# [21] Phenotypic Suppression Methods for Analyzing Intra- and Inter-Molecular Signaling Interactions of Chemoreceptors

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## Abstract

The receptors that mediate chemotactic behaviors in *E. coli* and other motile bacteria and archaea are exquisite molecular machines. They detect minute concentration changes in the organism's chemical environment, integrate multiple stimulus inputs, and generate a highly amplified output signal that modulates the cell's locomotor pattern. Genetic dissection and suppression analyses have played an important role in elucidating the molecular mechanisms that underlie chemoreceptor signaling. This chapter discusses three examples of phenotypic suppression analyses of receptor signaling defects. (i) Balancing suppression can occur in mutant receptors that have biased output signals and involves second-site mutations that create an offsetting bias change. Such suppressors can arise in many parts of the receptor and need not involve directly interacting parts of the molecule. (ii) Conformational suppression within a mutant receptor molecule occurs through a mutation that directly compensates for the initial structural defect. This form of suppression should be highly dependent on the nature of the structural alterations caused by the original mutation and its suppressor, but in practice may be difficult to distinguish from balancing suppression without high-resolution structural information about the mutant and pseudorevertant proteins. (iii) Conformational suppression between receptor molecules involves correction of a functional defect in one receptor by a mutational change in a heterologous receptor with which it normally interacts. The suppression patterns exhibit allele-specificity with respect to the compensatory residue positions and amino acid side chains, a hallmark of stereospecific protein–protein interactions.

## Introduction

Motile bacteria exhibit sophisticated chemotactic behaviors that are good models for exploring the molecular mechanisms that proteins use to detect and process sensory information about their chemical environment (reviewed by Armitage, 1999; Parkinson *et al.*, 2005; Sourjik, 2004; Wadhams and Armitage, 2004). *Escherichia coli*, the best-studied

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chemotactic organism, tracks very shallow gradients of attractant and repellent chemicals by continuously scanning for temporal changes in chemoeffector concentrations as it swims about. Favorable stimuli—for example, an increasing attractant level—suppress the likelihood of a directional change, thereby prolonging cell movement in the favorable direction. *E. coli* senses temporal concentration changes by comparing current conditions to those averaged over the past few seconds in its travels. Concentration changes as small as 0.1% can produce much larger changes in the rotational behavior of the flagellar motors, corresponding to a roughly 50-fold amplification of the input stimulus. An adaptation system that tunes the sensory machinery to match ambient chemoeffector levels maintains sensitive gradient detection over a nanomolar to millimolar concentration range.

Chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs) mediate these remarkable signaling feats (see reviews by Falke and Hazelbauer, 2001; Zhulin, 2001). MCPs are transmembrane homodimers with a periplasmic sensing domain and a cytoplasmic signaling domain (Fig. 1A). MCPs monitor chemoeffector levels through the occupancy state of their periplasmic ligand-binding domains. Past chemical conditions are recorded in the form of reversible covalent modifications at 4 to 6 specific glutamic acid residues in the MCP signaling domain. CheR, a methyltransferase, adds methyl groups to MCP molecules; CheB, a methylesterase, hydrolyzes MCP glutamyl-methyl esters back to glutamic acid. The MCP signaling domain forms stable ternary complexes with two cytoplasmic proteins, CheA, a histidine kinase, and CheW, which couples CheA to receptor control. The chemoreceptor signaling complex transmits phosphoryl groups to the response regulators CheB and CheY to control the cell's swimming behavior. Phospho-CheY interacts with the flagellar motor to enhance the probability of clockwise (CW) rotation, which causes random directional turns. Counterclockwise (CCW) rotation produces forward swimming, the default behavior. Phospho-CheB, the active form of the MCP methylesterase, is part of a feedback sensory adaptation circuit that adjusts the sensitive detection range of the receptors.

Although a receptor molecule can modulate the activity of its associated CheA partner over a 1000-fold range, this control alone is not responsible for the prodigious signal gain in the chemotactic signaling system. Rather, each receptor is able to regulate about 35 CheA molecules, most of which are probably physically associated with other receptor molecules (Sourjik and Berg, 2002). Signal amplification appears to arise through communication among receptor molecules in a cooperative signaling array (Sourjik and Berg, 2004). Receptor dimers form trimers of dimers that are, in turn, networked to other trimer-based receptor teams, possibly through shared

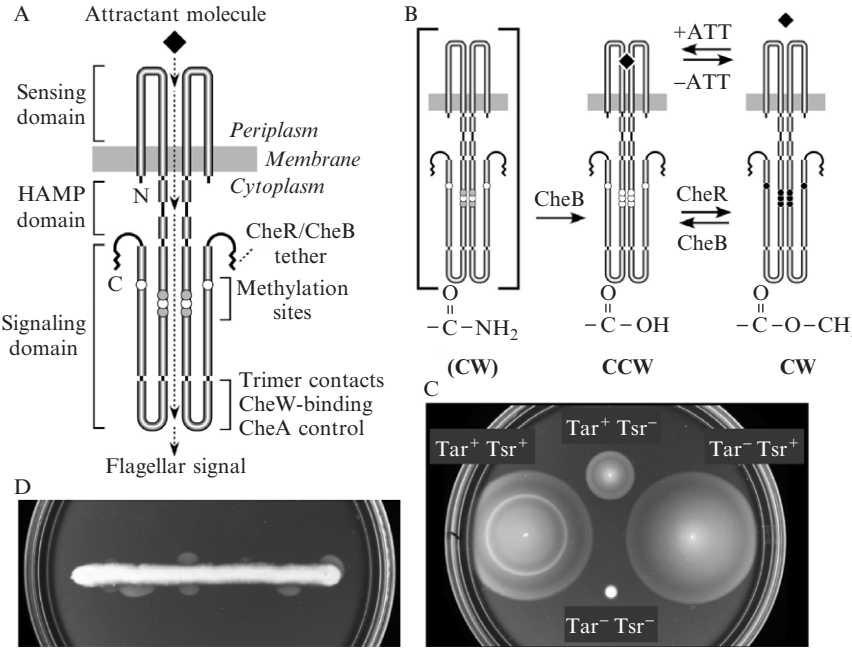


FIG. 1. Structure and function of MCP molecules. (A) Domain organization of transmembrane chemoreceptors of the MCP family. MCP subunits are ~550 residues in length; the native molecule is a homodimer. Thick segments denote polypeptide segments that have predominantly alpha-helical secondary structure. The periplasmic sensing domain contains two symmetric ligand-binding sites at the dimer interface (see Fig. 3D). Ligand-binding events trigger conformational changes that are transmitted through the membrane-spanning segments to the HAMP domain, which, in turn, regulates the activity of the signaling domain. The signaling domain helices of each subunit form anti-parallel coiled-coils that interact in the dimer to form a four-helix bundle. Conformational changes at the tip of the helix bundle regulate CheA activity, possibly by modulating receptor–receptor interactions in trimer-of-dimer signaling complexes. The modification status of the intervening methylation sites determines the sensitive range of the input–output connection. In *E. coli*, the high-abundance MCPs, Tar and Tsr, carry a pentapeptide sequence (NWETF) at their C-termini that interacts with the methylation (CheR) and deamidation/demethylation (CheB) enzymes. *E. coli* MCPs typically have two methylation sites (gray circles) that are synthesized as glutamine (Q) residues and subsequently converted to glutamic acid (E) by CheB-mediated deamidation. The remaining methylation sites (white circles) are synthesized as glutamic acid residues that are immediately competent to accept a methyl group, forming a glutamyl methyl ester. (B) Two-state model of receptor signaling and CheR/CheB-mediated modification reactions. MCP molecules can exist in CheA-activating (CW) or -deactivating (CCW) output states. The chemical structures of the methylation sites associated with each signaling state are shown beneath the molecules. Newly synthesized receptors (QEQE) have transient CW output until irreversible deamidation is complete (EEEE). In mature MCP molecules, the proportion of receptors in each signaling state is controlled by the interplay between ligand occupancy and modification state. (C) Chemotaxis phenotypes assayed on soft agar plates. The medium was tryptone broth

connections to their CheA and CheW signaling partners (Ames *et al.*, 2002; Studdert and Parkinson, 2004, 2005). The trimers can contain receptors of different detection specificities, any of which can modulate team signal output and relay sensory information to other receptor signaling teams (Parkinson *et al.*, 2005).

The signaling properties of a receptor team can be understood in terms of two alternative signaling states (Fig. 1B), a CW mode that activates CheA autophosphorylation and a CCW mode that deactivates CheA. The proportion of receptor molecules or signaling teams in each state and, consequently, the cell's behavior, reflect the interplay between ligand occupancy and methylation state averaged over the receptor population (Fig. 1B). Attractant increases, for example, drive receptor molecules toward the CCW signaling state, reducing CW motor rotation and initiating a signal-offsetting increase in MCP methylation state through feedback control of CheB activity. The cell ''remembers'' the chemoeffector change until sensory adaptation is complete.

## Genetic Analyses of Chemoreceptors

Genetic methods and logic compose a powerful tool for dissecting structure–function relationships in proteins. In bacterial chemotaxis, genetic studies of the *E. coli* system over the past 40 years have served to identify the various components of the signaling pathway and their overall

(10 g/l Difco tryptone, 5 g/l NaCl) containing ~3 g/l Difco bacto-agar. The firmness of agar varies from lot to lot; each new lot must be empirically calibrated (typically over a range of 2–3 g/l in agar concentration). Colonies were inoculated with cells (usually from a fresh overnight colony, but liquid cultures also work) on a sterile toothpick stabbed to the bottom of the plate. Strains: RP437 ($Tar^+$ $Tsr^+$); RP8604 ($Tar^+$ $Tsr^-$); RP8606 ($Tar^-$ $Tsr^+$); RP8611 ($Tar^-$ $Tsr^-$). Plates were incubated at 32.5° for 8 h. (D) Selection for chemotactic revertants on tryptone soft agar. Approximately 50 $\mu$l of liquid culture containing nonchemotactic parental cells and rare chemotactic revertant or pseudorevertant cells (at spontaneous or mutagen-induced frequencies) were gently spread on the surface of a soft agar plate with a plastic pipette tip so as to avoid tearing the surface of the agar. The plate was incubated overnight (~17 h) at 32.5°. The number of cells in the inoculum should be adjusted to obtain ~10 discrete revertant ''flares.'' If desired, nonmotile or nonchemotactic cells can be added to the inoculum to hasten formation of attractant gradients through growth in the cell streak. The ''helper'' cells should be incapable of reverting, for example, cells that have deletions of motility or chemotaxis genes. Note that the revertants in this example arose before placing the cells on the plate. This method can also be used to select spontaneous revertants that arise during growth on the soft agar plate, but the cell density of the inoculum and the overall incubation time will probably need to be increased to detect such reversion events. However, it is important to avoid prolonged incubation times (>24 h), which may yield undesirable multi-step revertants.

''wiring diagram'' (Parkinson, 1977, 1993) Genetic approaches have also provided valuable insights into the functional architecture of individual chemoreceptor molecules (Ames *et al.*, 1988) and, more recently, of trimers of dimers and chemoreceptor clusters (Ames and Parkinson, 2006; Ames *et al.*, 2002). Chemoreceptor defects that eliminate chemotactic ability in *E. coli* include lesions that affect maturation and stability of the native protein (Buron-Barral *et al.*, 2006; Butler and Falke, 1998; Danielson *et al.*, 1997; Ma *et al.*, 2005), ligand-binding determinants (Gardina and Manson, 1996; Lee and Imae, 1990; Wolfe *et al.*, 1988; Yaghmai and Hazelbauer, 1992, 1993), transmembrane segments (Chen, 1992; Jeffery and Koshland, 1994, 1999; Maruyama *et al.*, 1995; Nishiyama *et al.*, 1999; Oosawa and Simon, 1986), the HAMP domain (Ames and Parkinson, 1988), input/output control (Coleman *et al.*, 2005; Trammell and Falke, 1999), CheA control (Ames and Parkinson, 1994), sensory adaptation determinants (Nara *et al.*, 1996; Nishiyama *et al.*, 1997; Shapiro *et al.*, 1995; Shiomi *et al.*, 2000, 2002; Starrett and Falke, 2005), and trimer formation (Ames and Parkinson, 2006; Ames *et al.*, 2002). Most of these functional defects reflect loss-of-function lesions, but gain-of-function lesions that lock receptor output can also abrogate chemotactic responses (Ames and Parkinson, 1988; Mutoh *et al.*, 1986).

Knowing the primary structure change in a mutant protein (inferred from the DNA sequence of the mutant gene) may not shed much light on the nature of its functional defect. A powerful genetic approach that can provide insight into the functional nature of a mutant defect involves the isolation and characterization of secondary mutational changes that restore some measure of function to the mutant protein (Manson, 2000). Such second-site suppressors can arise within the original mutant gene (intragenic suppressors) or in some other gene (extragenic suppressors) whose product functionally interacts with the mutant protein. This chapter discusses three suppression case studies of chemoreceptor mutants to illustrate both the power and the pitfalls of this approach. The subjects of these studies are Tsr, the serine receptor, and Tar, the aspartate receptor, the predominant receptor types in *E. coli*. However, the lessons learned from Tar and Tsr are generally applicable to the low-abundance chemoreceptors (Aer, Tap, Trg) as well. We begin with a general discussion of the basic genetic tools available in the chemotaxis system.

### *Soft Agar Chemotaxis Assays*

*E. coli* chemoreceptor mutants exhibit distinctive colony morphologies on nutrient soft agar plates (Fig. 1C). At agar concentrations of ~3 g/l, cells can swim in the water-filled tunnels created by the agar matrix. As the colonies grow, the cells consume nutrients in the medium, such as aspartate

and serine, that are chemotattractants. If the cells are able to detect and respond to those chemoeffectors, the colony expands rapidly, consuming and following the attractant gradients. On complex tryptone medium, serine is the first attractant to be exhausted, so strains capable of serine chemotaxis ($Tsr^+$) form a band of cells at the colony margin that expands outward as they consume the serine (Fig. 1C). Cells left behind the serine pioneers no longer have free serine to eat and so consume aspartate, leading to a second ring of cells doing aspartate chemotaxis. $Tsr^+$ strains that lack the aspartate receptor ($Tar^-$) expand equally fast, but lack the inner aspartate ring (Fig. 1C). In contrast, $Tar^+$ colonies lacking Tsr function expand more slowly than does the aspartate ring inside wild-type colonies because the $Tsr^-$ $Tar^+$ strain must still consume serine before it can establish an aspartate gradient (Fig. 1C). Strains lacking both of these major chemoreceptors ($Tar^-$ $Tsr^-$) are generally nonchemotactic ($Che^-$) because the remaining low-abundance receptors (Tap, dipeptides; Trg, ribose and galactose; Aer, aerotaxis) cannot by themselves sufficiently activate CheA to establish a suitable balance of running and tumbling behavior (Fig. 1C). Mutant strains lacking any of the shared components of the chemotaxis signaling pathway also exhibit $Che^-$ phenotypes. Those cells are motile, but either constantly running (CCW-biased: $CheA^-$, $CheW^-$, $CheR^-$, $CheY^-$) or constantly tumbling (CW-biased: $CheB^-$ and $CheZ^-$) and, consequently, cannot track chemoeffector gradients.

Colonies on soft agar plates are usually inoculated with a toothpick carrying cells from fresh colonies growing on hard agar plates, ideally composed of the same growth medium. The toothpicks are stabbed nearly to the bottom of the soft agar plate, which is then incubated until colonies are sufficiently large to score their chemotaxis phenotypes (typically, 6–10 hours at 30–35°). To select chemotactic revertants from a nonchemotactic parent, the mutant cells are generally inoculated in a stripe across the surface and incubated overnight or longer, depending on the frequency of reversion events. Chemotactic revertants appear as small ''flares'' that emanate from the border of the parental stripe (Fig. 1D).

### *The Pros and Cons of Plasmids*

The case studies described in the following text were carried out with plasmid-borne chemoreceptor genes, an experimental approach that facilitates many aspects of suppression analyses: targeted mutagenesis, control of gene expression levels, large-scale revertant hunts, DNA sequencing analyses, and rapid transfer of mutant genes to new genetic backgrounds. However, plasmids can also complicate genetic analyses and it is important to understand and appreciate their limitations.

*Expression level effects*. Optimal chemotactic behavior depends on proper stoichiometry of the chemotaxis signaling components. Owing to relatively high copy numbers, plasmid-borne genes may express too much product for optimal performance. If the plasmid-borne gene is accompanied by its native promoter, it may titrate positive regulatory factors needed for transcription of other flagellar and chemotaxis genes, leading to a significant disparity in the expression levels of plasmid and chromosomal gene products. Foreign promoters that do not compete for shared transcription factors can alleviate this problem, but may result in high basal expression levels of the plasmid-borne gene(s), depending on the tightness of their regulatory controls. Our criterion for choosing plasmid constructs for genetic studies is that the plasmid-borne gene be able to produce optimal chemotactic ability at an intermediate induction level. This ensures that its uninduced expression level is below that of its chromosomally encoded counterpart and obviates aberrant stoichiometry effects.

*Genotypic and phenotypic lag*. A cell containing a multicopy plasmid is quite slow to produce mutant offspring following a loss-of-function mutation in one of the plasmid molecules. The long lag in mutant appearance is due to two factors: (1) the need to dilute away the functional nonmutant gene products present in the parental cell (phenotypic lag), and (2) the need to generate a progeny cell with all mutant plasmids (genotypic lag). Genotypic lag is the more severe problem because plasmids usually partition randomly, but more or less equally, into daughter cells at division. A single mutant plasmid among many nonmutant ones in a cell will need many generations of random segregation and genetic drift before it becomes fixed in the plasmid population of a descendant cell. Genotypic lag is further exacerbated by recombination or replication events that create multimeric plasmids with several tandem copies in the same molecule.

Segregation lag should not be a significant factor when selecting for gain-of-function revertants from a mutant parental plasmid. Any mutation that restores function should be expressed phenotypically as soon as it arises, provided that multiple copies of the mutant gene do not interfere with that function. However, subsequent analysis of the gain-of-function mutation may be confounded by the dominant nature of the revertant phenotype. The revertant cell will probably not carry a genetically homogeneous plasmid population. Plasmid dimers are especially insidious because they can remain heterozygous through single-plasmid transformations of new host cells.

*Plasmid maintenance*. Plasmids represent an added genetic load to their host cell and must be maintained by positive selection, most often for a plasmid-encoded antibiotic resistance trait. Growth in the presence of antibiotics seems to have a generalized dampening effect on chemotactic

performance in soft agar plates, perhaps owing to slowed growth rates. To ameliorate this effect as much as possible, we routinely halve the usual concentration of an antibiotic for use in soft agar plates.

All of these plasmid-related problems can be overcome or circumvented. For example, plasmid dimers can be eliminated by linearizing a population of plasmid molecules with a restriction enzyme that cuts once per monomer and religating the linear products at low DNA concentration to "clone" monomeric forms of the plasmid. Similarly, two different copies of a mutant gene can be carried on compatible plasmids for complementation analyses, using recombination-deficient host cells to avoid recombination events between the multicopy parental plasmids. Thus, with foreknowledge and appropriate precautions, the technical advantages of manipulating chemoreceptor genes on plasmids vastly outweigh the potential drawbacks. The plasmid-specific details of these manipulations will not be explicitly discussed in the case studies to follow.

## Balancing Suppression: Methylation-Independent Chemoreceptors

According to the two-state signaling model, chemoreceptor molecules with low methylation states have CCW output; those with high methylation states produce CW output (Fig. 1B). Receptors shift between these two signaling modes upon changes in ligand occupancy and upon subsequent compensatory changes in methylation state. Control of receptor methylation level occurs in two ways, through stimulus-induced changes in the substrate properties of the receptor molecules for the CheR and CheB enzymes and through feedback regulation of CheB activity by phosphorylation. Cells lacking CheR and/or CheB function are generally nonchemotactic because they cannot adjust their chemoreceptor methylation levels. The receptor molecules in CheB mutants are fully methylated (CW-signaling); those in CheR mutants are fully demethylated (CCW-signaling) (Fig. 2A). These defects, respectively, cause incessantly tumbling or constantly running swimming behaviors that preclude gradient tracking in soft agar plates. Thus, in adaptation-defective cells, wild-type receptors have locked output signals and cannot mediate chemotaxis.

In CheR-deficient cells, the serine receptor (Tsr) readily acquires the ability to promote chemotaxis-like colony expansion on soft agar plates (Fig. 2B). Single amino acid replacements in many parts of the Tsr molecule can create CheR-independent receptors (designated Tsr!): near the ligand-binding sites in the sensing domain, in the membrane-spanning segments, in the HAMP domain, near the methylation site residues, and near the tip of the cytoplasmic signaling domain (Ames, unpublished results). In $CheR^+$ cells, most Tsr! receptors mediate robust serine chemotaxis, demonstrating

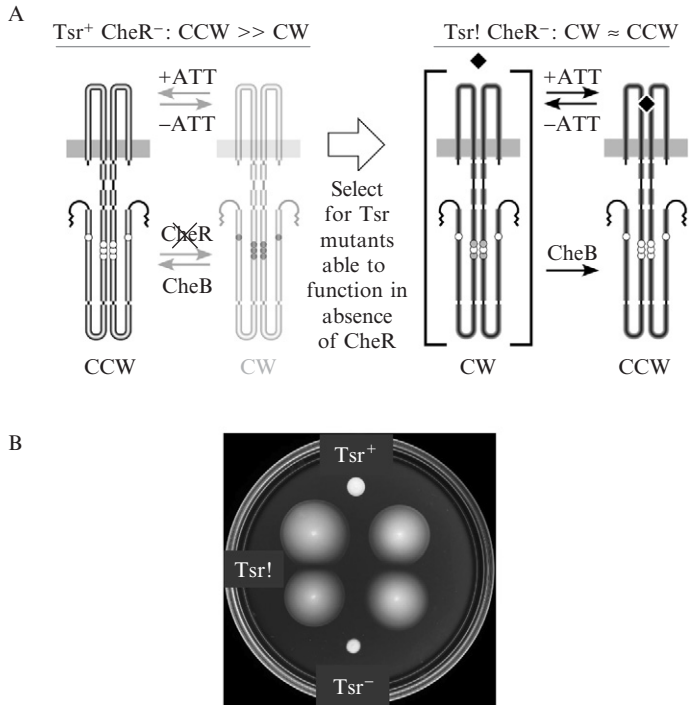


FIG. 2. Gain-of-function suppression of a methyltransferase defect. (A) Summary of mutant phenotypes and suppressor selection. Wild-type Tsr molecules cannot function properly in the absence of CheR function because the mature receptors are trapped in the unmethylated (CCW) signaling state. Selection for chemotactic ability yields Tsr! mutations that most likely extend the lifetime or enhance the CW-signaling activity of undeamidated Tsr molecules. (B) Examples of Tsr phenotypes in the absence of CheR function. Various Tsr-bearing plasmids were transferred to a Δ*tsr* Δ*cheR* recipient strain and tested for chemotactic ability on tryptone soft agar. Plasmids expressing wild-type Tsr ($Tsr^+$) or no Tsr ($Tsr^-$) cannot support chemotaxis in a CheR-deficient host, whereas Tsr! receptors do. Tsr! alleles were, reading clockwise from upper left: L291F, E304K, A495T, A498T. The plate was incubated at 30° for 15 h.

that their signaling properties are quite similar to those of wild-type Tsr under adaptation-competent conditions. However, in $CheR^-$ cells, Tsr! mutations probably shift the unmethylated receptor molecules toward the CW-signaling state, resulting in a more balanced CCW/CW distribution of signaling states in the receptor population (Fig. 2A). Evidently, subtle structural changes in many parts of the Tsr molecule can accomplish this balancing act, conceivably by a variety of mechanisms. Ligand-binding and HAMP alterations might attenuate the attractant-bound (CCW) signaling

state or mimic a repellent-sensing (CW) condition. Structural alterations near the adaptation sites might mimic the methylated (CW) conformation. Alterations in the signaling tip might directly enhance the stability of the CheA-activating (CW) output state.

Do Tsr! suppressors restore true chemotaxis in the absence of a methylation system? Probably not. We know that colony expansion by Tsr! CheR$^-$ strains on soft agar plates involves serine sensing because serine-binding lesions abrogate that behavior. However, Tsr! receptors mediate rather slow colony expansion in CheR$^-$ strains relative to CheR$^+$ strains, implying an inefficient gradient-tracking strategy. Moreover, efficient chemotaxis most likely requires a system for sensory adaptation and it is difficult to imagine how CheB alone could *reversibly* modulate the CCW/CW equilibrium of a Tsr! receptor population in CheR$^-$ cells. Nevertheless, Tsr! CheR$^-$ ''chemotaxis'' remains CheB-dependent, suggesting that CheB-mediated deamidation reactions, the only ones possible in the absence of methylation, play some role in colony expansion. One behavioral scenario is that Tsr! CheR$^-$ colonies expand, albeit inefficiently, by modulating down-gradient rather than up-gradient cell movements. Owing to their intrinsic CW bias, unmethylated Tsr! molecules should be more sensitive than their wild-type counterparts to decreasing serine levels, which would tend to shift the receptor population toward the CW output state and elevated tumbling probability (Fig. 2A). Subsequent CheB-mediated deamidation of nascent MCP molecules might serve as a sensory adaptation mechanism by restoring a more balanced CCW/CW distribution in the receptor population. Thus, outward expansion of Tsr! CheR$^-$ colonies might occur through a difference in average cell path lengths between random up-gradient excursions and stimulus-enhanced tumbling during down-gradient travels.

Shiomi *et al.* (2002) demonstrated a similar type of balancing suppression in the aspartate receptor, Tar. The C-termini of Tsr and Tar molecules carry a pentapeptide sequence (NWETF) to which the CheR and CheB adaptation enzymes can bind (Fig. 1A). Binding tethers the adaptation enzymes to the receptor cluster and enhances their activities on Tar and Tsr molecules as well as on their low-abundance neighbors (Tap and Trg), which lack the NWETF sequence. Tar and Tsr mutants that lack the tethering sequence are inefficiently methylated and confer phenotypes very similar to those of wild-type receptors in a CheR-defective strain. Shiomi *et al.* isolated pseudorevertants of a Tar mutant lacking the NWETF sequence and showed that they contained second-site mutations in the Tar signaling domain that imparted a CW output bias.

The sensory adaptation capacity of MCP molecules makes balancing suppression a very common mechanism for restoring receptor function. Most loss-of-function receptor lesions shift the equilibrium between CCW

and CW signaling states beyond the control range of the sensory adaptation system, resulting in excessively CCW or CW signal output in the absence of stimuli. Such receptor mutants can readily regain function through a variety of second-site mutations that create an offsetting bias change to bring the CCW-CW equilibrium back into the control range of the adaptation system. Because so many parts of the receptor molecule influence its signal output, the initial bias alteration and its suppressor need not affect the same step in signal production. For example, a receptor mutant with a binding site lesion that mimics ligand occupancy could most likely be phenotypically suppressed by a mutational change in one of the methylation sites. Obviously, the ligand-binding and methylation sites do not interact directly with one another, but rather both regions influence the receptor's signal output. In conclusion, balancing suppression is a good way to isolate mutations that cause a particular type of output bias, but reveals little about their structural and functional relationship to the initial signaling defect of the mutant receptor.

### Conformational Suppression within Receptor Molecules

Second-site suppressors in a mutant receptor gene can also identify direct structural interactions within receptor molecules, but it may be difficult to distinguish a compensatory structural interaction from less direct balancing suppression mechanisms. Here, we describe a well-studied example of what appears to be conformational suppression between mutations in a receptor molecule, which nevertheless defies an entirely satisfactory mechanistic explanation.

MCP subunits have two membrane-spanning segments, one (TM1) leading to the periplasm and a second (TM2) that returns the polypeptide to the cytoplasm (Fig. 3A). In a seminal attempt to investigate the signaling role of these transmembrane segments, Oosawa and Simon (1986) engineered a loss-of-function lesion in TM1 of the aspartate receptor, Tar, and then isolated and characterized second-site suppressor mutations in the mutant gene. The starting mutation, A19K, in a residue thought to lie near the middle of TM1, eliminated aspartate chemotaxis on soft agar plates even though the mutant protein was membrane-localized and bound aspartate with normal affinity. Cells containing Tar-A19K as their sole receptor exhibited CCW-biased signal output, consistent with a substantial defect in activating the CheA kinase.

To explore the basis for the Tar-A19K signaling defect, Oosawa and Simon selected chemotactic pseudorevertants as spontaneous cell flares on soft agar medium (see Fig. 1D). A number of second-site mutations were found to restore function to Tar-A19K (Fig. 3B), including one in TM1 (V17E) and four in TM2 (W192R, A198E, V201E, V202L). Based on these

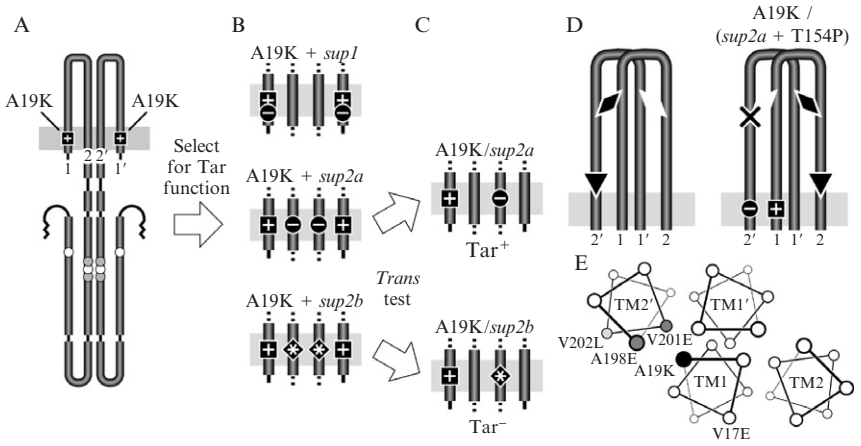


FIG. 3. Intragenic, second-site suppression of a transmembrane segment lesion. (A) A mutant Tar homodimer carrying the A19K mutation (" + " symbol) in the first transmembrane segment (TM1) of each subunit cannot mediate chemotactic responses to aspartate. (B) Selection for chemotactic pseudorevertants of Tar-A19K yielded second-site suppressor mutations in TM1 (*sup1)* or in the second transmembrane segment TM2 (*sup2*). Some of the suppressors introduced an acidic residue ("−" symbols); other suppressors had nonacidic amino acid replacements ("*" symbols). (C) *Trans* test of *sup2* action. A19K and *sup2* subunits were coexpressed to determine whether heterodimeric Tar molecules were functional. The *sup2* suppressors with acidic residue replacements were able to act in *trans*; the other *sup2* suppressors were not. To ensure that both types of mutant homodimers (A19K/A19K and *sup2* + T154P/*sup2* + T154P) were nonfunctional, the *sup2* mutations were combined with an aspartate binding site lesion (T154P), described in panel D. (D-left) The piston model of transmembrane signaling. The periplasmic sensing domains of Tar and Tsr have two symmetric, negatively cooperative ligand-binding sites at the dimer interface. Binding of one ligand molecule (black diamond) to either site induces a small downward movement of the TM2 segment in one of the subunits, determined by ligand orientation at the binding sites. (D-right) Use of a binding site lesion (T154P) to control the route of the transmembrane conformational signal in heterodimeric receptor molecules (Tar-A19K/*sup2a* + T154P). T154P subunits cannot function as homodimers, but contribute aspartate-binding determinants that allow heterodimers to sense aspartate ligands in one orientation, resulting in transmission of a transmembrane piston motion through the subunit that does not carry the T154P lesion (in this case, the A19K subunit). (E) Cross-section of the TM segments in a Tar dimer viewed from the periplasmic side. For each helix, the largest residue circles and thickest connecting lines are those closest to the periplasm. Note that A19 in one subunit is close to A198 and V201 in the other subunit. In contrast, V202L, which does not suppress A19K in *trans*, is not as closely aligned. Another suppressor that cannot act in *trans*, W192R, is not shown, but lies two turns above and just clockwise of the V202 position, even further out of line with residue 19 in TM1.

results, Oosawa and Simon suggested that the positively charged side-chain of the lysine replacement in Tar-A19K might distort the structure or position of TM1 through interaction with the negatively charged head groups in the membrane phospholipid. This TM1 structural change, in turn, must

somehow alter the signaling state of the Tar output domain. Oosawa and Simon suggested that the V17E change in TM1 and the A198E and V201E changes in TM2 might suppress A19K through formation of a salt bridge that effectively satisfied the offending positive charge to restore correct TM1 structure. They offered no specific explanation for the suppressor mutations in TM2 with non-acidic amino acid replacements (W192R and V202L), but these residue changes could conceivably compensate the A19K lesion through a direct structural interaction of a different sort. This reversion study provided the first evidence for an important role of the TM segments in MCP signaling and suggested that TM1 and TM2 might interact structurally, as well.

In a subsequent study, Umemura *et al.* (1998) obtained strong evidence for TM1–TM2 interactions and demonstrated that the TM2 suppressors of Tar-A19K fell into two distinct functional groups (Fig. 3B). Through cleverly designed experiments, they asked whether, in heterodimeric Tar molecules, a TM2 alteration in one subunit could suppress an A19K lesion in the other. To perform the *trans* test, Umemura *et al.* exploited information about the structure of the Tar ligand-binding domain and the transmembrane signaling mechanism that had emerged since the original study by Oosawa and Simon.

X-ray structures of the aspartate-bound form of the Tar sensing domain had revealed two symmetric binding sites at the dimer interface, each relying on side chain contacts from residues in both subunits (Fig. 3D). The critical binding-site determinants involve residues in the helical extension ($\alpha 1$) adjoining TM1 from one subunit and the helical extension ($\alpha 4$) adjoining TM2 in the other subunit. Ligand binding at the two sites is negatively cooperative; except at very high ligand concentrations, aspartate can only occupy one site per dimer. Comparisons of the ligand-occupied and the unliganded X-ray structures of the Tar sensing domain had also revealed a small ($\sim$2 Å) downward displacement of the $\alpha 4$ helix in one subunit of the aspartate-bound receptor (Chervitz and Falke, 1996). Subsequent cysteine crosslinking studies of several MCPs confirmed that ligand binding induces a modest piston motion in the TM2 segment that shifts the receptor signaling domain toward the CCW output state (Falke and Hazelbauer, 2001). Thus, aspartate binds to either one of two symmetric sites in the Tar periplasmic domain, inducing a downward movement of one of the TM2 segments in the dimer to modulate its signal output.

To set up A19K/*sup2* heterodimers for the *trans* tests, Umemura *et al.* coexpressed A19K and *sup2* subunits in the same cell. However, at comparable subunit expression levels, both types of homodimers will also form. Although A19K homodimers have no Tar function, *sup2* homodimers exhibited Tar function. To ensure that only heterodimers were capable of

furnishing Tar function, Umemura *et al.* introduced a binding site lesion (T154P) into the *sup2* subunit (Fig. 3D). Under these conditions, they found that the acidic (*sup2a*) suppressors (A198E, V201E) could act in *trans*, whereas the non-acidic (*sup2b*) suppressors (W192R, V202L) could not (Fig. 3C). In the test configuration, the T154P binding site lesion in the *sup2a* subunit constrains the ligand-induced piston motion to travel through the TM2 segment of the A19K subunit, demonstrating that the TM1 lesion does not interfere with the piston transmembrane signaling mechanism in the heterodimer.

### *Possible Mechanisms of Tar-A19K Suppression*

The spatial arrangement of Tar TM segments in the membrane, established through cysteine-directed crosslinking and molecular modeling studies (Chervitz and Falke, 1995; Milburn *et al.*, 1991; Pakula and Simon, 1992), suggests a simple explanation for *trans* action of the *sup2a* suppressors (Fig. 3E). The four TM segments in a Tar dimer form a bundle in which the TM1 segments pack against one another at the dimer interface. The TM2 segments, in contrast, are far from one another and loosely associated with both TM segments. In the Tar TM bundle, residue A19 in TM1 lies close to residues A198 and V201 in TM2 of the opposing subunit (Fig. 3E). This spatial arrangement supports the idea that acidic replacements at the TM2 residues could form a salt bridge with the lysine replacement in TM1 of the opposing subunit to suppress the A19K defect in *trans*. Moreover, it implies that the productive TM interactions in A19K + *sup2a* homodimers also occur in *trans,* that is, between rather than within subunits. This predicts that a Tar heterodimer with one wild-type and one A19K + *sup2a* subunit might not function. The *cis* arrangement could be tested by introducing complementary binding-site lesions into the two different subunits to prevent both types of homodimers from functioning. This experiment has not, to our knowledge, been done. However, Umemura *et al*. provided strong support for the salt-bridge model by constructing additional acidic replacements at TM2 residues that were predicted to face residue 19 in TM1 of the opposing subunit (Umemura *et al.*, 1998). Replacements at residues most closely aligned with A19K (e.g., V201D, L205D, L205E) suppressed well in *trans*, whereas those with less optimal alignments suppressed poorly (I204D, I204E) or not at all (A208D, A208E). Interestingly, although A198E was a good suppressor, A198D failed to suppress, perhaps owing to its shorter side-chain.

In the context of the piston model for Tar transmembrane signaling, the A19K lesion most likely produces a downward movement of TM2 because it causes CCW-biased signal output. This could happen by any of several

different mechanisms: (1) The A19K alteration might destabilize the TM1/TM1′ interface, leading to a conformational change in the periplasmic domain that mimics the ligand-occupied state. Indeed, basic replacements at nearby residues (G22R, S25R) in Tar-TM1 peptides have been shown to impair their dimerization (Sal-Man and Shai, 2005). (2) Interaction of the A19K side-chains with the lipid head groups at the cytoplasmic or periplasmic interface could shift the membrane position of the mutant TM1 segments, in turn triggering TM2 displacement through conformational changes in the periplasmic domain. (3) The mutant TM1 segment might perturb the membrane position of TM2 through a direct structural interaction. The trans-acting *sup2a* suppressors are consistent with all three mechanisms, but perhaps most compatible with a direct effect of TM1-A19K on the membrane position of TM2′, because the interacting TM segments are not covalently connected. In this scenario, it is easy to imagine how the postulated salt-bridge could resist the downward movement of TM2′ caused by the TM1-A19K lesion, thus restoring the ability to produce a downward piston movement in response to ligand-binding events.

It is less obvious how the acidic suppressor in TM1 (V17E) operates because this residue position is not close to A19 in either subunit (Fig. 3E) and has not been tested in *trans*. Conceivably, the TM1/TM1′ interaction is sufficiently malleable to allow salt bridge formation between V17E in one subunit and A19K in the other. This could serve to stabilize the TM1-TM1′ interface to alleviate a deleterious interaction of the TM1-A19K side-chain with TM2′. These suppression mechanisms predict that the V17E suppressor should be able to act in *trans*. If the acidic TM1 suppressor cannot act in *trans*, the suppression mechanism could be altogether different. For example, the A19K signaling defect might be caused by attraction of the basic side-chain toward the lipid head groups. The V17E acidic side-chain in *cis* might create a countering repulsive force that restores a more normal membrane position to the doubly mutant TM1.

The two *cis*-acting TM2 suppressors (W192R and V202L) probably work by different mechanisms. Owing to its aromatic side-chain, Tar-W192 is positioned at the lipid-head group interface at the periplasmic side of the membrane. Miller and Falke (2004) showed that Tar-W192R ''superactivated'' CheA in the absence of aspartate but was still able to inhibit kinase activity upon aspartate binding. They proposed that the arginine side-chain is attracted to the polar head groups, thereby shifting TM2 toward the periplasmic interface. This CW-biased conformational change should serve to offset the CCW-biased downward shift of TM2 caused by the A19K lesion. The failure of W192R to act on A19K in *trans* indicates that the compensatory membrane shift must occur in the subunit in which TM2 is displaced downward by the mutant TM1. The other

cis-acting suppressor (V202L) may also counteract the downward piston displacement caused by the A19K lesion, but the underlying compensatory mechanism is less apparent. V202 lies midway in TM2, roughly equidistant from the periplasmic and cytoplasmic interfaces, so it is unclear how a valine to leucine change at this position would preferentially cause an upward shift of TM2. However, V202L in one subunit might be close enough to A19K in the other subunit for their side-chains to interact (Fig. 3E), which might serve to shift TM1 and/or TM2′ toward the periplasmic side of the membrane. Although this is a *trans* interaction, perhaps it must occur in both subunits to achieve a significant suppression effect.

Further *cis-trans* tests of the A19K suppressors should serve to eliminate some of the proposed structural compensation mechanisms. However, the complex suppression behaviors in this relatively simple system illustrate the difficulty of reaching explicit molecular explanations for second-site reversion effects. To do so really requires accompanying high-resolution structural information for the original mutant protein, for its suppressor alteration, and for the doubly mutant revertant protein.

## Conformational Suppression Between Receptor Molecules

Initial genetic evidence for collaborative signaling interactions between receptors of different types came from studies of Tsr trimer contact mutants that had amino acid replacements in residues thought to promote trimer-of-dimer formation (Ames *et al.*, 2002). The principal trimer contact residues are identical in all *E. coli* MCPs and most single amino acid changes at any of these positions abolished receptor function. However, some Tsr trimer contact mutants exhibited interesting functional effects when coexpressed with wild-type aspartate receptors: some regained the ability to mediate chemotactic responses to serine (rescuable Tsr defects); others blocked Tar function (epistatic Tsr defects) (Fig. 4A,B). The rescue and epistasis effects are consistent with the idea that receptors function in mixed, higher-order signaling teams. In this view, rescuable receptors benefit from association with normal team members, whereas epistatic members spoil the function of the entire team. Presumably, the conformations and/or dynamic motions of the mixed receptor teams are instrumental in both effects. Accordingly, we reasoned that it might be possible to find mutant forms of Tar (designated Tar^) that ''rescued'' the function of epistatic Tsr defects (designated Tsr*) by imparting a compensatory conformational change to a mixed Tar^/Tsr* signaling team.

To look for Tar^ suppressors of Tsr* defects, we induced random mutations in a wild-type Tar expression plasmid by passage through a *mutD* host. Independently mutagenized plasmid pools were transformed

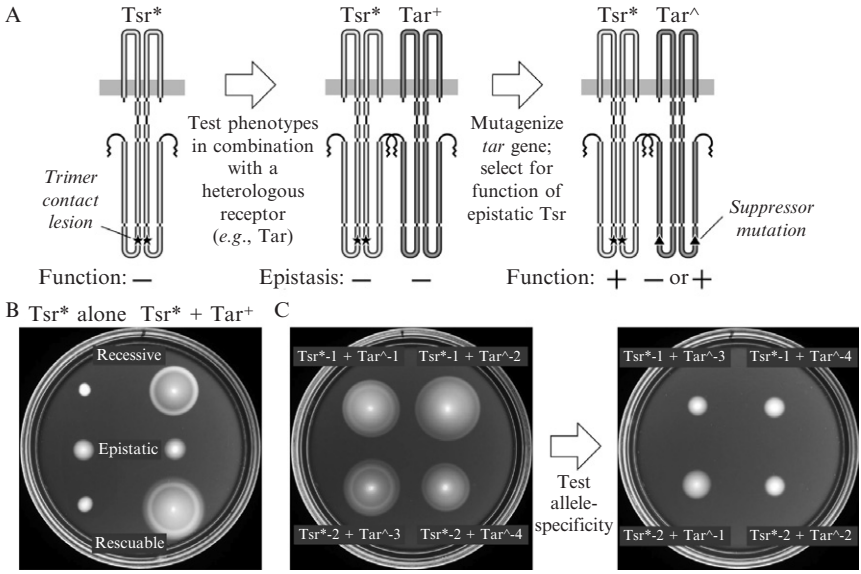


FIG. 4. Intergenic, conformational suppression of epistatic trimer contact lesions. (A) Scheme for selecting mutant Tar receptors (Tar^) that suppress epistatic Tsr trimer contact defects (Tsr*). Cells expressing Tsr* and Tar$^+$ receptors cannot mediate chemotactic responses to either serine or aspartate. Tar^ receptors were selected from such cells as Tsr$^+$ pseudorevertants on tryptone soft agar. Some Tar^ receptors exhibited Tar function in the presence of Tsr* receptors; others did not. (B) Phenotypes produced by Tsr* receptors alone and in combination with wild-type Tar receptors. Recessive Tsr* lesions (illustrated here by a vector control) have no effect on Tar function; rescuable Tsr* lesions (Tsr-R388A, in this example) regain Tsr function in the presence of wild-type Tar; epistatic lesions (Tsr-F373W, in this example) block the function of wild-type Tar molecules. The plate was incubated at 32.5° for 10 h. (C) Phenotypes and allele-specificity of Tar^ Tsr* suppression effects. Mutations: Tsr-F373W (Tsr*-1); Tsr-L380A (Tsr*-2); Tar-A380V (Tar^-1); Tar-V397M (Tar^-2); Tar-E389G (Tar^-3); and Tar-R386H (Tar^-4). The host strain (UU1250) carried chromosomal deletions of all MCP genes; Tar^ and Tsr* were supplied from compatible, independently regulatable expression plasmids. The plates were incubated at 32.5° for 10 h.

en masse into a receptorless recipient strain carrying a compatible, independently regulatable Tsr* plasmid and chemotactic cells in the transformation mix were selected as flares from a cell streak on a soft agar plate, as illustrated in Fig. 1D. The Tar^ plasmids were then purified from independent revertants and further characterized.

In all, we obtained 19 different Tar^ suppressors from four different Tsr* mutations. Ten more epistatic lesions at other Tsr trimer contact residues yielded no Tar^ suppressors. We subsequently showed that none

of the recalcitrant Tsr* mutations were suppressible by any of the Tar^ plasmids, implying that their structural defects were somehow different from those of the suppressible Tsr* receptors. All Tar^ Tsr* revertants had regained serine chemotaxis ability; some had also regained aspartate responsiveness, whereas others were still defective for aspartate chemotaxis. These findings implied, consistent with our working hypothesis of collaborative signaling in trimer-based receptor teams, that single-step mutational changes cannot create ''epistasis-resistant'' Tar proteins, whereas they can create Tar alterations that correct Tsr* functional defects, often with a concomitant loss of Tar function.

The amino acid changes in all Tar^ receptors, inferred from DNA sequence analysis of the mutant plasmids, occurred within or very close to the trimer contact region of Tar, including three with alterations at a trimer contact residue. Not surprisingly, all 19 Tar^ mutants exhibited functional defects similar to those of their Tsr* counterparts. In the absence of their Tsr* partner, most Tar^ receptors were defective in promoting aspartate chemotaxis. Moreover, most of the nonresponsive Tar^ receptors exerted a strong epistatic effect on wild-type Tsr function. Presumably, the similar functional alterations of Tar^ and Tsr* receptors arise through similar structural alterations of trimers of dimers.

To test the proposition that Tar^ suppressors act by creating a compensatory conformational change in a mixed Tar^ Tsr* signaling team, we tested the allele specificity of Tar^-Tsr* interaction. Allele specificity is the litmus test for conformational suppression between directly interacting proteins, reflecting the underlying stereospecificity of the interaction. Accordingly, we tested each Tar^ receptor for ability to restore function to each of the four suppressible Tsr* receptors. The suppression pattern revealed six major interaction classes: three groups of Tar^ suppressors acted productively with only a single, different Tsr* partner; another two groups suppressed different pairs of partners; and one Tar^ group suppressed three of the four Tsr* receptors. Some examples of the test results are shown in Fig. 4C. Tar-A397M (Tar^-2) and Tar-R386H (Tar^-4) represent two of the highly specific suppressor classes, acting, respectively, on Tsr-F373W (Tsr*-1) and Tsr-L380A (Tsr*-2). Tar-A380V (Tar^-1) and Tar-E389G (Tar^-3) represent the two group-specific suppressor classes. Their Tsr* target groups are represented by Tsr-F373W and Tsr-L380A, respectively.

The Tar^-Tsr* test matrix showed that many Tar^ suppressors discriminated among Tsr* changes at different residue positions (e.g., F373 and L380). However, true allele-specific suppressors should also be able to distinguish different amino acid changes at the same position in an interacting protein. To explore the side-chain specificity of Tar^-Tsr*

suppression, we isolated additional epistatic lesions by all-codon mutagenesis of Tsr residues F373 and L380, then compared the suppression patterns of the new epistatic alleles with those of the original Tsr* alleles (F373W and L380A) (Ames, unpublished results). For both residues, some of the Tar^ alleles that suppressed the Tsr* prototypes also suppressed some of the new epistatic lesions at the same residue position. The F373W suppressors fell into two groups, one that could suppress F373K and F373M and one that could not. The L380A suppressors fell into three groups, one that could suppress L380F, L380M, L380Q, and L380Y; a second that could suppress all of these except L380M; and a third group that could not suppress any of the new alleles. None of the new Tsr* alleles were suppressible by a Tar^ allele that could not act on the corresponding Tsr* prototype. However, if we were to repeat the suppressor selection by starting with the new Tsr* alleles, we would expect to find novel Tar^ alleles that would not suppress the original Tsr* mutations.

The highly specific pattern of Tar^-Tsr* suppression effects, both with respect to residue position and side-chain character, is fully consistent with the receptor team hypothesis. The location and nature of the various residue changes provide tantalizing clues about the underlying suppression mechanism(s). Productive Tar^-Tsr* combinations might arise through two types of compensatory structural interactions. Bulky amino acid replacements at a Tsr trimer contact site (e.g., F373W) that most likely distort the trimer interface might require a correspondingly reduced side-chain volume in their Tar^ partner to restore a functional trimer shape. In contrast, a small amino acid replacement at a Tsr trimer contact site (e.g., L380A), would most likely destabilize the trimer, perhaps increasing its dynamic motions. Tar^ alterations that enhance dimer–dimer packing interactions or that reduce receptor dynamics might correct these sorts of Tsr* defects. Only structural studies of the interacting proteins and their complexes can eliminate this guesswork.

### *General Guidelines for Intermolecular Conformational Suppression Studies*

In theory, any direct protein–protein interaction should be amenable to conformational suppression analysis. One needs only a strong selection for restored function and methods for analyzing the genetic changes in the revertants. However, the success of such endeavors is critically dependent on starting with the right mutants; some of them must carry lesions that affect a contact surface for the interaction. Alterations that do not cause a structural change at the site of protein–protein interaction are not likely to be conformationally suppressed by a structural change in a partner protein. The key to a successful suppression study is to focus on the types of mutants

that are most likely to have interaction site lesions. Most randomly isolated loss-of-function mutations will not fall within protein–protein interaction determinants, but rather at the many sites critical for proper protein folding, maturation, and stability. Thus, mutant proteins that do not have wild-type expression level and stability should be excluded from the reversion analysis. They may yield revertants that have a second-site suppressor mutation, but they will probably not identify an interacting partner protein.

The second key to a successful conformational suppression study is to work with a large enough set of suppressible mutations and suppressors to build a compelling case for allele-specificity. The mutation set can be expanded in several ways. A putative interaction determinant that has been identified by a suppressible mutation can be explored by constructing additional mutational changes that may affect the same docking surface and isolating suppressors of those mutations as well. Obviously, knowledge about the structure of the starting protein is helpful, but not essential, in guiding such approaches. It is also possible to expand the mutation set by using any suppressor mutations that are functionally defective with a wild-type partner as the starting point for additional rounds of reversion (Liu and Parkinson, 1991).

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