Genetic Approaches for Signaling Pathways and Proteins

John S. Parkinson
Biology Department
University of Utah
Salt Lake City, UT 84112

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Bacterial signaling proteins are built from modular components: input sensing domains; output effector domains; and transmitter and receiver domains for promoting protein-protein communication. Signaling circuits are assembled by "wiring" these elements in various configurations. This chapter discusses some genetic approaches for studying signaling pathways and for elucidating the molecular mechanisms of information processing by modular signaling proteins.

The components of a signaling pathway can be identified through genetic dissection, much like a conventional biochemical pathway. Given an appropriate phenotypic handle, brute-force screens will yield a more representative spectrum of mutants than will elegant, but less general, selection schemes. The multifunctional nature of signaling proteins can lead to complex mutant phenotypes and complementation properties, but information about the nature of the mutational lesion will help in relating mutant behaviors to functional defects. Finally, epistasis tests among signaling mutants can establish the sequence of steps in the signaling pathway.

Domain surgeries (ablation, scission, and transplantation) can provide clues to the mechanisms of interdomain communication in signaling proteins. Liberated communication modules can disrupt normal signaling by quenching input signals, by jamming output elements with inappropriate signals, or by shielding output elements from their input signals. These pathological effects can be exploited to identify the targets of inhibition and the binding determinants that mediate signaling interactions within and between proteins.

Comparative sequence analyses and site-directed mutation can provide initial clues to the importance of particular structural features in transmitter and receiver modules. Screens and selections can be devised to look for mutants with defects in the various functional activities of communication modules (phosphorylation, dephosphorylation, and input or output control). Reversion analyses of such mutants, either through bypass or conformational suppression, can also provide valuable insight into the structure-function organization of signaling proteins.

SENSORY SIGNALING IN BACTERIA

Bacteria live in precarious environments. Nutrient and toxin levels, acidity, temperature, osmolarity, humidity, and many other conditions can change rapidly and unexpectedly. To survive, the cells must constantly monitor external conditions and adjust their structure, physiology, and behavior accordingly. Given strong selective pressures such as these, it is no surprise that bacteria have devised sophisticated signaling systems for eliciting adaptive responses to their environment. (For recent reviews see Bourret et al., 1991; Parkinson, 1993; Parkinson and Kofoid, 1992; Stock et al., 1990.) They readily detect minute fluctuations in many chemical and physical conditions, which in turn trigger changes in gene expression or motility that enhance survival prospects. The sensory machinery underlying these behaviors handles signaling tasks fundamental to all cell sensory systems: stimulus detection; signal processing, including amplification and integration of sensory inputs; and production of appropriate output responses. The sensory systems of prokaryotes provide tractable models for exploring these events in molecular detail and have begun to reveal general principles of cellular signaling mechanisms.

Bacterial signaling systems are amenable to detailed genetic and biochemical analyses. This chapter focuses on genetic methods; biochemical studies are covered separately (see Chapter 3). Readers should appreciate that these tools are not mutually exclusive but rather complementary. A combination of genetic and biochemical approaches undoubtedly offers the most incisive experimental strategy for elucidating sensory pathways and signaling mechanisms. Genetic methods are uniquely valuable, however, for identifying the components of a signaling pathway and for determining the sequence in which they act. Moreover, simple genetic tests can shed considerable light on the information-processing mechanisms of signaling proteins. In this chapter, rather than reviewing genetic studies of specific signaling systems, which are amply covered elsewhere in this book, some general strategies for using genetic methods to study sensory pathways and signaling proteins are discussed.

Two-Component Signal Transduction, edited by James A. Hoch and Thomas J. Silhavy, *pp. 9-24*

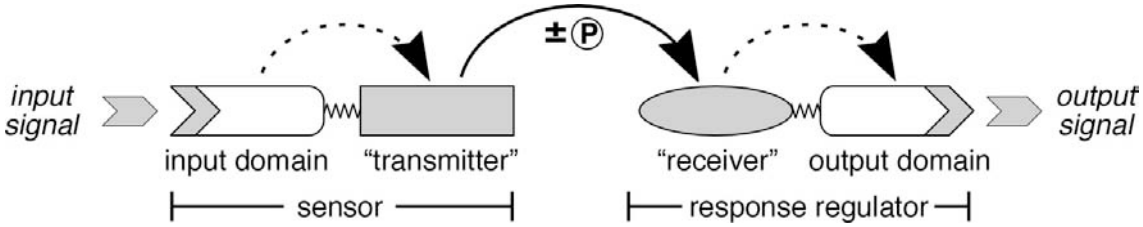

FIGURE 1. Two-component" paradigm for sensory signaling via communication modules. Sensory information flows through noncovalent controls exerted by one domain on another (dashed arrows) and through phosphorylation reactions between transmitter and receiver domains. The convention of representing transmitters by rectangles and receivers by ovals is used in all subsequent figures.

TWO-COMPONENT PARADIGM

Many signaling proteins, from both gram-positive and gram-negative bacteria, contain characteristic "transmitters" and "receivers," domains that promote information transfer within and between proteins (Parkinson and Kofoid, 1992). Similar communication modules are now turning up in eukaryotic signaling proteins, indicating that this could be a fundamental and widespread strategy for building signaling circuits. Transmitters and receivers function in combination with a variety of input and output domains and can be arranged in different configurations to build signaling circuits of many types. The simplest circuits have two protein components—a sensor, often located in the cytoplasmic membrane, that monitors some environmental parameter; and a cytoplasmic response regulator that mediates an adaptive response, usually a change in gene expression (Fig. 1). Sensors typically contain a C-terminal transmitter module coupled to an N-terminal input domain. Response regulators typically contain an N-terminal receiver module coupled to one or more C-terminal output domains. On detecting a stimulus, the input domain of a sensor modulates the signaling activity of its associated transmitter to communicate with its response regulator partner. The receiver domain of the response regulator detects the incoming sensor signal and then alters the activity of its associated output domain to trigger the response.

Phosphorylation Activities of Transmitters and Receivers

The only demonstrated mechanisms of transmitter-receiver communication involve phosphorylation and dephosphorylation reactions (Fig. 2). Transmitters have an autokinase activity that attaches phosphate groups from ATP to a histidine residue. This reaction is readily reversible. The product phosphohistidine serves as a high-energy intermediate for subsequent transfer of the phosphate group to an aspartate residue in the receiver. Although formally reversible, the phosphotransfer step is effectively unidirectional. The receiver probably catalyzes the transfer reaction, with the transmitter phosphohistidines simply serving as substrates. Receivers also catalyze hydrolytic loss of their phosphate groups, with half-lives ranging from a few seconds to many minutes. Finally, transmitters can also have an apparent phosphatase activity toward their cognate receivers. It is not yet clear whether this reaction is catalyzed by the transmitter or whether the transmitter acts as an allosteric effector to enhance the intrinsic dephosphorylation ability of its target receiver.

Signaling Properties of Transmitters and Receivers

Most transmitter-containing proteins are located in the cytoplasmic membrane, with their transmitters projecting into the cell. They typically have two membrane-spanning segments flanking their input domain, which is consequently deployed in the periplasmic space between the inner membrane and cell wall. Sensor input domains differ broadly in primary structure, reflecting the variety of chemical and physical stimuli they detect. Some have demonstrable ligand binding functions, but most are still poorly characterized, often because the exact nature of the stimulus is unknown. Communication with the cytoplasmic transmitter domain involves propagation of sensory information across the cytoplasmic membrane, presumably via stimulus-induced conformational changes. A few sensor proteins are soluble and contain N-terminal domains that may serve comparable input roles.

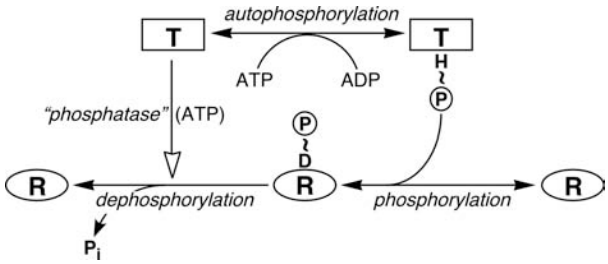

FIGURE 2. Phosphorylation activities of transmitters and receivers. Abbreviations: T, transmitter; R, receiver; H, histidine, D, aspartic acid); P_i, inorganic phosphate. Details of the phosphorylation reactions are discussed in the text. ATP is required for the "phosphatase" activity exhibited by some transmitters but is not hydrolyzed in the reaction.

Receiver-containing proteins are generally cytoplasmic. In most cases, their output domains have DNA binding or other regulatory functions that provide transcriptional control over one or more target genes. The receiver and output domains in response regulators are often joined by flexible linkers, suggesting that pliable connections may be important in enabling a receiver to exert control over its adjoining output domain.

Transmitters and receivers are ideally suited as circuit elements for assembling signaling pathways. The interplay of kinase and phosphatase activities in a transmitter is subject to input control, enabling the transmitter to regulate the phosphorylation state of its cognate receiver in response to sensory signals. The phosphorylation state of the receiver in turn controls the activity of an adjoining output domain to trigger the behavioral response. The signaling characteristics of module-based circuits thus depend on several parameters. The most critical ones include the basal and stimulated phosphotransfer rates between transmitter and receiver; the lifetimes of activated transmitters and receivers; the ways in which these elements are wired together; and the extent of crosstalk from other signaling pathways.

Although the signaling attributes of transmitters and receivers are now apparent, their activities are still poorly understood at the molecular level. Consequently, the overall logic and information-processing properties of many module-based signaling circuits remain mysterious. Even the simplest sensor-response regulator pathways must carry out four discrete communication transactions (Fig. 3): stimulus detection by the input domain (Fig. 3a); input control over transmitter activity (Fig. 3b); transmitter-receiver communication (Fig. 3c); and receiver control over output activity (Fig. 3d). None of these events is well understood:

- How does stimulus detection alter the conformation of the input domain? How does the stimulated input domain communicate with the adjoining transmitter? Is the segment linking the input and transmitter domains important in this process?
- How do transmitter conformational changes alter its kinase, phosphotransfer and phosphatase activities? Does the receiver catalyze the phosphotransfer reaction? Which component catalyzes the "phosphatase" reaction?
- What confers signaling specificity to transmitter-receiver transactions? How do matching modules recognize one another? How do they avoid unwanted crosstalk? Does phosphorylation modulate their binding interactions?
- How does phosphorylation alter the conformation of the receiver domain? How does this conformational change regulate the activity of the adjoining output domain? Does the receiver directly contact the output domain? What structural features determine the lifetime of the phosphorylated receiver?

Genetic approaches can provide mechanistic answers to these sorts of questions. First, general strategies for identifying and ordering the components of a signaling pathway are described. Then, some specific experimental schemes for elucidating the communication transactions within and between signaling proteins are outlined. The ensuing discussion and illustrative examples deal almost exclusively with an idealized two-component signaling circuit such as that shown in Fig. 3, in which the sensor detects a small-molecule stimulus and the response regulator controls the expression of a nonessential gene.

GENETIC ANALYSIS OF SIGNALING PATHWAYS

Signaling circuits are amenable to genetic dissection in much the same manner as conventional biochemical pathways. In both cases, gene products participate in an ordered series of reactions whose end product, an essential metabolite on the one hand or a behavioral response on the other, influences the organism's phenotype. A defect at any step in the sequence should block the pathway and alter its phenotypic outcome, permitting identification of the responsible gene products through mutants. However, as discussed below, the inherent complexity of biological signaling processes poses some special challenges to genetic analysis.

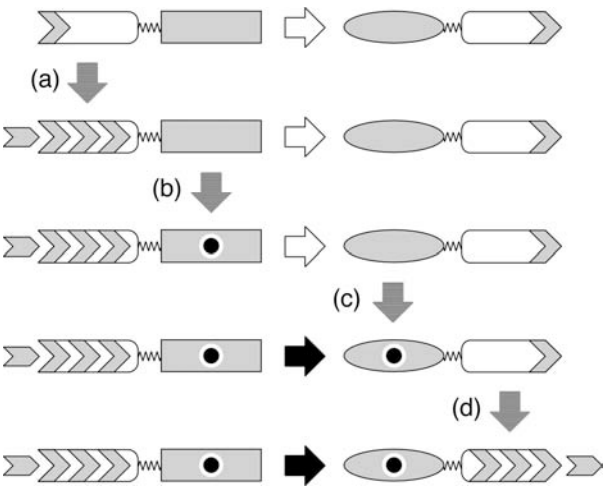

FIGURE 3. Signaling transactions in senso-response regulator circuit. (a) Generation of a conformational change in the sensor input domain upon detection of an input signal; (b) Modulation of the autophosphorylation-phosphatase activities of the transmitter by the stimulated input domain; (c) Communication between transmitter and receiver via specific docking and phosphotransfer; (d) Stimulation or inhibition of the response regulator output domain on a change in phosphorylation state of the receiver.

Identifying the Pathway Elements

GETTING A GRIP ON BEHAVIOR

The phenotypic consequences of a signaling process are often far removed from the underlying molecular events. Some signaling systems elicit discrete regulatory responses to relatively simple stimuli [e.g., a change in porin synthesis upon osmolarity shift (see Chapter 7), but others trigger elaborate developmental programs in response to complex stimuli [e.g., induction of sporulation by starvation conditions (see Chapter 8)]. The apparent speed of the overall signaling process can range from fractions of a second [e.g., locomotor responses to chemotactic signals (see Chapter 6)] to many hours [e.g., fruiting body formation in *Myxococcus* (see Chapter 27)]. Simple, easily assayed phenotypes can greatly facilitate isolation and characterization of mutants with behavioral defects. For example, gene expression reporters (e.g., promoter fusions to β-galactosidase) provide especially convenient phenotypic handles for following the consequences of regulatory signaling transactions. Unfortunately, these tools are not often applicable to tactic behaviors, which depend on a locomotor apparatus for phenotypic expression.

MUTANT SCREENS OR SELECTIONS?

Selection schemes simplify the process of isolating mutants, but are not necessarily the most effective way to dissect a signaling pathway. Selections based on a special attribute of the desired mutant phenotype could easily bias the kinds of mutants obtained. For example, *Escherichia coli* mutants that cannot tumble while swimming are nonchemotactic. Because they always swim forward, such mutants move faster down a vertical race course than wild-type cells, which tumble fairly frequently. However, taking the winners does not yield a representative spectrum of nonchemotactic mutants because those with other swimming patterns (e.g., excessively tumbly) get overlooked. Brute-force screens based on a more general phenotype (e.g., colony morphologies on motility plates) afford more comprehensive mutant hunts. As a general rule, mutant screens are preferable to selections whenever the desired mutants are reasonably frequent or there is an abundant supply of labor.

Most adaptive behaviors are not essential for viability, at least in the laboratory. In general, then, mutations that block a response pathway should not be lethal, unless the signaling components play other vital roles. Therefore, it should be possible to identify many of the key elements in a signaling pathway through loss-of-function mutants induced by transposon insertions or other knockout mutations. However, to reconstruct the pathway through epistatic analysis (see below), some gain-of-function mutants are needed. If null mutants fail to respond to the stimulus, gain-of-function mutants would show constitutive, stimulus-independent responses. Mutations that activate a signaling pathway will probably be relatively uncommon, but their potential value justifies devising special screens or selections to get them

Determining the Functional Defects of Signaling Mutants

CAUTION: MULTIFUNCTIONAL PROTEINS

The phenotypes of signaling mutants may provide misleading clues about their underlying functional defects. Signaling proteins must perform several different functions to serve as information-processing devices. These include recognition and docking with other signaling components, catalysis of phosphorylation or dephosphorylation reactions, presentation of the substrate sites involved in those reactions, and control of these activities in response to input signals. Because signaling proteins are inherently multifunctional, genetic lesions can cause a variety of functional defects. Knockout or null mutants would be expected to exhibit complete loss-of-function phenotypes, but missense mutants may well have residual activities that confound their defective phenotypes. In attempting to relate mutant phenotypes to functional defects, it is extremely helpful to know the nature of the structural lesion in the mutant protein. This can be easily determined by DNA sequencing or sometimes inferred from the mutagenic agent used. Without this information, it is foolhardy to offer more than a superficial interpretation of mutant phenotypes when dealing with signaling proteins.

COMPLEMENTATION ANALYSIS OF SIGNALING MUTANTS

The complementation behavior of a mutant reflects the severity of its functional defect. Null mutants should be recessive, gain-of-function mutants should be dominant. The multifunctional nature of signaling proteins is likely to complicate the complementation properties of signaling mutants. Defects in any one subfunction could lead to dominant negative behavior caused by the residual activities in the mutant protein. A catalytic defect, for example, might not prevent docking interactions with signaling partners, enabling the mutant protein to act as a "spoiler" that interferes or competes with its normal counterpart in complementation tests. A similar spoiling effect can occur on interaction of mutant and wild-type protomers to form inactive oligomers. The severity of dominance should depend on the relative stoichiometry of the mutant subunits, so it may be possible to alleviate much of the effect by adjusting gene dosages.

Partially dominant mutants can provide unique subjects for subsequent mutational studies. If a mutant protein disrupts signaling in wild-type cells, it should be possible to look for second-step mutants that are more or less dominant. In the example above, a

signaling protein with a catalytic defect might block signal propagation by titrating a target protein. Mutant proteins that block signaling at lower expression levels might have enhanced affinity for their target. Conversely, mutant proteins with diminished effectiveness might have reduced affinities. Affinity mutants could serve to identify the structural determinants involved in docking interactions and signaling specificity (see below).

Reconstructing the Signaling Pathway

LITTLE HOPE FOR PATHWAY INTERMEDIATES

It is often possible to elucidate the stepwise reactions in a conventional biochemical pathway by identifying the precursor compounds that accumulate in different mutants. In principle, pathway intermediates should also accumulate in a blocked signaling circuit. For example, a lesion in the receiver module of a response regulator should, in the presence of an activating stimulus, cause a buildup of the autophosphorylated form of the transmitter module in the sensor. Such intermediates might be ephemeral and, in any case, would be difficult to detect unless they conferred an aberrant phenotype, for example, through inappropriate crosstalk. So, unlike biosynthetic pathways, signaling intermediates would probably be of little use in deducing the sequence of transactions in a communication pathway.

EXPLOITING EPISTASIS

Epistasis is a dominance relationship between nonallelic genes, assessed at the level of their mutant phenotypes. The order in which gene products act in a signaling pathway can be determined from their epistatic interactions. The test is simple: if mutations in two different genes produce two different phenotypes, which phenotype prevails in a double mutant? In signaling pathways, where the scored phenotype is an output response, the test is typically performed by combining gain-of-function and loss-of-function defects (Fig. 4). First, consider an activating mutation in the sensor that enables it to generate transmitter signals with no stimulus input, causing a constitutive output response. When combined with a null defect in the cognate response regulator, the aberrant output ceases, demonstrating that the null mutation affects a component required for the constitutive response (i.e., a later step in the signaling pathway) (Fig. 4a). By contrast, an activating mutation in the response regulator should enable it to generate a constitutive output signal independent of its cognate sensor, demonstrating that the sensor defect blocks an earlier step in the signaling pathway (Fig. 4b).

With caution, the logic of epistatic analysis can be extended to more elaborate signaling pathways that have branches, feedback loops, etc. However, it is essential at the outset to know the general nature of the mutant defects (gain- or loss-of-function?). If loss-of-function mutations in different genes produce dissimilar phenotypes, either the pathway or the phenotypic handle must be more complicated than the simple example shown in Fig. 4.

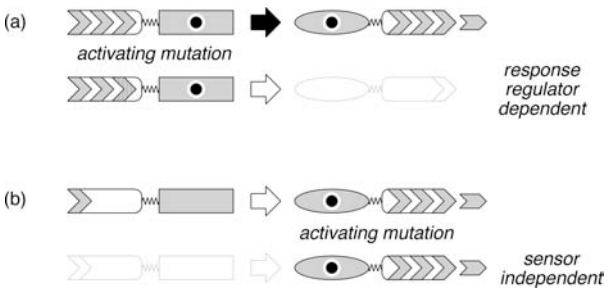

FIGURE 4. Ordering components of signaling pathways by epistasis tests. (a) An activating mutation in the sensor requires a functional response regulator to produce a constitutive output signal. (b) An activating mutation in the response regulator does not require a functional sensor to produce a constitutive output signal.

DOMAIN INTERACTIONS IN SIGNALING PROTEINS

Input-Output Communication Within Signaling Proteins

The primary input and output functions of sensors and response regulators are carried out by different domains joined through flexible linkers. How do the input and output modules within a signaling protein communicate with one another? On the one hand, the input domain might make specific direct contact with the output domain to stimulate or inhibit its activity. On the other hand, the input domain might control output activity indirectly by manipulating the subunit organization or overall conformation of the protein. Indirect control mechanisms would not require specific contacts between domains, although the segment connecting them might play an important role.

The modular design of signaling proteins suggests three conceptually simple genetic tests for distinguishing between direct and indirect mechanisms of interdomain communication (Fig. 5). All three approaches involve wholesale surgery on the signaling protein: ablation of the input domain (Fig. 5a); scission of the input and output domains (Fig. 5b); and transplantation of foreign domains (Fig. 5c). These genetic alterations can be readily accomplished by in vitro methods but may lead to postoperative complications. In domain ablation and scission experiments, a change in expression level or stability of the modified proteins could confound interpretation of their signaling properties. In domain transplantation experiments, the length and flexibility of the linker segment might prove critically important for proper domain interactions. Because such experiments could

fail for a variety of reasons, it would be unwise to draw conclusions from any surgical operations that produce negative results.

ABLATIONS

Ablation experiments can determine the general manner in which an input domain exerts control over an adjoining output domain (Fig. 5a). If the control mechanism is negative, removal of the input domain might lead to a stimulus-independent output response. This would imply that the input domain normally inhibits output activity and that interaction of the stimulus with the input domain alleviates the inhibition. Negative controls appear to be common in sensor proteins because removal of their input domain often leads to activation of transmitter kinase activity. However, this control strategy seems to be less prevalent in response regulators, where removal of the receiver seldom leads to activation of the output domain.

SCISSIONS

Scission experiments can establish whether the control mechanism requires a physical connection between the input and output domains (Fig. 5b). If the input domain makes direct specific contact with the output domain, those binding interactions might still occur when the linker is severed, provided that the concentrations of the untethered domains can be adjusted to compensate for the loss of a covalent connection. By contrast, domain scission would definitely abrogate control mediated through nonspecific mechanisms such as monomer-dimer transitions. Indirect controls are probably common in sensors, many of which are transmembrane proteins whose input domain is in the periplasmic space, presumably incapable of contacting the cytoplasmic transmitter. Few scission experiments have been reported with response regulators, and those have been negative. Whether receivers can communicate with adjoining domains through specific contacts is still an open issue.

TRANSPLANTATIONS

Transplantation experiments also address the mechanism of communication between input and output domains but can provide information complementary to that from scission approaches (Fig. 5c). Input and output domains that fail to communicate when physically disconnected could conceivably interact through nonspecific mechanisms. If so, combinations of heterologous domains that use the same control strategy might communicate properly. This approach has succeeded with the EnvZ sensor, which modulates OmpR phosphorylation state in response to changes in medium osmolarity. When coupled to the sensing domain of the aspartate-maltose (Tar) or ribose-galactose (Trg) chemoreceptor, the EnvZ transmitter is able to modulate OmpR activity in response to the appropriate chemoeffector (Baumgartner et al., 1994; Utsumi et al., 1989). Because EnvZ, Tar, and Trg are transmembrane proteins, their input and output domains cannot contact one another directly but instead must communicate through the membrane-spanning segments of the protein. The signaling properties of the hybrid proteins imply that the chemosensing domains of Tar and Trg use the same conformational control mechanisms as the osmosensing input domain of EnvZ. There have been several transplantation attempts with response regulators, all with negative outcomes.

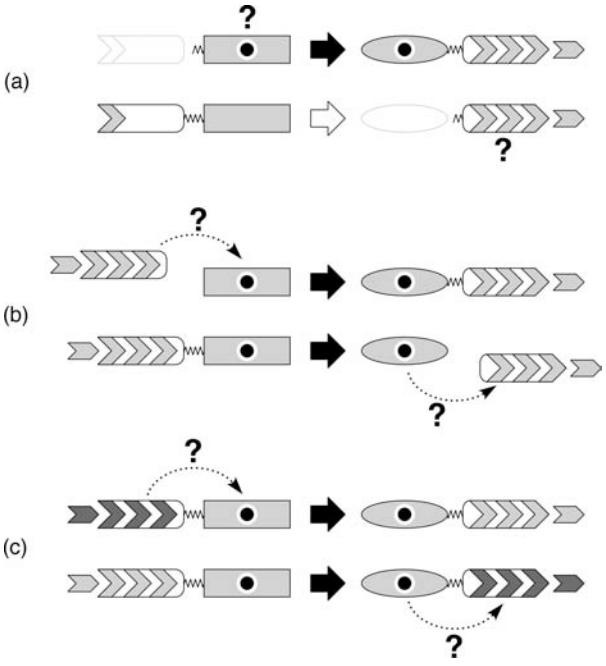

FIGURE 5. Analyzing intraprotein communication mechanisms by domain surgery. (a) Does ablation of the input domain activate the adjoining output domain? (b) Does scission of input and output domains disrupt signal propagation? (c) Does transplantation of foreign domains disrupt signal propagation?

Signaling Transactions Between Transmitters and Receivers

E. coli probably contains at least 50 transmitter-receiver pairs and nearly as many signaling circuits. Inappropriate crosstalk between them is minimal, implying that receivers are precisely tuned to their cognate transmitters. High fidelity signaling presumably derives from specific binding interactions between transmitters and receivers, but their structural basis is not yet understood. Domain liberation approaches can provide useful experimental subjects for exploring the process of target recognition and the ensuing phosphotransfer reactions in molecular detail.

Domain liberation is a general method of identifying functional subdomains within proteins (Morrison and Parkinson, 1994). The approach is based on the premise that protein domains invariably function

through specific interactions with some partner, either a small molecule, another macromolecule, or another part of the same protein. When subcloned and overexpressed, a liberated domain should compete with its counterpart in the intact protein, disrupting its activity. This could happen in several ways, for example, through formation of nonfunctional hetero-oligomers with the parent protein, through stoichiometric titration of a common interaction target, or through creation of an aberrant or unregulated catalytic activity. In the case of two component signaling pathways, liberated transmitter or receiver domains could conceivably disrupt communication in three different ways (Fig. 6).

QUENCHING

Liberated receiver domains could attenuate communication between a sensor and its response regulator target by intercepting incoming transmitter signals (Fig. 6a). To quench signal flow in this manner, the freed receivers must be incapable of exerting control over the activity of the output domain in the response regulator. This appears to be the case in the EnvZ-OmpR system, where expression of the OmpR receiver leads to disruption of the wild-type regulatory responses (Nakashima et al., 1991).

JAMMING

Removal of the input domain from a transmitter may lead to activation of its output signal. If so, the liberated transmitter should interfere by overloading its response regulator target with inappropriate signals, causing a constitutive behavioral response (Fig. 6b). The resultant phenotype should resemble that of sensor or response regulator mutants with an activating mutation. It should be possible to identify liberated transmitters that are capable of signal jamming by simply looking for transmitter clones that cause a constitutive output phenotype in wild-type cells. This approach has been used to identify the signaling domain of Tsr, the serine chemoreceptor in *E. coli* (Ames and Parkinson, 1994). When liberated, the wild-type Tsr signaling domain exhibits constitutive signaling activity that jams chemotactic responses.

SHIELDING

Transmitters that are inactive on liberation could still block signal flow by docking with target receivers in response regulators, thereby shielding them from their sensor partners (Fig. 6c). Liberated transmitters need only retain the ability to recognize and bind to their receiver targets to disrupt communication by this mechanism. For example, mutant signaling domains from the Tsr chemoreceptor that cannot activate the signaling pathway still block chemotaxis by shielding intracellular signaling components from interactions with other chemoreceptors (Ames and Parkinson, 1994).

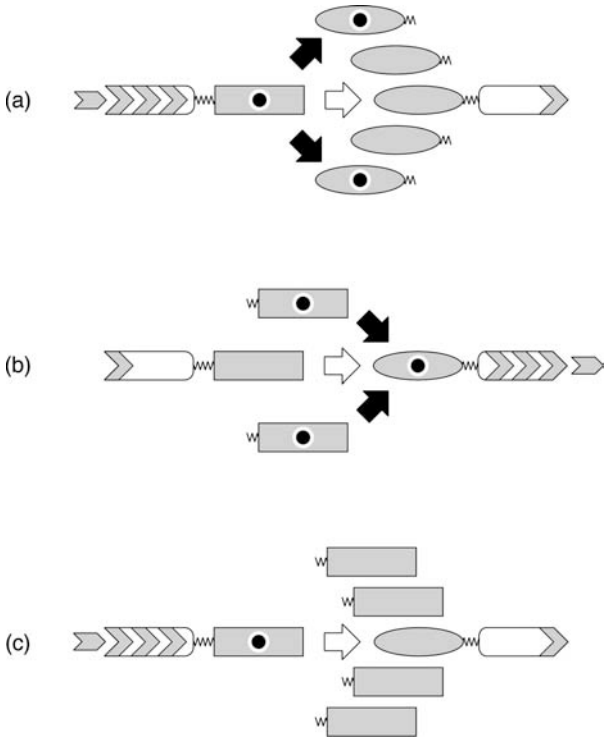

FIGURE 6. Analyzing transmitter-receiver interactions by domain liberation. (a) Quenching of transmitter signals by liberated receivers; (b) jamming signals from liberated, constitutively active transmitters; (c) shielding of receivers by liberated quiescent transmitters.

USING LIBERATED DOMAINS

The pathological effects produced by a liberated signaling domain provide a genetic handle for exploring its normal signaling role. The clone that expresses the liberated domain can be treated much like any other gene, except that its function is to disrupt normal lines of communication in the cell. The component that serves as the target of the inhibitory effect can be identified through gene dosage and epistasis tests. Also, mutations that alter the inhibitory properties of the liberated domain could be isolated to identify the structural determinants involved in its interaction with target proteins. If the inhibitor is under regulatable control, its expression could be reduced to look for mutations that enhance potency, or increased to look for mutations that reduce potency. Several uses for such docking affinity mutants are described in the next section. Clones that express functional domains from a signaling protein also provide useful material for biochemical studies (e.g., measurements of binding and catalytic activities and structural determinations).

STRUCTURE-FUNCTION STUDIES OF COMMUNICATION MODULES

Although their structures are undoubtedly very different, transmitters and receivers have remarkably similar functional properties that enable them to serve

as signal-processing devices. First, both types of modules have enzymatic activities, primarily autophosphorylation but sometimes dephosphorylation as well. To carry out these reactions they must have determinants for substrate binding and catalysis and an autophosphorylation site composed of the target residue and the determinants that promote its interaction with the catalytic center. Second, these enzymatic activities are subject to input control and, in turn, regulate output activities. The input determinants of a transmitter modulate its autophosphorylation activity in response to stimulus-induced conformational changes in an adjoining input domain. Its output determinants enable it to communicate with its receiver partner through docking and phosphotransfer transactions. The input determinants of a receiver promote recognition and docking with its transmitter partner. On phosphorylation, its output determinants trigger conformational changes that enable it to control an adjoining output domain. Thus, both transmitters and receivers possess determinants for modulating autophosphorylation activity in response to input signals and for producing output signals that are regulated by phosphorylation state.

The communication functions of transmitters and receivers are still poorly understood in molecular terms. The genetic approaches outlined below can serve to identify structural features important for signal input, processing, and output activities.

Sequence Analyses

The amino acid sequence of a transmitter or receiver, inferred from DNA sequence data, can provide useful clues to its structural and functional organization. The sequence data base of "two-component" systems contains hundreds of transmitter- and receiver-containing proteins. Sequence comparisons within these extended module families have revealed conserved residues and other motifs that probably play important roles in their signaling functions (Fig. 7).

Transmitters are about 240 amino acids in length. Essentially nothing is known about their secondary or tertiary structures, but their primary structures exhibit several blocks of nearly invariant residues (Fig. 7). The histidine phosphorylation site is typically located near the N terminus of the module. The residues flanking the phosphorylation site are not highly conserved, suggesting they may not play important roles in interactions with the catalytic center. Rather, these flanking sequences and other variable blocks in the amino half of the transmitter define transmitter subfamilies whose receiver partners are also similar to one another. These regions might contain the specificity determinants for properly identifying receiver targets, but there is no experimental evidence bearing on this idea. Four blocks of residues in the carboxyl half of the transmitter may comprise the

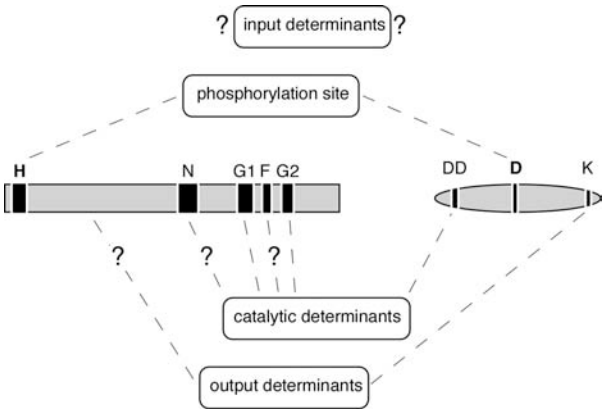

FIGURE 7. Structure-function relationships in transmitters and receivers. Sequence motifs characteristic of transmitters and receivers are indicated by black bars whose widths are proportional to the lengths of the motifs. Each sequence tract is labeled with a letter or two indicating their most prominent amino acid residue. Possible functions for some of these structural features are discussed in the text.

catalytic center. Two of these segments (G1 and G2) are glycine-rich and resemble nucleotide binding motifs seen in other proteins. The sequences of the other two segments (N and F) reveal little about their possible structures or functions.

Receiver modules are roughly 120 amino acids in length. Unlike transmitters, receiver structure is well understood (see Chapter 4). Extrapolating from X-ray and nuclear magnetic resonance studies of CheY, all receiver modules are probably α/β barrels with five sets of alternating β strands and α helices. The β strands align to form a hydrophobic inner core with the α-helical segments wrapped around the outside of the molecule. Prominent sequence features of receivers include a pair of aspartates near the amino terminus, a lysine near the carboxyl terminus, and a centrally located aspartate (Fig. 7). The three aspartate residues comprise an acid pocket at one end of the barrel, into which the side chain of the conserved lysine protrudes. The central aspartate is the site of phosphorylation, whereas the amino-terminal pair are probably important for catalysis. The conserved lysine may be involved in effecting the phosphorylation-induced conformational changes that regulate output activity.

The primary structures of communication modules have furnished few clues about their input determinants. In receivers, it seems likely that residues in or near the acid pocket could be involved in docking interactions with transmitters, but distal residues might also play roles in input specificity. The external α-helices, for example, could contain additional contact sites for transmitter binding. The input determinants in transmitters are even less apparent but could be involved in controlling interactions between transmitter subunits. Transmitters probably function as dimers, with the catalytic site of one subunit phosphorylating

the acceptor site in the other. The fact that many sensors are transmembrane proteins suggests that transmitters are designed to be controlled by long-distance conformational changes rather than by direct contact with an input domain. Conformational changes that alter the spatial orientation of the two subunits could provide the basis for input control of transmitter autophosphorylation.

Mutant Modules

To ascribe communication functions to particular structural features in a transmitter or receiver, mutants are needed that affect specific signaling activities while leaving others intact. They are likely to be partially dominant, possibly leaky, and relatively rare, necessitating a variety of isolation schemes to obtain a representative spectrum of functional defects. Single amino acid replacements, generated by site-directed or random mutagenesis, are best suited for this purpose, but in special circumstances, more drastic structural changes (e.g., deletions and substitution chimeras) might also be informative. The ensuing discussion pertains mainly to missense mutants and assumes the existence of phenotypic or biochemical assays for the various functions under study (input control, catalysis, output control, and so on.

Site-directed mutation, often the genetic equivalent of turning gold into lead, is nevertheless a gratifying way to obtain an initial collection of signaling mutants. For example, alterations can be engineered to test the functional importance of particular structural features in a transmitter or receiver. Popular targets for amino acid replacements are the conserved residues believed to comprise the substrate or catalytic sites (Fig. 7). Such mutant proteins invariably fail to function in vivo and, if examined in vitro, cannot be phosphorylated. This demonstrates that the target residue is important but not what its functional role might be. The amino acid change could conceivably affect the synthesis, folding, stability or activity of the protein. Distinguishing these possibilities calls for additional biochemical tests. Perversely, site-directed mutation reveals more about a protein when it fails to yield an expected loss-of-function result. If the mutant protein still supports normal in vivo behavior, then the altered residue must not be important for any of its signaling functions. In principle, one could identify all the protein's functionally important residues in this manner, but there are better ways to go about this.

It should be possible to devise isolation schemes for obtaining particular kinds of signaling mutants. When seeking mutants with a specific functional change, try to exploit the anticipated properties and residual activities of the mutant protein to eliminate unwanted types (e.g., complete loss-of-function). Or start the hunt with a mutant that limits the spectrum of possible functional changes. A few general examples will illustrate the possibilities. (i) To identify structural determinants involved in protein-protein interactions, first look for mutants with increased, rather than reduced, target binding affinity. Lesions of this sort should enhance the inhibitory potency of liberated modules or module parts (e.g., phosphorylation site peptides) and might cause dominant signaling defects in intact proteins. They can provide a structural foothold for exploring the interaction surface by conformational suppression (see below). (ii) To identify structural determinants involved in input control, look for dominant mutants with stimulus-independent constitutive output signals. (iii) To identify structural determinants involved in autophosphorylation or phosphotransfer activity, begin with a constitutively active mutant and look for mutations that amplify its output signals. If the constitutive alteration fully short-circuits input control, any increases in output activity should come through enhanced efficiency of a subsequent signaling function.

Reversion Analysis

The ways in which a mutant protein regains function can reveal a lot about its structure-function organization. In addition to back mutation and informational suppression, which are irrelevant in the present context, a variety of functional suppression mechanisms could conceivably alleviate a missense defect in a signaling protein. The suppressors either restore activity to the mutant protein or create an alternative function that bypasses the defective signaling step. Here only the most salient features of these two general suppression mechanisms and some specific examples of each are discussed.

BYPASS SUPPRESSION

Bypass suppressors are gene-specific but not allele-specific. They act on knockout mutations (e.g., deletions) as well as less drastic lesions because they make no use of the defective protein. Consider, for example, a null defect in a sensor that mediates a regulatory response to a particular stimulus, say osmolarity changes. "Revertants" that exhibit appropriate behavioral responses to osmolarity shifts could conceivably arise through bypass mutations in other signaling proteins. Another osmosensor might acquire the ability to communicate with the response regulator partner of the missing sensor, or the response regulator of another osmosensing circuit might acquire the ability to control the signal output of the defective pathway. The mechanisms of bypass suppression are as varied as the imagination. Except for general principles, lessons learned with one signaling pathway will not apply to another. However, understanding how a particular bypass suppressor operates can provide fresh insights into a signaling pathway and its elements.

CONFORMATIONAL SUPPRESSION

Conformational suppressors restore activity to the mutant protein through compensatory structural alterations. They cannot correct null defects because the mutant gene product is essential to the suppression mechanism. Conformational suppressors act through stereospecific contacts within and between proteins and, consequently, are highly allele-specific. These types of compensatory changes can arise at secondary sites within the mutant protein or in another protein that interacts with it. The example in Fig. 8 shows the kinds of conformational suppressors that might be obtained by reverting a mutant with a missense defect in a transmitter module.

Transmitter defects in the catalytic center, phosphorylation site, or other regions important for autokinase activity might be most easily corrected by additional structural changes within the transmitter domain (Fig. 8a). For example, a mutation that distorts the catalytic center might regain autophosphorylation ability through a nearby change that alleviates the distortion, through alterations of the phosphorylation site that enable it to access the mutant catalytic center, or through other conformational changes that influence the interaction between the phosphorylation site and catalytic center. Transmitter defects in input or output determinants should also be correctable by structural changes within the domain. However, alteration of the communication partner might also compensate for such defects. Input defects might be suppressed by structural changes in the adjoining input domain (Fig. 8b); output defects might be suppressed by changes in the receiver domain of the response regulator target (Fig. 8c).

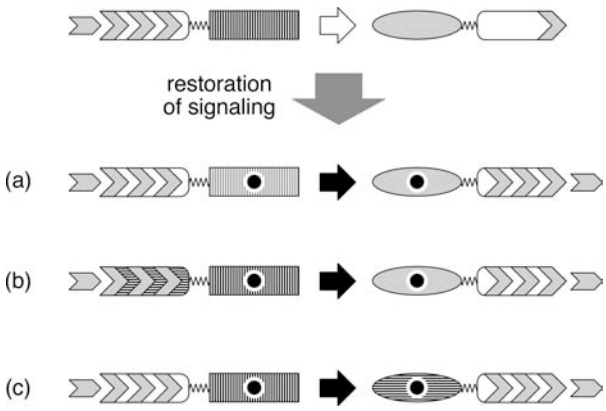

FIGURE 8. Functional suppression of signaling defects. The starting circuit has a missense mutation in the transmitter that interrupts the signal pathway. Three different suppression mechanisms that may be represented among phenotypic revertants are shown: (a) mutations within the mutant domain that compensate for a defect in folding, stability, or signaling function; (b) mutations in another domain of the same protein that restore normal communication with the mutant domain; (c) mutations in another signaling component that restore normal communication with the mutant protein.

TAKE-HOME LESSON

Genetic approaches can provide considerable insight into the operation of signaling pathways and proteins. Even though actual signaling circuits are unlikely to be as simple as the two component examples in this chapter, the same basic principles should apply. Extension of these ideas to real-world situations is left as an exercise for the reader.

REFERENCES

Ames, P., and J. S. Parkinson. 1994. Constitutively signaling fragments of Tsr, the *E.* coli serine chemoreceptor. *J. Bacteriol.* **176:**6340-6348.

Baumgartner, J. W., C. Kim, R. R. Brissette, M. Inouye, C. Park, and G. L. Hazelbauer. 1994. Transmembrane signalling by a hybrid protein: communication from the domain of chemoreceptor Trg that recognizes sugar-binding proteins to kinase/phosphatase domain of osmosensor EnvZ. *J. Bacteriol.* **176:**1157-1163.

Bourret, R. B., K. A. Borkovich, and M. I. Simon. 1991. Signal transduction pathways involving protein phosphorylation in prokaryotes. *Annu. Rev. Biochem.* **60:**401-441.

Morrison, T. B., and J. S. Parkinson. 1994. Liberation of an interaction domain from the phosphotransfer region of CheA, a signaling kinase of *E. coli*. *Proc. Natl. Acad. Sci. USA* **91:**5485-5489.

Nakashima, K., K. Kanamaru, H. Aiba, and T. Mizuno. 1991. Osmoregulatory expression of the porin genes in Escherichia coli: evidence for signal titration in the signal transduction through EnvZ-OmpR phosphotransfer. *FEMS Microbiol. Lett.* **66:**43-47.

Parkinson, J. S. 1993. Signal transduction schemes of bacteria. *Cell* **73:**857-871.

Parkinson, J. S.,.and E. C. Kofoid. 1992. Communication modules in bacterial signaling proteins. *Annu. Rev. Genet.* **26:**71-112.

Stock, J. B., A. M. Stock, and J. M. Mottonen. 1990. Signal transduction in bacteria. *Nature* **344:**395-400.

Utsumi, R., R. E. Brissette, A. Rampersaud, S. A. Forst, K. Oosawa, and M. Inouye. 1989. Activation of bacterial porin gene expression by a chimeric signal transducer in response to aspartate. *Science* **245:**1246-1249.