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Determinants of specificity in two-component signal transduction systems

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Abstract

Maintaining the faithful flow of information through signal transduction pathways is critical to the survival and proliferation of organisms. This problem is particularly challenging as many signaling proteins are part of large, paralogous families that are highly similar at the sequence and structural levels, increasing the risk of unwanted cross-talk. To detect environmental signals and process information, bacteria rely heavily on two-component signaling systems comprised of sensor histidine kinases and their cognate response regulators. Although most species encode dozens of these signaling pathways, there is relatively little cross-talk, indicating that individual pathways are well insulated and highly specific. Here, we review the molecular mechanisms that enforce this specificity. Further, we highlight recent studies that have revealed how these mechanisms evolve to accommodate the introduction of new pathways by gene duplication.

Introduction

Two-component signal transduction is a prevalent bacterial mechanism for sensing and responding to the environment. These signaling pathways typically consist of a sensor histidine kinase and a cognate response regulator. In response to a particular stimulus the kinase autophosphorylates on a conserved histidine residue and then transfers the phosphoryl group to its cognate regulator, which typically elicits an appropriate cellular response by modulating gene expression [1]. Strikingly, most bacteria encode dozens, if not hundreds, of two-component pathways for responding to a diverse range of signals [2,3]. The faithful coupling of input signals to desired cellular outputs requires tight enforcement of pathway specificity [4]. Here we review recent progress in elucidating the molecular basis of this specificity. Integral to the discussion is a consideration of the evolutionary pressures that influence two-component signaling pathways after gene duplication, the primary means by which these large paralogous protein families have expanded so dramatically. Our focus is on the specificity and evolution of phosphotransfer, the defining and most widely conserved element of two-component signaling pathways. Other important aspects of two-component signaling specificity have been reviewed elsewhere [5-9].

Mechanisms ensuring specificity in two-component signaling pathways

Before transferring its phosphoryl group, an autophosphorylated histidine kinase must somehow discriminate its cognate response regulator from a sea of non-cognate partners. This ability to avoid deleterious cross-talk is critical to the faithful transmission of signals inside bacterial cells. There are three key mechanisms for ensuring the specificity of two-component pathways at the level of phosphotransfer: molecular recognition, phosphatase activity, and substrate competition.

The predominant mechanism for enforcing specificity is molecular recognition, the intrinsic ability of an autophosphorylated histidine kinase to recognize its cognate partner to the exclusion of all possible non-cognate partners (Fig. 1). Early kinetic studies with the *Enterococcus* kinase VanS demonstrated that it preferentially phosphorylates its cognate regulator VanR relative to the *E. coli* regulator PhoB. The k_{cat}/K_M ratio, or specificity constant, for transfer to VanR is 10^4-fold higher than to PhoB [10]. More recently, systematic analyses of phosphotransfer from a given kinase to all possible regulators encoded in a genome have demonstrated that histidine kinases typically harbor a global and strong kinetic preference for their cognate response regulator *in vitro* [11]. This ability to discriminate cognate from non-cognate partners in the absence of other cellular components, such as scaffolds, indicates that

specificity is encoded primarily at the molecular level. The recognition of the cognate partner is driven by a small set of residues located primarily in one alpha helix of each molecule [12,13], and is discussed in the next section.

The specificity of two-component pathways is further reinforced *in vivo* through the phosphatase activity of histidine kinases. Most histidine kinases are bifunctional as they can drive phosphorylation of their cognate response regulators and act as phosphatases that stimulate dephosphorylation of the cognate partner [14-16] (Fig. 1b). The phosphatase reaction serves, in part, to modulate the level of pathway output and to inhibit the pathway after an activating signal has subsided [17]. Importantly, the phosphatase activity of a histidine kinase also serves to minimize unwanted cross-talk by dephosphorylating the cognate response regulator when it is inappropriately phosphorylated by another kinase or a small molecule phosphodonor (Fig. 2a). Many response regulators can be nonspecifically phosphorylated by the cellular pool of acetyl-phosphate [18-20]; by acting as phosphatases for their cognate response regulators, histidine kinases effectively clear this spurious, signal-independent phosphorylation [21]. Consequently, mutations that eliminate the phosphatase activity of a histidine kinase, including deletion of the histidine kinase gene, can lead to the inappropriate activation of the kinase's cognate response regulator under non-inducing conditions (Fig. 2a) [21].

Specificity is further enhanced by the relative cellular concentrations of histidine kinases and their cognate response regulators, and by competition between regulators for phosphorylated kinases (Fig. 2b). For most two-component pathways, abundance of the response regulator likely exceeds that of the cognate kinase. The well-characterized *E. coli* kinase EnvZ and its partner OmpR are found at a ratio of about ~1:35, and other pathways are reported to have similar ratios [22,23]. The higher abundance of the response regulators creates a scenario in which a given regulator effectively outcompetes non-cognate regulators for binding to a cognate kinase, further preventing unwanted phosphotransfer events. Consequently, deleting a given response regulator can lead to inappropriate cross-talk from its cognate kinase to other response regulators (Fig. 2b) [21,24].

In addition to these three mechanisms, specificity could also arise through temporal or spatial restriction of pathways. For example, in *Rhodobacter capsulatus* the subcellular localization of chemotaxis proteins to either polar or mid-cell clusters helps prevent cross-talk [25]. Although the expression of different pathways at different times could help to prevent unwanted cross-talk, to our knowledge there are no clear examples of this mechanism.

Collectively, three primary mechanisms – molecular recognition, phosphatase activity, and substrate competition – ensure that two-component signaling pathways are insulated from one another at the level of phosphotransfer. In most cases these mechanisms enforce specific, one-to-one relationships between kinases and their cognate regulators. There are, however, some cases of branched pathways with physiologically-relevant one-to-many or many-to-one connectivity [4].

Identification and characterization of specificity residues

The ability of histidine kinases and response regulators to preferentially recognize their cognate partners relies on a limited set of amino acids in each protein. These specificity-determining residues were identified initially through computational analyses of amino acid covariation in large sets of cognate, co-operonic two-component proteins [12,26,27] (Fig. 3a). This statistical approach identifies pairs of amino acids that covary, or change in a concerted manner over the course of evolution, to maintain the interaction between the partner proteins [28]. In some cases, these pairs are located in the same protein, where they make intramolecular contacts necessary for structural integrity or for promoting certain protein conformations. In other cases, the amino acids are located in opposite proteins, and likely have coevolved to preserve the interaction of a cognate kinase and regulator pair (Fig. 3a).

These intermolecular, coevolving residues were subsequently demonstrated to be critical specificity determinants. Mutating these residues in a model histidine kinase, *E. coli* EnvZ, to match those found in other *E. coli* kinases was sufficient to endow EnvZ with the ability to specifically phosphorylate other *E. coli* response regulators rather than its usual cognate partner, OmpR [12]. Similarly, response regulators have been rationally rewired to receive phosphoryl groups from non-cognate kinases [27,29], solidifying the notion that these coevolving amino acids are indeed specificity-determining residues.

Strikingly, the phosphotransfer specificity of EnvZ can be rewired to match the specificity of the *E. coli* kinase RstB through just three substitutions [12]. Subsequent analysis of the three single and three double mutant intermediates separating EnvZ and RstB indicated that different intermediates harbor substantially different specificities. Of the three double mutants, one does not phosphorylate either OmpR or RstA (the cognate partner of RstB), one still phosphorylates only OmpR, albeit weakly, and the third robustly phosphorylates both regulators [27]. These findings imply that individual sites do not contribute in simple, additive ways to specificity.

Instead, the effects of individual substitutions on specificity are highly context dependent, being influenced by the surrounding interfacial residues.

The first solved crystal structure of a histidine kinase in complex with its cognate regulator, the *Thermatoga maritima* pair HK853-RR468, demonstrated that the phosphotransfer specificity residues lie mainly at the interface formed by these proteins and reside on the surface of an alpha helix in each protein [13]. For HK853 this alpha helix is part of the dimerization and histidine phosphotransfer (DHp) domain and for RR468 it is the first of five alpha helices in the phosphoaccepting receiver domain. The docking of these helical surfaces and the inter-digitation of specificity residues promotes an orientation of the kinase and regulator in which the conserved histidine and aspartate side-chains are ideally positioned for phosphotransfer or dephosphorylation (Fig. 3a). Two other structures of kinases and response regulators in complex confirm the central position of the specificity residues at the interaction interface [30,31].

Structural studies are also being combined with mutagenesis studies to provide atomic-level insight into specificity. The *T. maritima* HK853-RR468 system has been rewired to harbor the specificity residues of *E. coli* PhoR-PhoB by introducing three and four substitutions into the kinase and regulator, respectively (A. Podgornaia, M. Laub, unpublished). Subsequent characterization of all possible mutational combinations demonstrated that substitutions that disrupt phosphotransfer often introduce bulkier residues; the consequent steric clashes can be alleviated by mutations that introduce smaller residues in the cognate protein. More generally, kinase-regulator interfaces appear to be mediated primarily by hydrophobic and van der Waals interactions that promote steric, rather than charge, complementarity. Consistently, the distribution of amino acid frequencies for the specificity residues in >6500 histidine kinases indicates a preponderance of small, hydrophobic residues and a relative paucity of bulky and charged residues (Fig. 3b).

The residues in histidine kinases critical for phosphotransfer specificity are also leveraged by kinase inhibitors. For example, in *B. subtilis* the small proteins Sda and KipI each bind to the specificity residues of the sporulation kinases, thereby blocking phosphotransfer to their cognate partner, Spo0F, and preventing the initiation of spore formation [32-34]. These small inhibitors represent exciting templates for future protein design efforts and the development of specific inhibitors of other two-component systems.

Covariation analysis has also been used to identify residues critical to other aspects of two-component signaling, including homodimerization and autophosphorylation. Histidine kinases

are constitutive dimers, and a small set of coevolving residues at the base of the DHp domain is critical to promoting homodimerization while preventing heterodimerization [35]. Coevolution studies have also guided the identification of residues that mediate autophosphorylation, a reaction in which the CA (catalytic and ATP binding) domain of the kinase transfers the γ-phosphoryl group from ATP to a conserved histidine within the DHp domain. This work enabled a rational rescue of autophosphorylation in chimeric kinases harboring incompatible DHp and CA domains [36].

Evolution of two-component signaling specificity

Why do specificity residues in two-component signaling proteins covary in the first place? The answer appears, in many cases, to be gene duplication events and the birth of new pathways [3,37]. Phylogenetic analyses indicate that, for most species, the majority of new two-component pathways emerge through gene duplication [38,39]. Immediately after duplication of a kinase-regulator pair, the two signaling pathways are identical, such that each kinase can interact with each regulator. After the pathways diverge with respect to signal inputs and downstream outputs, there is a need to avoid cross-talk via changes in the specificity residues of one or both of the recently duplicated kinases (Fig. 4a). Such mutations must then be compensated through mutations in the cognate response regulators. This intermolecular coevolution enables the insulation of the two new pathways while maintaining phosphotransfer within each system. Evidence for this model comes from the inspection of specificity residues in two-component signaling proteins derived from a relatively recent gene duplication [37]. For instance, while there is a single copy of EnvZ-OmpR in γ-proteobacteria, there are two copies in most α-proteobacteria (Fig. 4b). These two systems are insulated from one another at the level of phosphotransfer and have different specificity residues. Importantly, the specificity residues of each system are well conserved, indicating that once insulated following duplication, there is likely strong purifying selective pressure on these residues.

The insulation of recently duplicated pathways may also require changes in other existing two-component pathways [37]. For instance, in α-proteobacteria a duplication of the NtrB-NtrC system produced the NtrY-NtrX system, and the specificity residues of NtrY-NtrX subsequently diverged from those of NtrB-NtrC to yield two insulated pathways. However, the accumulated changes in NtrY-NtrX likely led to cross-talk with the PhoR-PhoB system in α-proteobacteria, driving adaptive substitutions in the specificity residues of that system to insulate it from NtrY-NtrX. Reverting these putative adaptive substitutions in the PhoR of an extant α-

proteobacterium, *Caulobacter crescentus*, leads to cross-talk with NtrX and a significant fitness disadvantage relative to the wild-type strain. Thus, the avoidance of cross-talk between pathways appears to be a major selective pressure driving the diversification of specificity residues following gene duplication events.

This process of pathway insulation following duplication has resulted in extant organisms harboring large sets of two-component pathways that can transduce signals without significant cross-talk [4,11,40]. Consistently, an examination of the specificity residues in two-component proteins from an individual organism typically reveals significant differences in almost all possible pairwise comparisons of kinases or regulators [37]. In rare cases, the specificity residues of some two-component proteins are similar, indicating potentially advantageous cross-regulation [41]. For instance, the sporulation kinases in *B. subtilis*, KinA/B/C/D/E, have similar specificity residues and each phosphorylates Spo0F, effectively integrating different signals into a common response – the initiation of sporulation [42].

The selective pressure to diversify specificity residues following gene duplication is relaxed for so-called hybrid histidine kinases. For these kinases, which comprise ~25% of all histidine kinases, a canonical histidine kinase containing DHp and CA domains is fused to a receiver domain, similar to that found in stand-alone response regulators. The physical proximity of the covalently attached receiver domain significantly increases its effective concentration relative to all other regulators in a cell such that the kinase will engage almost exclusively in intramolecular phosphotransfer [43,44]. As a consequence of this spatial arrangement, duplicated hybrid kinases are under less pressure to diversify their phosphotransfer specificity residues as each kinase will preferentially transfer intramolecularly, even immediately after duplication [43]. These findings also suggest that fusing non-cognate partners may be a means of rewiring information flow inside cells. A recent study reported some success, although the successful fusions involved proteins that had some basal level of molecular recognition as separate proteins [45].

Concluding remarks

Twenty-five years after the initial discovery of bacterial histidine kinases [46], the field has entered an exciting new era that integrates evolutionary, computational, and systems-level approaches. Although analyses of amino acid coevolution have helped to identify the critical specificity determining residues for two-component pathways, major challenges and intriguing questions remain. (1) Developing methods to predict interaction partners. Some algorithms have been developed [47,48], but making reliable and accurate predictions, particularly for orphan

signaling proteins, remains a significant challenge. (2) Adapting the ability to rewire two-component pathways for synthetic biology efforts. Methods for rationally redirecting information flow within bacteria combined with advances in engineering histidine kinases with desired sensory capabilities [49-51] will enable the construction of sophisticated new signaling circuits. (3) Understanding how a small set of amino acids determines the interaction specificity of two-component signaling proteins. How does a set of specificity residues enable a given kinase to recognize certain regulators and not others? Because individual residues do not contribute to specificity additively, traditional mutagenesis approaches that examine one position at a time will be of limited value so new, combinatorial methods are needed. High-throughput, deep sequencing-based approaches have recently been applied to other sequence-phenotype relationships and should prove powerful when applied to two-component signaling [52,53]. Such work promises to help inform prediction algorithms and bioengineering efforts, and will provide important new insights into the specificity and evolution of two-component signaling pathways.

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Highlights

- two-component signaling pathways are highly specific, exhibiting minimal cross-talk
- specificity stems from molecular recognition, phosphatases, substrate competition
- specificity-determining residues in kinases and their substrates coevolve
- specificity residues change following a duplication to insulate the new pathways
- avoiding cross-talk is a major selective pressure acting on two-component proteins
- tethering a kinase to a substrate relaxes pressure to diversify specificity residues

Figure Legends

Figure 1. Canonical two-component signal transduction system. (a) When activated by an input signal, canonical histidine kinases use ATP to autophosphorylate on a conserved histidine. The phosphoryl group is transferred to a conserved aspartate on the cognate response regulator, which can then effect an output response by changing cellular physiology or gene expression. (b) Most histidine kinases are bifunctional such that, in the absence of an input signal, a histidine kinase will drive dephosphorylation of its cognate response regulator, thereby suppressing an unwanted output.

Figure 2. Multiple mechanisms ensure the specificity of two-component signaling pathways. (a) In addition to molecular recognition, phosphotransfer specificity is enforced by the phosphatase activity of histidine kinases. Unwanted cross-talk from a non-cognate kinase (HK2) to a response regulator (RR1) is normally eliminated by the phosphatase activity of the cognate kinase (HK1). Deleting a kinase (greyed out HK1) can, consequently, lead to spurious activation of a pathway. (b) Competition between response regulators can further enhance the specificity of phosphotransfer. When a kinase (HK1) is autophoshorylated, its cognate response regulator (RR1) will better recognize, and hence outcompete, other response regulators for phosphotransfer. Deleting a regulator (greyed out RR1) can therefore allow its cognate kinase to phosphorylate a non-cognate substrate (RR2).

Figure 3. Amino acid residues important for phosphotransfer specificity identified by covariation analysis. (a) Residues that strongly coevolve in cognate pairs of histidine kinases and response regulators are shown on a crystal structure of the *T. maritima* HK853 in complex with RR468 (PDB: 3DGE). Only the DHp domain of HK853 is shown. Specificity residues on the kinase and regulator are shown with space-filling spheres in orange and red, respectively. The conserved histidine and aspartate that participate in phoshotransfer are shown as sticks. (b) Histogram showing amino acid frequencies for the six key specificity residues from α-helix 1 of the kinase and for all residues from α-helix 2, which does not play a prominent role in specificity. Frequencies were computed using a sequence alignment of > 6500 histidine kinases.

Figure 4. The process of pathway insulation following gene duplication has resulted in two-component pathways without significant cross-talk. (a) Duplication of a two-component pathway initially produces two identical pathways that engage in cross-talk. To insulate the new pathways from one another, the specificity residues in one or both histidine kinases must change, along with compensatory changes in their cognate response regulators, or *vice versa*.

(b) The EnvZ-OmpR system, present in single copy in γ-proteobacteria, was duplicated in an ancestor of the α-proteobacteria. The duplicates subsequently became insulated at the level of phosphotransfer specificity. Sequence logos of the specificity residues for each group of α-EnvZ and α-OmpR orthologs indicate the changes that likely led to insulation; logos for γ-EnvZ and γ-OmpR orthologs are included for comparison.

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