation at a magnitude nearly as high as that observed with the homologous AgrC-I_{R238H}, *His* / AgrC-I_{R238H}, *Kin* pairing (Figure 6C). With the reciprocal combination, involving AgrC-I_{His} and AgrC-I_{R238H}, *Kin* (Figure 6B), there was again activity in the absence of AIP-I (Figure 6C), consistent with symmetric signaling. In this case, the activity was only about one third of that seen with the

reciprocal dimer (AgrC-I_{R238H, His} / AgrC-I_{Kin}), indicating that the symmetry was imperfect. Nonetheless, an activating conformational change imparted by one point mutation was transferred between protomers of an AgrC dimer.

Discussion

In this study, we have directly probed the activation mechanism of the polytopic, quorum-sensing HPK, AgrC, a receptor that is critical to the biology of the pathogenic organism *S. aureus*. By demonstrating complementation between receptor mutants lacking kinase or histidine phosphorylase activity, we have confirmed for AgrC the well-established paradigm for HPK signaling in which each subunit *trans*-autophosphorylates the other. Since this clearly represents a bimolecular reaction between the cytoplasmic HK domains of the receptor, it is clear that the functional unit of the HK domain is a dimer. Although we have also demonstrated by co-immunoprecipitation that receptor complex formation is ligand-independent, we have not addressed the possibility that the entire receptor forms a higher order multimer. This possibility is deferred until later. For this part of the discussion, we continue to refer to the functional receptor complex as a dimer. The moderately reduced maximal activity and potency of the AgrC-I_{His}/AgrC-I_{Kin} complementary pair compared to WT AgrC is consistent with the prediction that the mutants form a statistical mixture of dimers in which only half of the AgrC molecules form functional heterodimers, each containing only one out of two viable phosphorylation sites. The concentration of agonist-bound receptor required to elicit a half-maximal response, K_E , is thereby increased; and, according to Black and Leff's operational model of agonism, both efficacy and potency decrease as K_E increases (Black & Leff, 1983).

To address the mechanism of receptor activation, we used several strategies in which the configuration of the sensor domain was varied. By pairing a truncated receptor lacking extracellular loop regions necessary for ligand recognition (Geisinger et al., 2008) with an intact one, we observed ligand-induced activation, suggesting that a single receptor sensor domain is sufficient. The activation here was rather weak, probably owing to suboptimal localization at the membrane. To obtain optimal membrane localization with an essentially non-functional receptor, we used a mutant AgrC-I with three site-directed substitutions in the second extracellular loop, causing a ~50-fold reduction in response to AIP-I (Geisinger et al., 2008). With this mutant receptor, we observed nearly full complementation with the WT receptor in the heterodimeric configuration, again consistent with activation of signaling by a single functional receptor molecule. Although these findings suggest that a single receptor protomer forms a functional ligand-binding pocket and is sufficient for activation, it is conceivable that the functional unit of the entire receptor is a higher order oligomer, *e. g.*, a tetramer, and that the ligand-binding pocket is formed by two sensor domains. If this were the case, then the tetramer of two truncated and two intact receptors would have two intact sensor domains and would form a normal binding pocket, generating normal activity. However, the activity of the complex is weak, meaning that the full ligand-binding pocket would have to be composed of four sensor domains. A tetrameric receptor is not formally ruled out by our data, and it would obviate the concept of symmetrical signaling, if it were not definitively supported by the constitutive receptor mutants (see below).

The receptor mutants also enabled a test of the intuitive hypothesis that the activation signal is transmitted directly by the receptor to its own kinase domain, which then *trans*-phosphorylates the contralateral histidine. This hypothesis predicts that signaling depends on whether the ligand-bound sensor domain is linked to an active or an inactive kinase: if the linked kinase is functional, activation will be robust; if the linked kinase is inactive, the receptor will be activated very poorly or not at all. In this test, and in a parallel test with heterologous sensors of AgrC-I and AgrC-II, both heterodimers had essentially the same

activity; therefore, the linear signaling pathway hypothesis is clearly incorrect. Instead, signaling within the HK domain must be symmetric; that is, ligand binding to either protomer must induce or stabilize a conformational change in the HK domain that enables activation of kinase activity, whether it is connected to the functional or the non-functional sensor. In the case of the heterologous pairings, the maximum activation levels were considerably lower than for the homologous mutant pair. This result could possibly represent the transmission of a weak inhibitory signal by the noncognate AIP, based on the independent finding that both of these AIPs show inverse agonism with certain constitutive mutants of the respective non-cognate receptors (Geisinger et al., 2009). It is also possible that heterodimerization of AgrC-I and AgrC-II is less favorable than homodimerization of AgrC-I.

Since our data do not definitively establish whether AgrC is a dimer or higher order oligomer, and therefore studies of receptor domain variants do not *per se* support the concept of symmetrical signaling unequivocally, we tested for symmetrical signaling in the absence of sensor domain function by using a constitutive receptor mutant. This mutant has a histidine replacing the conserved arginine at receptor position 239 and is fully activated in the absence of any ligand (Geisinger *et al.*, 2009). Here again, we used HK mutants lacking either kinase activity or the active site histidine. When paired with a receptor lacking the constitutive mutation, we observed complementation in the absence of AIP ligand, although the activity was about three times stronger when the constitutive receptor contained the functional kinase. Nonetheless, these results demonstrate that an activating signal generated by one protomer was transduced across the dimer interface to the sister protomer, in support of the idea that signaling is symmetrical.

On the basis of these results, we have developed the following working model of AgrC signal transduction, here again, for the sake of simplicity, basing the discussion on the receptor as a functional dimer. Cognate ligand binding to one or both transmembrane sensor domains of an AgrC homodimer induces or stabilizes an active conformation in the corresponding cytoplasmic DHp subdomain(s). Intermolecular interactions across the coiled-coil dimer interface induce or stabilize functionally parallel conformations in both protomers whether or not they are ligand-bound. The concerted formation of the activated conformational state in both protomers leads to phosphorylation of each histidine by the contralateral kinase subdomain. It seems intuitively likely, though there is no explicit evidence, that with the WT receptor, both histidines are phosphorylated more or less simultaneously, and there is no reason to think this would not also be the case when only one receptor protomer is activated. It is also possible that only one of the histidines is phosphorylated, and that the choice could be random. While the nature of the conformational changes responsible for symmetric signaling by AgrC is yet to be determined, they are likely mediated by coiled-coils in the predicted dimerization (DHp) subdomain. Recently, several constitutively active AgrC mutants were isolated with single point mutations in the putative coiled-coil region of the DHp subdomain (Geisinger et al., 2009), suggesting that this region of AgrC plays an important role in the activation mechanism. Furthermore, constitutive mutants of SppK of *Lactobacillus sakei* and ComD of *Streptococcus pneumoniae*, two other quorum sensing receptors of the HPK₁₀ family, were found to contain point mutations in or near the predicted DHp coiled-coils (Mathiesen *et al.*, 2006, Martin *et al.*, 2000). These findings suggest that critical conformational changes involving the coiled-coil dimer interface, and by inference symmetrical signaling, may be a general mechanism by which the quorum sensing HPKs transmit their signals.

Although not present in class 10 HPKs, the HAMP linker, an additional coiled-coil domain found in many other signaling proteins, was shown in a previous analysis to be critical for symmetrical signaling in chimeric mutants of the *E. coli* aspartate receptor Tar and the

osmosensor EnvZ. In these hybrid dimeric receptors, homologous HAMP domain pairings enabled symmetric signal transduction through the receptors, whereas heterologous HAMP pairings resulted only in linear, asymmetric receptor signaling (Zhu & Inouye, 2004). In a subsequent study, structural analysis of an archaeal HAMP domain by NMR implied that its signaling involves a concerted, gear-like rotational transition between distinct helical packing modes (Hulko *et al.*, 2006), a mechanism that could feasibly explain how a conformational shift is transferred between protomers within a dimeric receptor. A rotational activation mechanism has also been suggested for the *A. tumefaciens* VirA and yeast Sln1 HPKs, in which the effects of altering the coiled-coil domain helical register were analyzed using N-terminally fused leucine-zipper insertions (Gao & Lynn, 2007, Tao *et al.*, 2002).

In the eukaryotic G-protein coupled receptors (GPCRs), another class of polytopic receptors in which dimerization is important (Hendrickson, 2005), conformational changes transduced across the dimer interface have been recently observed. In analyses of purified leukotriene B4 GPCR dimers labeled with spectroscopic probes, intermolecular conformational changes were shown to be transmitted between a ligand-bound protomer and an unbound one (Damian *et al.*, 2006), although these conformational changes were not sufficient to produce activation in the G-protein associated with the unliganded protomer. In another study, FRET experiments used to track specific ligand-induced conformational changes within μ -opioid and α_{2A} -adrenergic receptor heterodimers revealed that interprotomer interactions resulted in inhibition of G-protein activation (Vilardaga *et al.*, 2008). Finally, co-expression of two inactive somastatin-5 (SSTR5) receptor mutants, one that hampered ligand binding and one that disabled signaling, restored activity (Rocheville *et al.*, 2000), indicative of symmetric signaling across the dimer interface.

It is possible that interprotomer conformational changes through coiled-coil dimerization motifs, enabling symmetric signal transduction, may represent a “default” mechanism inherent to this class of receptors. Whether asymmetric modes of signaling by natural HPKs also exist is unclear. Also unknown is the biological significance of symmetrical HPK activation. Regarding AgrC dimers in the staphylococci, while additional investigations are needed, it is possible that this mode of signaling results in positive cooperativity of ligand binding and/or signal amplification, outcomes that would potentially be beneficial to the physiological process of quorum sensing governed by this receptor.

Experimental procedures

Bacterial strains and growth conditions

The *S. aureus* strains used in this study (Table S1) are derivatives of NCTC8325. RN7206 is a derivative of our standard *agr* group I laboratory strain, RN6734, in which the *agr* locus has been replaced by *tetM*. Cloning was performed using *E. coli* strain DH5 α . All clones were first transformed into RN4220, our standard recipient for *E. coli* DNA, before transduction to other strains. *S. aureus* cells from overnight plates containing the appropriate selective antibiotics (chloramphenicol, 10 mg/ml and/or erythromycin, 10 mg/ml) were used as inocula for all experiments. Subsequent growth in CYGP or MH broth without antibiotics was performed at 37 °C with shaking. Cell density was determined using a ThermoMax microplate reader (Molecular Devices) to measure the OD₆₅₀ of 100 μ L culture samples.

Plasmid and reporter construction

The plasmids used in this study (Table S1) were prepared by cloning PCR products obtained from oligonucleotide primers (Integrated DNA Technologies, Coralville, Iowa; Table S2). Clones were sequenced by the Skirball DNA Sequencing Core Facility or Genewiz. Plasmid pRN9231 was used as the backbone vector for WT *agrC* and G-box mutant constructs and

contains the *agrP2* promoter, an erythromycin resistance cassette, and the pT181 replicon (Geisinger et al., 2008). Compatible plasmid pEG54, which served as the backbone vector for H-box mutant *agrC* constructs, was created by replacing the resistance cassette and replicon of pRN9231 with a chloramphenicol resistance cassette and the pC194 replicon, cloned with *ApaI* and *NarI* sites. These plasmids contain an insertion site for *agrC*, formed by *PstI* and *KpnI* restriction sites, such that *agrC* expression is driven by *agrP2*. Point mutations in *agrC* were introduced via QuikChange (Stratagene) or by two-step PCR. *agrC* truncation and deletion mutants were constructed by inverse PCR on pUC18 subclones, closing on silent *AflII* or *BglII* sites. A C-terminal AgrC translational fusion to GFP was constructed using an in-frame *XbaI* site, and subsequent GFP-tagged mutants were created either by site-directed mutagenesis (as above) or using a *ClaI* site endogenous to *agrC* to swap in the mutant sequences. A chromosomal *agr* locus derivative lacking *agrC*, *B*, and *D* was constructed by deleting *agrB* and *D* from pRN9254.

Synthesis of AIPs

AIPs were synthesized via solid phase Fmoc-based chemistry using a hydroxy cysteine linker, as described (George *et al.*, 2008).

Western blot analysis and fluorescence microscopy

For western blots, cells were grown to OD₆₅₀ 0.7-0.9, pelleted, and washed with buffer containing 10mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 1.1 M sucrose (wash buffer). Cells were lysed by treatment with 100 µg/mL lysostaphin (Broos *et al.*) in wash buffer containing protease inhibitors (Roche), rocking for 10 minutes at 37 °C, followed by high speed spin (30 minutes, 8,000g), removal of supernatant, and resuspension in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% NP-40, 5 mM MgCl₂; or 50 mM Tris-HCl, pH 7.4, 1.0% NP-40, 0.25% DOC, 150 mM NaCl, and 1 mM EGTA) containing 10 µg/mL DNase (Sigma) and protease inhibitors. Lysates were incubated on ice 10 minutes, and the soluble fractions were removed following 30 minute spin at 10,000g. Total protein concentrations of the soluble fractions were determined by BCA assay (Pierce). SDS loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 1 mg/mL bromophenol blue, 10% glycerol) was added to samples, which were then acidified with Immuno Elution buffer (Pierce) prior to loading on 10-20% Criterion Tris-HCl gel (Bio-Rad). Protein transfer to nitrocellulose membrane was followed by immunoblotting with mouse anti-GFP (Roche), mouse anti-HA (Covance), or rabbit anti-Sortase (Abcam) primary antibodies and anti-mouse HRP (Bio-Rad) or anti-rabbit HRP (Bio-Rad) secondary antibodies. Visualization was carried out with ECL or ECL plus (Amersham).

For fluorescence imaging, cells were grown to OD₆₅₀ 0.65-0.8, pelleted, and re-suspended in PBS, concentrating the cells up to 50-fold relative to the liquid culture density. 5 µL aliquots of cells were added to poly-lysine (Sigma) coated coverslips (Fisher) and placed on glass slides (Fisher). Imaging was immediately carried out on a DeltaVision image restoration microscope (Applied Precision/Olympus). Images were deconvolved with SoftWoRx (Applied Precision).

Immunoprecipitation

Derivatives of RN10306 containing GFP and/or HA tagged AgrC were grown to OD₆₅₀ 0.7-0.8. Each culture was split into two subcultures, one treated with 1 µM AIP-I and one with buffer. All subcultures were grown for an additional 15 minutes. Cells were then pelleted, washed, and lysed as for western blotting. 250 µg aliquots of total protein, diluted with lysis buffer to uniform final volumes, were added to 25 µL aliquots of anti-HA affinity matrix (Covance) washed with lysis buffer. Samples were incubated 1 hour at 4 °C, with mixing by slow rotation. Unbound material was removed following centrifugation. Beads

were washed gently 3 times with lysis buffer, and bound proteins were eluted with acidic elution buffer (Pierce) by gentle mixing for 3 minutes. Analysis of samples was completed by western blot.

Reporter assays

Derivatives of strain RN10829 containing plasmid-borne *agrC* and chromosomal P3-*blaZ* were grown to mid-exponential phase and transferred to microtiter plates. In experiments involving constitutive mutants, growth proceeded without transfer to microtiter plates. Assay of β -lactamase activity was performed by the nitrocefin method as described (Lyon et al., 2002b). Assay data were normalized to percent maximal activation and plotted as initial β -lactamase reaction velocity versus log peptide concentration. PRISM 4.0 (GraphPad, San Diego) was used to fit individual agonist or antagonist dose-response curves via nonlinear regression to the following four-parameter logistic equation:

$$E = \text{basal} + \frac{E_{\text{max}} - \text{basal}}{1 + 10^{\log EC_{50} - \log [A]^{n_H}}}$$

in which E denotes effect, $[A]$ denotes the agonist concentration, n_H denotes the midpoint slope, EC_{50} denotes the midpoint location parameter, and E_{max} and basal denote the upper and lower asymptotes, respectively. For inhibition curves, the midpoint location parameter from the above equation reflects the IC_{50} . Each data point represents two or three replicates, and error bars represent standard error measurements. All curves shown in the same graph correspond to experiments performed on the same day.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Alison North of the Rockefeller University Bio-Imaging Resource Center for assistance with fluorescence microscopy, the Rout and Kapoor labs for antibody samples, and Kyle Chiang, Matt Pratt, and Peter Moyle for helpful discussions. This work was supported by the U. S. National Institutes of Health (Grant R 501 AI42783).

REFERENCES

- Black JW, Leff P. Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci.* 1983; 220(1219):141–162. [PubMed: 6141562]
- Broos J, Strambini GB, Gonnelli M, Vos EP, Koolhof M, Robillard GT. Sensitive monitoring of the dynamics of a membrane-bound transport protein by tryptophan phosphorescence spectroscopy. *Biochemistry.* 2000; 39:10877–10883. [PubMed: 10978174]
- Damian M, Martin A, Mesnier D, Pin JP, Baneres JL. Asymmetric conformational changes in a GPCR dimer controlled by G-proteins. *Embo J.* 2006; 25:5693–5702. [PubMed: 17139258]
- Dufour P, Jarraud S, Vandenesch F, Greenland T, Novick RP, Bes M, et al. High genetic variability of the *agr* locus in *Staphylococcus* species. *J Bacteriol.* 2002; 184:1180–1186. [PubMed: 11807079]
- Dutta R, Qin L, Inouye M. Histidine kinases: diversity of domain organization. *Molec Microbiol.* 1999; 34:633–640. [PubMed: 10564504]
- Gao R, Lynn DG. Integration of rotation and piston motions in coiled-coil signal transduction. *J Bacteriol.* 2007; 189:6048–6056. [PubMed: 17573470]
- Geisinger E, George EA, Muir TW, Novick RP. Identification of ligand specificity determinants in *AgrC*, the *Staphylococcus aureus* quorum-sensing receptor. *J Biol Chem.* 2008; 283:8930–8938. [PubMed: 18222919]

- Geisinger E, Muir TW, Novick RP. agr receptor mutants reveal distinct modes of inhibition by staphylococcal autoinducing peptides. *Proc Natl Acad Sci U S A*. 2009; 106:1216–1221. [PubMed: 19147840]
- George EA, Muir TW. Molecular mechanisms of agr quorum sensing in virulent staphylococci. *ChemBiochem*. 2007; 8:847–855. [PubMed: 17457814]
- George EA, Novick RP, Muir TW. Cyclic peptide inhibitors of staphylococcal virulence prepared by Fmoc-based thiolactone peptide synthesis. *J Am Chem Soc*. 2008; 130:4914–4924. [PubMed: 18335939]
- Grebe TW, Stock JB. The histidine protein kinase superfamily. *Adv Microb Physiol*. 1999; 41:139–227. [PubMed: 10500846]
- Hendrickson WA. Transduction of biochemical signals across cell membranes. *Q Rev Biophys*. 2005; 38:321–330. [PubMed: 16600054]
- Hirschman A, Boukhvalova M, VanBruggen R, Wolfe AJ, Stewart RC. Active site mutations in CheA, the signal-transducing protein kinase of the chemotaxis system in *Escherichia coli*. *Biochemistry*. 2001; 40:13876–13887. [PubMed: 11705377]
- Hulko M, Berndt F, Gruber M, Linder JU, Truffault V, Schultz A, et al. The HAMP domain structure implies helix rotation in transmembrane signaling. *Cell*. 2006; 126:929–940. [PubMed: 16959572]
- Janzon L, Arvidson S. The role of the delta-lysin gene (hld) in the regulation of virulence genes by the accessory gene regulator (agr) in *Staphylococcus aureus*. *Embo J*. 1990; 9:1391–1399. [PubMed: 2328718]
- Jarraud S, Lyon GJ, Figueiredo AM, Gerard L, Vandenesch F, Etienne J, et al. Exfoliatin-producing strains define a fourth agr specificity group in *Staphylococcus aureus*. *J Bacteriol*. 2000; 182:6517–6522. [PubMed: 11053400]
- Jensen RO, Winzer K, Clarke SR, Chan WC, Williams P. Differential recognition of *Staphylococcus aureus* quorum-sensing signals depends on both extracellular loops 1 and 2 of the transmembrane sensor AgrC. *J Mol Biol*. 2008; 381:300–309. [PubMed: 18582472]
- Ji G, Beavis R, Novick RP. Bacterial interference caused by autoinducing peptide variants. *Science*. 1997; 276:2027–2030. [PubMed: 9197262]
- Lina G, Jarraud S, Ji G, Greenland T, Pedraza A, Etienne J, Novick RP, Vandenesch F. Transmembrane topology and histidine protein kinase activity of AgrC, the agr signal receptor in *Staphylococcus aureus*. *Mol Microbiol*. 1998; 28:655–662. [PubMed: 9632266]
- Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. *Science*. 1991; 252:1162–1164. [PubMed: 2031185]
- Lyon GJ, Wright JS, Christopoulos A, Novick RP, Muir TW. Reversible and specific extracellular antagonism of receptor-histidine kinase signaling. *J Biol Chem*. 2002; 277:6247–6253. [PubMed: 11733525]
- Lyon GJ, Wright JS, Muir TW, Novick RP. Key determinants of receptor activation in the agr autoinducing peptides of *Staphylococcus aureus*. *Biochemistry*. 2002; 41:10095–10104. [PubMed: 12146974]
- Martin B, Prudhomme M, Alloing G, Granadel C, Claverys JP. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. *Mol Microbiol*. 2000; 38:867–878. [PubMed: 11115120]
- Mascher T, Helmann JD, Uuden G. Stimulus Perception in Bacterial Signal-Transducing Histidine Kinases. *Microbiol. Molec. Biol. Rev*. 2006; 70:910–938. [PubMed: 17158704]
- Mathiesen G, Axelsen GW, Axelsson L, Eijssink VG. Isolation of constitutive variants of a subfamily 10 histidine protein kinase (SppK) from *Lactobacillus* using random mutagenesis. *Arch Microbiol*. 2006; 184:327–334. [PubMed: 16283250]
- Milligan DL, Koshland DE Jr. Site-directed cross-linking. Establishing the dimeric structure of the aspartate receptor of bacterial chemotaxis. *J Biol Chem*. 1988; 263:6268–6275. [PubMed: 2834370]
- Ninfa EG, Atkinson MR, Kamberov ES, Ninfa AJ. Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NRII or NtrB): transphosphorylation between subunits. *J Bacteriol*. 1993; 175:7024–7032. [PubMed: 8226644]

- Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, et al. The agr P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. *Mol Gen Genet*. 1995; 248:446–458. [PubMed: 7565609]
- Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *Embo J*. 1993; 12:3967–3975. [PubMed: 7691599]
- Pan SQ, Charles T, Jin S, Wu ZL, Nester EW. Preformed dimeric state of the sensor protein VirA is involved in plant–*Agrobacterium* signal transduction. *Proc Natl Acad Sci U S A*. 1993; 90:9939–9943. [PubMed: 8234338]
- Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC, Patel YC. Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. *J Biol Chem*. 2000; 275:7862–7869. [PubMed: 10713101]
- Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. *Annu Rev Biochem*. 2000; 69:183–215. [PubMed: 10966457]
- Swanson RV, Bourret RB, Simon MI. Intermolecular complementation of the kinase activity of CheA. *Mol Microbiol*. 1993; 8:435–441. [PubMed: 8326858]
- Szurmant H, White RA, Hoch JA. Sensor complexes regulating two-component signal transduction. *Curr Opin Struct Biol*. 2007; 17:706–715. [PubMed: 17913492]
- Tao W, Malone CL, Ault AD, Deschenes AJ, Fassler JS. A cytoplasmic coiled-coil domain is required for histidine kinase activity of the yeast osmosensor, SLN1. *Mol Microbiol*. 2002; 43:459–473. [PubMed: 11985722]
- Vilardaga JP, Nikolaev VO, Lorenz K, Ferrandon S, Zhuang Z, Lohse MJ. Conformational cross-talk between α 2A-adrenergic and μ -opioid receptors controls cell signaling. *Nat Chem Biol*. 2008; 4:126–131. [PubMed: 18193048]
- Yang Y, Inouye M. Intermolecular complementation between two defective mutant signal-transducing receptors of *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1991; 88:11057–11061. [PubMed: 1662380]
- Zhu Y, Inouye M. The role of the G2 box, a conserved motif in the histidine kinase superfamily, in modulating the function of EnvZ. *Mol Microbiol*. 2002; 45:653–663. [PubMed: 12139613]
- Zhu Y, Inouye M. The HAMP linker in histidine kinase dimeric receptors is critical for symmetric transmembrane signal transduction. *J Biol Chem*. 2004; 279:48152–48158. [PubMed: 15316026]

Figure 1. Signaling by the *agr* locus and prediction of AgrC domains and alignment

(A) The AgrD propeptide is processed and secreted, in part, by AgrB to form the mature AIP. At sufficient concentrations, AIP binding to the sensor domain of the receptor histidine kinase, AgrC, results in autophosphorylation and phosphotransfer to AgrA. Phosphorylated AgrA activates transcription at the *agr* P2 and P3 promoters, resulting in an autoinduction of the P2 transcript and expression of the P3 transcript, RNAIII, which regulates expression of many downstream *agr*-related target genes.

(B) AgrC-I is a 430 amino acid protein with an N-terminal, polytopic transmembrane sensor domain and a C-terminal, cytoplasmic HK domain. The transmembrane (TM) segments are indicated as previously determined (Lina *et al.*, 1998). Three sensor domain residues critical for activation by AIP-I are indicated. The HK domain can be subdivided into two functionally distinct subdomains: the dimerization/histidine phosphotransfer (DHp) subdomain and the catalytic/ATP-binding (CA) subdomain. Conserved catalytic motifs within the HK domain are represented as black boxes. Gray rectangles denote predicted β -helical regions in the sensor domain and DHp subdomain. The hatched rectangle denotes a region with high parallel coiled-coil propensity as predicted using the COILS program (Lupas *et al.*, 1991).

(C) Alignment of H-box, N-box, and G-box motifs in representative HPK₁₀ subfamily members and divergent histidine kinases EnvZ and CheA of *E. coli*. Highly conserved residues mutated in this study are highlighted in yellow, with other conserved residues in turquoise.

Figure 2. Characterization of AgrC-GFP constructs

(A) Western blot of AgrC-GFP fusions expressed in RN10829, a β -lactamase reporter strain that does not produce AIP (– AIP), RN10828, an AIP-I producing strain (+ AIP), or EG61, an AIP-I producing strain with an *agrA* deletion (no *agrA*). The loading control, Sortase, is a membrane protein that is not known to be affected by *agr* activation. (B–I) Fluorescence microscopy of the same AgrC-GFP fusions analyzed in (A). Scale bars equal 2 μ m.

Figure 3. Co-expression of complementary mutants

(A) Schematic of a mutant dimer formed between AgrC-I_{His} and AgrC-I_{Kin}.

(B) β-lactamase activity of reporter cells expressing the indicated AgrC-I construct(s) following incubation with increasing concentrations of AIP-I. Percent activity is relative to maximal activation of WT AgrC-I ± SEM.

(C) Activity of reporter cells expressing both AgrC-I_{His} and AgrC-I_{Kin} following incubation with increasing concentrations of AIP-III in the presence of a constant concentration (150 nM) of AIP-I. Percent activity is relative to maximal activation of AgrC-I_{His} /AgrC-I_{Kin} by 150 nM AIP-I ± SEM.

Figure 4. Co-immunoprecipitation of AgrC-I-GFP and AgrC-I-HA

(A) Activity of reporter cells expressing AgrC-I-GFP_{His} and AgrC-I-HA_{Kin} following incubation with increasing concentrations of AIP-I. Data are presented as percent maximal activation of this AgrC-I pair ± SEM.

(B) Immunoprecipitation of lysates from cells co-expressing WT AgrC-I-GFP and AgrC-I-HA or AgrC-I_{R238H}-GFP and AgrC-I_{R238H}-HA using an α-GFP affinity matrix. Asterisk indicates IgG bands. Bracket indicates AgrC-HA bands.

Figure 5. Analysis of AgrC mutant dimers containing sensor domains with different ligand specificities

(A) Schematic of AgrC-I_{His}/AgrC-I_{Sensor, Kin} mutant dimer with a WT sensor domain linked to the functional kinase domain.

(B) Schematic of AgrC-I_{Kin}/AgrC-I_{Sensor, His} mutant dimer with a dysfunctional sensor domain linked to the functional kinase domain.

(C) Activity of reporter cells expressing AgrC-I_{His}/AgrC-I_{Sensor, Kin} as in (A) and AgrC-I_{Kin}/AgrC-I_{Sensor, His} as in (B) following incubation with increasing concentrations of AIP-I as indicated. Percent activity is relative to maximal activation of WT AgrC-I ± SEM.

(D) Activity of reporter cells in (C) following incubation with increasing concentrations of AIP-III in the presence of a constant concentration (1 ± M) of AIP-I. Percent activity is relative to maximal activation of AgrC-I_{His}/AgrC-I_{Sensor, Kin} by 1 μM AIP-I ± SEM.

(E) Schematic of AgrC-I_{His}/AgrC-II_{Kin} mutant heterodimer that contains receptors from two different *agr* groups.

(F) Activity of reporter cells expressing AgrC-I_{His}/AgrC-II_{Kin} as in (E) following incubation with increasing concentrations of AIP-I or AIP-II as indicated. Percent activity is relative to maximal activation of WT AgrC-I ± SEM.

(G) Activity of reporter cells in (F) following incubation with increasing concentrations of AIP-III in the presence of a constant concentration (150 nM) of AIP-I. Percent activity is relative to maximal activation of AgrC-I_{His}/AgrC-II_{Kin} by 150 nM AIP-I ± SEM. Dotted curves corresponding to AgrC-I_{His}/AgrC-I_{Kin} dimers in (C), (F), and (G) are shown for reference.

Figure 6. Ligand-independent signaling by AgrC mutant dimers containing constitutive mutation R238H

(A) Schematic of AgrC-I_{R238H, His} / AgrC-I_{Kin} mutant dimer in which one protomer contains both a constitutive mutation and a WT kinase domain.

(B) Schematic of AgrC-I_{His} / AgrC-I_{R238H, Kin} mutant dimer in which one protomer contains both a constitutive mutant and an inactive kinase domain.

(C) Activity of reporter cells expressing the indicated AgrC-I constructs following incubation with or without 1 μ M AIP-I at 1 μ M, as indicated. Data are presented as percent maximal activation of WT AgrC-I $\pm$ SEM.

Table 1EC₅₀s and IC₅₀s for described AgrC variants.

WT AgrC	AgrC _{His} (H239Q)	AgrC _{Kin} (G394A, G396A)	AIP-I EC ₅₀ (nM), (95% CI)	AIP-II EC ₅₀ (nM), (95% CI)	AIP-III IC ₅₀ (nM), (95% CI)
AgrC-I			10, (8.7-13)	-	
	AgrC-I		-	-	
		AgrC-I	-	-	
	AgrC-I	AgrC-I	40, (35-45)	-	18, (13-25) *
AgrC-I-GFP			16, (14-17)	-	
	AgrC-I-GFP		-	-	
		AgrC-I-GFP	-	-	
	AgrC-I-GFP	AgrC-I-GFP	36, (31-42)	-	
	AgrC-I _{Sensor}		-	-	
		AgrC-I _{Sensor}	-	-	
	AgrC-I _{Sensor}	AgrC-I _{Sensor}	6100, (4200-9000)	-	
	AgrC-I	AgrC-I _{Sensor}	200, (170-240)	-	140, (88-230) [#]
	AgrC-I _{Sensor}	AgrC-I	310, (260-380)	-	320, (150-650) [#]
AgrC-II			-	2.9, (2.3-3.6)	
	AgrC-II		-	-	
		AgrC-II	-	-	
	AgrC-I	AgrC-II	21, (18-25)	17, (13-22)	15, (9.8-21) *
	AgrC-II	AgrC-I	17, (16-19)	4.5, (3.6-5.6)	17, (13-22) *

“-” indicates no activation by any AIP concentration tested.

Parentheses following an EC₅₀ or IC₅₀ value denote the 95 percent confidence interval.

* 150 nM AIP-I added.

[#] 1 μM AIP-I added.