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Large Roles of Small Proteins

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Abstract

Bacterial proteins of ≤50 amino acids, denoted small proteins or microproteins, have been traditionally understudied and overlooked, as standard computational, biochemical, and genetic approaches often do not detect proteins of this size. However, with the realization that small proteins are stably expressed and have important cellular roles, there has been increased identification of small proteins in bacteria and eukaryotes. Gradually, the functions of a few of these small proteins are being elucidated. Many interact with larger protein products to modulate their subcellular localization, stabilities, or activities. Here, we provide an overview of these diverse functions in bacteria, highlighting generalities among bacterial small proteins and similarly sized proteins in eukaryotic organisms and discussing questions for future research.

Keywords

microproteins; ion transport; antibiotic resistance; competence; sporulation; degradation

INTRODUCTION

Proteins, the fundamental building blocks of life, play a pivotal role in driving all biological processes. However, most techniques for protein analysis, such as gel electrophoresis and mass spectrometry, have been biased toward larger-molecular-weight proteins. Consequently, small proteins of ≤50 amino acids often have not been detected in biochemical studies. Due to challenges in annotation, they also have been dismissed as noise in bioinformatic analyses. Once identified, the characterization of small proteins is additionally hampered by limitations in obtaining tagged derivatives that are functional.

Nevertheless, small protein discovery is rapidly expanding due to genome reevaluation and advances in technology such as transcriptome-wide analysis of ribosome association with RNA (denoted ribosome profiling or ribo-seq). Hundreds of previously overlooked small proteins have been identified in a wide variety of bacteria (reviewed in 33). Similarly,

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there has been a dramatic increase in the discovery of proteins of ≤100 amino acids, frequently denoted microproteins (reviewed in 95), in eukaryotes. With the identification of numerous new small proteins, there also has been increasing evidence that these small proteins/microproteins have regulatory activities, often part of feedback loops, in diverse biological processes. However, despite these advances, the molecular characterization of small proteins is challenging.

Here we summarize the general functions that have been uncovered for small proteins in bacteria to set the stage for further characterization of this important group of molecules (Supplemental Table 1). We focus on what has been discovered for bacterial proteins but point out parallels to functions found for microproteins in eukaryotes. For the purposes of this discussion, we are grouping the small proteins into categories but acknowledge that some proteins may ultimately be miscategorized or belong to more than one group, since information about function and mechanism of action is still incomplete. Many small proteins with a known function form a single α-helix that can be a single transmembrane helix or an amphipathic helix; we first turn our attention to these proteins.

PROTEIN RECRUITMENT TO MEMBRANES

The proper subcellular localization of proteins or protein complexes can be critical to their activities. One function of small proteins is the recruitment of larger proteins to membranes.

Recruitment by an Amphipathic α-Helix Protein

Amphipathic α-helices, which lie parallel to and partially embedded in membranes, are emerging as one recruitment mechanism (Figure 1a).

SpoVM anchor for spore coat formation.—*Bacillus subtilis* employs sporulation as a developmental strategy to survive in adverse conditions, forming metabolically dormant spores that resist stressors like UV radiation and heat (reviewed in 87). A player in this process is SpoVM, a 26–amino acid protein expressed during sporulation, which was identified through genetic screens for sporulation defects (63, 98). Mutations in *spoVM* led to immature spores stuck at sporulation stage IV-V with compromised cortex and coat development as well as heightened sensitivity to heat, organic solvents, and lysozyme (63).

SpoVM is a kinked, amphipathic α-helix with one positively charged surface, one hydrophobic surface, and an internal proline residue (80). Remarkably, SpoVM preferentially associates with convex membranes via its hydrophobic residues (78, 85). Individual SpoVM proteins rapidly bind the positively curved membranes and then recruit additional SpoVM molecules in a manner termed dash-and-recruit (56). This allows the small protein to sense a larger surface despite its small size. Once bound, SpoVM acts as a binding site for SpoIVA, an ATPase, which upon ATP hydrolysis forms the spore basement layer (78, 84). In *Clostridium difficile*, another spore-forming bacterium, SpoVM also binds SpoIVA, but the lack of SpoVM has only a minor effect on spore formation (86), raising questions about the reasons for the differential requirement for the small protein.

AzuC modulator of GlpD activity and membrane association.—Like SpoVM, the 28–amino acid *Escherichia coli* protein AzuC is an amphipathic α-helix containing proline residues (44, 82). AzuC was shown to associate with the membrane in both cell fractionation experiments and microscopic detection of a green fluorescent protein (GFP)-tagged derivative. The introduction of charged residues into the hydrophobic face of the protein eliminated the membrane association (82). Expression of AzuC is repressed by the cyclic AMP receptor protein (CRP), such that AzuC levels are highest when the preferred carbon source glucose is present (44). Additionally, AzuC levels are high in cells grown in low-pH medium; some of this induction is through CRP, though other regulatory factors also appear to be involved (44).

Consistent with the regulation, copurification and biochemical assays revealed that AzuC binds the aerobic glycerol 3-phosphate dehydrogenase GlpD (82). Assays of cell extracts showed that lysates from wild-type cells had higher GlpD activity than those from Δ*azuC* cells. This increased activity is likely due to GlpD recruitment to the membrane given the increased membrane association of fluorescently tagged GlpD in wild-type cells compared with Δ*azuC* cells and previous reports that full GlpD activity requires cytoplasmic membranes or reconstitution with phospholipids or other amphipathic compounds (89, 100). The specific residues involved in AzuC binding to GlpD are still unknown. It is also not known whether AzuC preferentially associates with specialized membranes like SpoVM. The observations that the similarly sized *B. subtilis* SpoVM and *E. coli* AzuC proteins have comparable functions suggest that recruitment of proteins to the membrane may be a widespread role of small, amphipathic α-helix proteins.

The AzuC RNA also acts as a base-pairing sRNA (AzuR) that represses target gene expression and, in fact, was first identified as a regulatory sRNA (19). Indeed, sRNAs that harbor a small open reading frame are increasingly being reported and are commonly termed dual-function RNAs (reviewed in 3, 83). AzuC is not broadly conserved, raising the possibility that the gene arose de novo, whereby a randomly occurring small open reading frame becomes expressed and retained because it confers a selective advantage (reviewed in 92, 126).

Recruitment by a Small Transmembrane Protein

An integral membrane protein that traverses the cell membrane can be another mode by which a small protein can recruit larger proteins to the membrane (Figure 1b).

Prli42 anchor for stressosome complex.—This second mechanism is illustrated by the 31–amino acid Prli42 protein of *Listeria monocytogenes*. Prli42 was identified in a screen for translation initiation sites by N-terminal combined fractional diagonal chromatography (COFRADIC) and is quite broadly conserved in *Firmicutes* (now denoted *Bacillota*) (49). The small protein resides in the membrane and, through basic amino acids in the cytoplasmic N terminus, interacts with acidic glutamate residues in the stressosome protein RsbR (49, 118). Super-resolution microscopy showed Prli42 and RsbR colocalize (106). These data support the model that Prli42 specifically recruits the RsbR–RsbS–RsbT stressosome complex to the membrane in response to stress, thereby activating the complex.

However, how activation occurs in response to stress, whether Prli42 is needed for only some stress responses, and whether Prli42 performs roles in addition to simply recruiting RsbR warrant further study. Two proposed hypotheses are that Prli42 forms a pore, which allows a signal to be transduced to the stressosome, and that Prli42 interacts with a channel, thereby colocalizing the stressosome to the position of signal import. There is still much to learn about Prli42, and there are likely other small single-transmembrane proteins that help recruit proteins to the membrane or anchor them there.

REGULATION OF PROTEIN STABILITY

Regulated protein degradation is an important mechanism for maintaining proper protein concentrations. Degradation also serves to remove proteins and conserve resources when the proteins are no longer needed or are misfolded. Nevertheless, given that the process is irreversible, degradation must be controlled. One form of regulation comes from protease adaptors, which form a ternary complex with the protease and substrate and allow for the selective degradation of specific substrates (reviewed in 67). There also are proteins that block degradation directly or block adaptor function, so-called antiadaptors. While much smaller than the proteases and the proteins targeted for degradation, the adaptors and antiadaptors characterized thus far generally fall outside the range of ≤50 amino acids. However, even smaller-sized proteins are emerging as potent regulators of protein degradation and may have similar adaptor and antiadaptor functions.

Modulating the Membrane Protease FtsH

The membrane protease FtsH degrades many membrane-associated proteins. Among the FtsH substrates is the *B. subtilis* SpoVM protein discussed above, and high levels of SpoVM can block the degradation of FtsH substrates, hinting at a possible second role for this 26–amino acid protein (23). However, a major function of three enterobacterial small proteins that are composed of a single transmembrane α-helix is to block or promote degradation by FtsH by binding to proteins that affect magnesium (Mg^{2+}) homeostasis (Figure 2a).

MgtR promotion of FtsH-mediated degradation of magnesium transporters.— Mg^{2+} is an essential enzyme cofactor that also stabilizes nucleic acids and macromolecular complexes and influences membrane permeability (reviewed in 41). Host cells take advantage of this need for appropriate Mg^{2+} concentrations by strategically limiting Mg^{2+} availability as a defense mechanism. One of the proteins induced in response to low Mg^{2+} in *Salmonella enterica* is the 30–amino acid protein MgtR, which is encoded in an operon with the MgtB Mg^{2+} importer and the MgtC virulence factor. Although the three inner membrane proteins are cotranscribed, during periods of Mg^{2+} starvation, MgtC was not detected by Western blot analysis despite the robust transcriptional induction via the low-Mg^{2+}-responsive PhoQ–PhoP two-component system (73). However, MgtC was seen in strains with a deletion of the *mgtR* region. This observation, together with findings that MgtR interacts with MgtC in vivo and that the presence of MgtR promotes MgtC degradation through FtsH (1), suggested that MgtR has an adaptor-like function for controlling MgtC levels.

MgtR also exerts posttranslational control over the activity of the MgtA Mg^{2+} importer through direct binding (21). Additionally, MgtR interacts with the MgtA paralog MgtB, an interaction that is contingent on the N terminus of MgtR (124). Although not tested directly, the prediction is that MgtR is acting as an FtsH protease adaptor for MgtC and the Mg^{2+} importers. These findings raise interesting mechanistic questions, such as how is MgtR promoting FtsH-mediated cleavage and how can a 30–amino acid protein recognize the different MgtC, MgtA, and MgtB proteins.

MgtU and MgtS inhibition of FtsH-mediated degradation of magnesium transporters.—Another small *S. enterica* protein, MgtU, which is 28 amino acids in length and is encoded downstream of *mgtR* on the *mgtBCRU* transcript, was identified through ribosome profiling and was found to bind and protect MgtB against FtsH-mediated degradation (124). This protective function is required for maintaining proper MgtB levels, which in turn are crucial for survival within macrophages and during periods of oxidative stress. How and why one small protein has a negative effect on stability while another small protein encoded in the same operon has a positive effect on stability are not clear.

MgtR and MgtU are not encoded by closely related *E. coli* strains. However, another small protein, the 31–amino acid protein MgtS, originally identified on the basis of nucleotide conservation and found to fractionate with the membrane (45, 116), protects *E. coli* MgtA against FtsH degradation. A role for MgtS in regulating Mg^{2+} homeostasis was first suggested by the observation that MgtS levels are strongly induced in low-Mg^{2+}-growth conditions in a PhoQ–PhoP-dependent manner (114). The finding that growth was reduced in low-Mg^{2+} medium allowed for the identification of a functionally tagged derivative of MgtS, which in turn was used to identify MgtA and FtsH as interacting proteins. Follow-up experiments examining MgtA levels in *mgtS* deletion and overexpression strains indicated that MgtS protected MgtA against FtsH-mediated degradation (114).

An observation that MgtS overexpression also led to the induction of genes by low phosphate through the PhoR–PhoB two-component system led to the discovery that MgtS also interacts with the PitA symporter (125). While PitA is annotated to be a phosphate transporter, MgtS blocks Mg^{2+} leakage through this protein. Although MgtS is only 31 amino acids long, the determinants for MgtA and PitA binding are at least partially distinct, but as for MgtR, it is not known how such a small protein can interact with different larger target proteins.

The findings that three different small proteins bind to the MgtA and MgtB P-type ATPase Mg^{2+} importers are interesting given that a closely related mammalian P-type ATPase is the sarcoendoplasmic reticulum calcium ATPase (SERCA), whose activity is also regulated by multiple small hydrophobic α-helical proteins: sarcolipin (31 amino acids), myoregulin (46 amino acids), DWORF (35 amino acids), and phospholamban (52 amino acids) (reviewed in 120). This pattern suggests that additional small protein regulators of P-type ATPases, and possibly other transporter families, are likely to be found and, given that P-type ATPases transport various critical ions, raises the possibility that small proteins present therapeutic opportunities.

Modulating Cytoplasmic Clp Proteases

Two small proteins in *B. subtilis* regulate the cytoplasmic Clp AAA+ family of proteases to affect two different biological processes.

CmpA promotion of SpoIVA degradation in sporulation quality control.— Another small protein that modulates *B. subtilis* sporulation is the 37–amino acid cortex morphogenetic protein A (CmpA) (28). The cortex functions as the spore's protective wall (reviewed in 6). CmpA was initially identified in a screen for suppressors capable of cortex formation in a cortex-deficient *spoVM* mutant strain (28, 99). Co-immunoprecipitation assays with tagged CmpA, SpoIVA, and ClpXP showed that these components formed a complex in vivo (Figure 2b). The deletion of CmpA also stabilized SpoIVA in the *spoVM* sporulation mutant background. On the basis of these observations, it was proposed that CmpA functions as an adaptor for ClpXP, promoting the degradation of SpoIVA in cells that exhibit flawed spore envelope assembly (104). It is unclear whether SpoIVA, CmpA, and ClpXP are sufficient to reconstitute the degradation in vitro or whether additional factors are required. Adaptors often deliver multiple cargo proteins to their dedicated proteases. Whether CmpA interacts with and delivers other substrates to ClpXP is another outstanding question that warrants further exploration.

ComS inhibition of MecA–ClpC degradation of the ComK competence regulator.—Bacterial competence allows bacteria to obtain external DNA that can be used as genetic material or as a source of nutrients (reviewed in 5). Some bacteria, such as *B. subtilis*, have high levels of natural competence. In *B. subtilis*, this is achieved in a subpopulation during stationary phase under specific nutrient conditions (reviewed in 50). A major player in the regulation of the competence state is the 46–amino acid protein ComS, encoded by the operon required for surfactin synthesis but, unusually, out-of-frame within *srfA* (24, 42). The mRNA encoding SfrA and ComS is transcribed in response to quorum sensing of bacterial cell density when the ComP–ComA two-component system, along with other regulators, is activated by the ComX pheromone (90, 102).

The initial characterization of ComS showed that the small protein releases the ComK transcription factor from the MecA adaptor–ClpC protease complex, thereby protecting ComK from degradation and allowing the transcription factor to bind DNA (107, 108). Further biochemical studies showed that both ComS and ComK interact with the N terminus of MecA (76, 79, 108). These findings suggest that ComS acts as an antiadaptor for ComK. Another small *B. subtilis* protein, the 30–amino acid protein ComI, encoded on a plasmid found in undomesticated strains of *B. subtilis* as well as in *Enterococcus* and *Streptococcus* species, limits competence though the mechanism is unknown (61).

The CmpA and ComS examples in *B. subtilis* prompt us to ponder how many other small proteins modulate proteolysis at different levels in all organisms. A recent screen for synthetic small proteins that prevent MazF toxicity led to the identification of random toxin of MazF (RamF), a 51–amino acid protein that titrates chaperones away from MazF, allowing the protein to be degraded (32).

We have focused on small proteins that modulate proteases and protein cleavage. However, recent studies of the 39–amino acid proteins SR1P and SR7P encoded by dual-function RNAs in *B. subtilis* showed that these small proteins bind components of the RNA degradosome and affect RNA cleavage (reviewed in 109). It is likely that other small proteins that modulate RNA stability by binding RNA or ribonucleases remain to be discovered.

ROLE IN COMPLEX ASSEMBLY AND STABILITY

The precise assembly of protein subunits is required to produce functional complexes. In this section, we discuss small proteins that are thought to play roles in complex assembly. While the proteins have been proposed to assist with assembly or to serve as structural components, it is also possible that they might function to regulate the activities of these larger complexes.

Stabilization of a Potassium Pump

Like Mg^{2+}, potassium (K^+) is crucial for the survival and optimal physiological function across all life forms. In *E. coli*, the KdpF–KdpA–KdpB–KdpC complex, another P-type ATPase, plays a pivotal role in preserving K^+ homeostasis, particularly under conditions of low external K^+ concentration (reviewed in 9, 14). Given that the 29–amino acid hydrophobic protein KdpF is encoded in the *kdpFABC* operon, it was perhaps not surprising that the small protein is part of the purified KdpA–KdpB–KdpC complex (35). The in vivo activity of the Kdp complex lacking KdpF was similar to that of the wild-type complex. However in vitro, the purified Kdp complex lacking KdpF had significantly lower activity, which could be complemented by the addition of purified KdpF (35). These observations led to the suggestion that the small protein has a role in stabilizing the Kdp complex, but further study of the KdpF function is warranted. Homologs of KdpF are associated with only some Kdp complexes (35), raising the issue of why a stabilizing protein may be needed for only some complexes or whether nonhomologous small proteins with analogous roles remain to be identified.

Components of Cytochrome Oxidase Complexes

Cytochrome oxidase complexes, the terminal enzymes of respiratory chains, provide an example of how different single-transmembrane domain α-helix proteins can be associated with the same complexes in a variety of bacteria. The first hint that small proteins could be part of cytochrome oxidases came from the observation that the 37–amino acid protein CydX and the 30–amino acid protein AppX, whose expression is induced under anaerobic conditions (44), are encoded in the same operons as the CydAB and AppBC cytochrome *bd* oxidases in *E. coli*. Strains lacking CydX had phenotypes similar to the strains lacking the CydA and CydB proteins, and copurification experiments documented that CydX interacts with the larger CydA and CydB proteins (110). The lack of CydX also leads to the loss of the di-heme active site, indicating CydX is needed for the assembly or stability of the di-heme center (48). Remarkably, structural studies of the CydAB cytochrome *bd* complex revealed that the complex contains an additional small subunit, the 29–amino acid protein CydH (also called CydY) (94, 105). The role of CydH is not clear. The small protein is

in a position to block an oxygen channel; however, no phenotypes related to cytochrome oxidase activity have been found for the lack of this protein subunit (38). Possibly, there is functional redundancy or CydH has a condition-specific role given that it is encoded at a genomic location separate from *cydABX*. Further, the AppX small protein is in the same position as CydX in the structure of the AppBC cytochrome *bd* oxidase, highlighting a possible conserved role of AppX and CydX in securing heme binding (40). Consistent with this observation, AppX can complement a *cydX* deletion when overexpressed (110).

Small protein genes associated with cytochrome *bd* oxidase operons can be detected in a broad swath of proteobacteria (2), and some of these proteins are important for oxidase function in *Brucella abortus* and *Shewanella oneidensis* (18, 103) and are associated with the larger cytochrome oxidase subunits in *Campylobacter jejuni* (31). However, the sequences of these small proteins can differ significantly, such that the core features required for activity are still not well understood, a conundrum that is underscored by the findings that *E. coli* CydX can be mutated extensively and still retain function (47).

Structural studies of the *Pseudomonas stutzeri* enzyme revealed that the 36–amino acid protein CcoM is associated with a cytochrome *c* oxidase (59). CcoM is not encoded within the same operon as the other oxidase subunits and its molecular function is not clear, but strains lacking the small protein show a significant growth lag in anaerobic denitrifying conditions. Another and slightly larger (62 amino acids) single-transmembrane domain protein, CcoQ, binds the *P. stutzeri* cytochrome *c* oxidase complexes as well (58). This protein has been proposed to be required for the assembly of the complexes but not for their activities. Two different single-transmembrane domain proteins interact with mammalian mitochondrial cytochrome oxidase complexes and regulate their activities (reviewed in 3), and many similarly small single-transmembrane domain proteins are components of photosystem complexes in a variety of organisms (39). We suggest that there is still much to discover about the presence and roles of small proteins in the large protein complexes critical for energy conversion.

MODULATION OF MEMBRANE PROTEIN ACTIVITY

In addition to modulating the stabilities and assemblies of membrane proteins, small proteins that are induced in response to specific environmental conditions can modulate the activities of transporters and other membrane proteins with functions as transporters or enzymes, in cell division, or in cell signaling.

Regulation of Transporter Activity

Stress-induced small proteins modulate various transporters with roles in expelling toxic compounds, facilitating nutrient uptake, and maintaining ion homeostasis.

AcrZ regulator of antibiotic specificity in conjunction with cardiolipin.— Synthesis and membrane association were documented for the 49–amino acid protein AcrZ in a study examining the expression of short open reading frames in the *E. coli* genome (45). Follow-up analysis showed that *acrZ* transcription is induced by the MarA, Rob, and SoxS transcription factors, which respond to a variety of toxic compounds. Additionally, the

tagged protein copurifies with AcrB, the inner membrane component of the AcrAB–TolC multidrug efflux pump, which is a member of the resistance-nodulation-division (RND) family of transporters (46). The genes for the components of the efflux pump are encoded distant from *acrZ*, but the *acrAB* operon is also regulated by MarA, Rob, and SoxS. The observation that cells lacking AcrZ had increased sensitivity to some but not all the antibiotics effluxed by AcrAB–TolC suggested that AcrZ regulates the activity or specificity of the pump (Figure 3a). Consistent with this suggestion, structural analysis revealed that AcrZ binding leads to an allosteric change in the drug binding pocket (25). The structure along with mutational analysis revealed that a proline-induced kink in the transmembrane α-helix is important for AcrZ activity.

Mutating AcrZ is synergistic with the effects of eliminating cardiolipin synthesis, indicating that the small protein acts in conjunction with the lipid environment (25), which also may be true for other small proteins acting on inner membrane proteins. Given that the family of RND transporters is large, we speculate that other members are also modulated by the binding of a small protein.

SgrT repression of glucose transport.—The *E. coli* 43–amino acid protein SgrT is encoded by the SgrS regulatory RNA (113), another example of a dual-function RNA (reviewed in 3, 83). Transcription of the *sgrST* RNA is induced in response to the presence of toxic sugar phosphates through derepression of the SgrR repressor encoded divergent from the dual-function RNA. The RNA base pairs with and regulates several target mRNAs, including the *ptsG* mRNA encoding a phosphoenolpyruvate phosphotransferase, to reduce intracellular sugar phosphate levels (reviewed in 11). SgrT, which does not have a transmembrane α-helix, colocalizes with the PtsG protein and inhibits the transporter (66). Thus, the regulatory RNA and the small protein function at two different levels to block PtsG, although time course experiments in *S. enterica* indicate that the RNA component acts before the small protein (8). Other assays showed that SgrT interacts with the linker and N-terminal domain of dephosphorylated PtsG (62, 66), but further work, including structural studies, are required to more fully elucidate the consequences of SgrT binding to PtsG.

MntS repression of manganese export.—Manganese (Mn) in various oxidation states is another metal that is needed for growth but is toxic at high levels. Consequently, Mn import and export are extensively regulated in bacterial cells (reviewed in 12). Among the regulators is the 42–amino acid protein MntS, initially identified on the basis of nucleotide sequence conservation (116). MntS is expressed in cells with low Mn levels but is repressed by MntR, encoded divergent from *mntS*, when Mn levels increase (117). This regulation and the observation that MntS overexpression leads to increased intracellular Mn levels (68) and Mn toxicity (117) suggested that MntS plays a role in Mn homeostasis. Subsequent studies showed that the detrimental effects of MntS overexpression require the Mn exporter MntP, to which MntS was shown to bind (119). These observations led to the model in which MntS blocks Mn export during transitions to low Mn. This results in a feedback loop whereby low Mn leads to MntS induction; increased levels of MntS inhibit MntP, reducing Mn export and increasing intracellular Mn levels, which ultimately lead to MntR-mediated repression

of *mntS*. At high intracellular Mn levels, MntS binds to itself, suggesting a potential shut-off mechanism when Mn concentrations increase.

MntS, which also does not have a transmembrane α-helix, shares an ancestral relationship with a signal peptide sequence present in certain SitA proteins, the Mn-binding subunits of the SitA–SitB–SitC–SitD Mn importer system, from a subset of organisms (119). In fact, the corresponding signal peptides can substitute for MntS in functional assays of the small protein. These discoveries raise the possibilities that other signal peptides may have cellular functions after being cleaved from larger membrane proteins and that other small protein regulators may have evolved from the truncation of a gene encoding a larger protein.

Regulation of Cell Division

Components and regulators of the machinery required for cell division, the divisome, are found in the inner membrane (reviewed in 17), representing an inviting target for small single-transmembrane domain proteins.

SidA inhibition of FtsW to block cell division.—One small protein that regulates cell division is SidA, a 29–amino acid inner membrane protein, identified because its expression is strongly induced by DNA damage as part of the SOS response in *Caulobacter crescentus* (72). SidA interacts with the FtsW membrane protein, blocking constriction of the cell division ring, a late step in cell division (72). Another and slightly larger protein of 71 amino acids, DidA, which also is induced by DNA damage but independent of the SOS response, inhibits cell division, in this case by binding FtsN, another component of the FtsW–FtsI–FtsN membrane complex. From a physiological point of view, it is advantageous to block or slow down cell division to provide time for DNA damage repair pathways to act and prevent deleterious propagation of DNA-damaged chromosomes. Thus, we predict that additional stress-induced single-transmembrane domain regulators of cell division will be discovered.

Regulation of Membrane-Associated Enzymes

While small proteins can be integral parts of large enzymatic complexes as described above, they can also regulate these membrane-associated enzymes.

PmrR inhibition of LpxT modification of the outer membrane.—Lipopolysaccharide (LPS), composed of lipid A, a core oligosaccharide, and the O-antigen polysaccharide chain, is a critical component of the outer leaflet of the outer membrane of gram-negative bacteria (reviewed in 101). LPS serves as a protective barrier but is also recognized by the host immune system and can be modulated in response to the environment. In *S. enterica*, the high-iron (Fe^{3+})-responsive PmrA–PmrB two-component system plays a role in regulating LPS modification (reviewed in 37). Among the genes upregulated by PmrA is *pmrR*, which encodes a 30–amino acid protein (55). The characterization of PmrR revealed that the small protein is membrane associated and interacts with and inhibits LpxT, a protein responsible for introducing an additional phosphate group to lipid A. The overexpression of PmrR led to decreased levels of the phosphorylated lipid A produced by LpxT (Figure 3b). These data support a negative

feedback loop model whereby the PmrA–PmrB two-component system is activated by Fe^{3+}, leading to induction of PmrR, which inhibits LpxT, reducing the negative charge and consequently Fe^{3+} binding to the cell, thus deactivating PmrA–PmrB (55).

In another synthetic library screen of random hydrophobic proteins of <50 amino acids, several of the synthetic peptides that conferred resistance to the antibiotic colistin [de novo colistin resistance (Dcr) peptides] bind to the PmrB response regulator (57). This interaction leads to the activation of the PmrA–PmrB two-component system and, again, to a change in the LPS, which reduced colistin uptake. These observations indicate that a variety of hydrophobic α-helices can affect the composition of the membrane.

Regulation of Sensor Kinases

We have already mentioned several two-component systems frequently composed of a sensor kinase in the membrane, which, in response to specific environmental signals, phosphorylates and activates a response regulator, usually a transcription factor (reviewed in 16). The two-component systems themselves can be regulated at multiple levels, including by small proteins, as already hinted by the synthetic Dcr peptides.

MgrB inhibition of PhoQ kinase.—The hydrophobic *E. coli* 47–amino acid protein MgrB was discovered in a deletion screen for negative regulators of the PhoQ–PhoP two-component system (64). MgrB was then shown to bind PhoQ, inhibiting the activity of the sensor kinase and leading to reduced PhoP phosphorylation (64, 96). This results in a negative feedback loop, since transcription of MgrB itself is induced by PhoQ and PhoP (Figure 3c). MgrB has been studied in some detail through mutational structures, which showed that the transmembrane region of MgrB, particularly Tyr-20, is critical for the interaction with PhoQ (121). The cytoplasmic N-terminal residues Lys-2 and Lys-3 also are important for MgrB activity. Cross-linking and protein dynamic simulations suggest that the MgrB–PhoQ interaction leads to structural rearrangements in PhoQ, which would lead to a movement of the PhoQ kinase domain (52). MgrB-mediated regulation might be particularly important under specific stress conditions, as activation of PhoQ by decreased periplasmic oxidation is dependent on MgrB, particularly on two periplasmic cysteines (65), and the small protein affects PhoQ activation by antibiotics in *E. coli* (52) and in *Klebsiella pneumoniae* (reviewed in 123). A somewhat larger membrane protein of 65 amino acids, SafA, whose expression is controlled by the acid-responsive EvgS–EvgA two-component system, activates PhoQ by binding to the sensor domain and triggering autophosphorylation (reviewed in 122).

UgtS inhibition of PhoQ kinase.—Additional regulators of the PhoQ–PhoP two-component system were found in *S. enterica*, where a transcript encoding the 34–amino acid UgtS and 132–amino acid UgtL membrane proteins also is induced by the PhoQ response regulator (97). Both UgtL and UgtS modulate PhoQ activation, with UgtL enhancing the PhoQ response through binding and promoting autophosphorylation (22) and UgtS antagonizing the effects of UgtL by binding both PhoQ and UgtL (97). UgtS and UgtL have somewhat different expression patterns, with UgtS expressed earlier after induction than

UgtL. At least part of this regulation is due to the presence of two different transcripts, one that can be translated to give both proteins and the other that encodes only UgtL.

Sda inhibition of KinA kinase.—Another sensor kinase regulated by a small protein is KinA of *B. subtilis*, which activates the Spo0A-dependent sporulation cascade in response to nutrient deprivation. The gene encoding the 46–amino acid protein Sda was first identified as a loss-of-function suppressor of a temperature-sensitive *dnaA* mutant, which is unable to sporulate (15). Wild-type Sda curtails the activity of KinA by binding the autokinase domain of KinA, consequently suppressing its autophosphorylation and phosphatase activities to block sporulation (15, 91). Unlike most of the small proteins discussed thus far, Sda forms a helical hairpin on which conserved residues interact with KinA, stabilizing the KinA dimer (91). Given that transcription of *sda* is induced by DnaA, connecting expression to DNA replication, this small protein provides another example of a checkpoint, like SidA, whereby a process is delayed by a small protein to first allow DNA repair to happen. Sda itself is a substrate of the ClpXP protease, providing a mechanism by which the inhibitor can be turned off (93).

How so many different small membrane proteins regulate PhoQ and whether other sensor kinases in addition to PhoQ and KinA are subject to regulation by multiple small proteins remain open questions. Given the hundreds of kinases found in eukaryotic cells, including membrane-associated kinases, it is also worth asking whether small protein regulators will be found for more complex organisms.

REGULATION OF SOLUBLE PROTEIN ACTIVITY

While most small proteins studied thus far are associated with the membrane, some characterized small proteins are found in the cytosol.

Regulators of Transcription Factor Activity

Transcription is a fundamental cellular process that is regulated at multiple levels, including by specific DNA binding factors that either recruit or block RNA polymerase binding to a promoter. There are several examples of very small proteins that regulate the activities of these transcription factors.

SpfP inhibition of the CRP transcription factor.—One example of a small protein regulator of transcription factor activity came from ribosome-profiling data that showed that the well-characterized *E. coli* Spot 42 sRNA is translated to give the 15–amino acid protein SpfP. Transcription of this dual-function RNA is regulated in response to carbon source availability: SpfP–Spot 42 levels are high in cells grown with glucose but repressed by cAMP-CRP when glucose is no longer available. Consistent with this regulation, Spot 42 base pairs with and represses numerous mRNAs encoding transporters and enzymes needed for the metabolism of nonpreferred carbon sources, which are not needed when glucose is available (reviewed in 27). To gain insight into the function of SpfP, researchers expressed the hydrophobic protein with an N-terminal nonnative amino acid that could be modified to allow for purification of the protein (4). Copurification experiments revealed that SpfP associates with CRP, and vice versa. Furthermore, SpfP overexpression led to

the downregulation of CRP-activated genes, particularly targets of Spot 42 base pairing activity such as operons required for galactose or maltose utilization (Figure 4a). The ribosome binding site for SpfP is within a stem in the RNA secondary structure. As a result, SpfP levels increase at elevated temperatures when the secondary structure is more open. This observation led to the model that SpfP reinforces the Spot 42 regulatory loop at elevated temperatures when base pairing is less effective. It was surprising to find that the extensively studied CRP bound a small protein; how many other well-characterized bacterial transcription factors bind difficult-to-detect small proteins of ≤15 amino acids?

Peptide pheromone regulation of regulator activity.—Another example of very small peptide regulators of transcription factor activity comes from the family of small proteins that are processed to give the peptide pheromones that bind the RRNPPA family proteins in gram-positive bacteria (reviewed in 75). The prototypical members of the RRNPPA family of proteins, defined as having a pheromone-binding domain, include the Rap phosphatases from *B. subtilis* and the transcription regulators Rgg from *Streptococcus*, NprR and PlcR from *Bacillus cereus*, and PrgX from *Enterococcus faecalis*. Many of these regulators control the expression of essential cellular processes, including the regulation of virulence, sporulation, biofilm production, competence, and conjugation. Another representative was added when it was realized that the AimR transcription factors from *Bacillus* phages, which control the decision between lysis and lysogeny, are also regulated by processed peptides and share structural homology (reviewed in 13). For all these examples, the bacteria synthesize propeptides of 30 to 60 amino acids, which are commonly encoded adjacent to the regulators or, in the case of *aimR* genes, are overlapping. After synthesis, the proteins are released into the surrounding environment as processed peptides of 5 to 10 amino acids. Once in the external environment, the pheromones are imported by the bacterial cell, where they interact with their respective RRNPPA sensors, thus initiating a regulatory change.

Given the important roles of the RRNPPA sensors, there have been extensive structural studies of how the peptide pheromones bind and of the specificity and the consequences of the binding, possibly setting the stage for how other small proteins might be able to modulate transcription factor activity. For example, binding of the AimP pheromone to AimR induced a conformational change that shifted the positions of the DNA-binding domains, thus preventing AimR binding at the DNA operator site (34). However, despite this mechanistic progress, given the many different RRNPPA family members and corresponding peptide pheromones, there is still much to learn. In *Drosophila*, the proteins of 11 to 32 amino acids encoded by the *pri/tal* locus affect the activity of the Shavenbaby transcription factor (60). Thus, very small proteins might modulate transcription regulators in all organisms.

Regulation of Cell Division

In addition to SidA (and DidA) described above, a cytoplasmic small protein also regulates cell division.

MciZ inhibition of FtsZ.—In most bacteria, the tubulin-like FtsZ protein plays an indispensable role in cell division by forming the Z-ring required for cell constriction (reviewed in 17). In *B. subtilis*, the 40–amino acid MciZ inhibitor of FtsZ activity was identified in a bacterial two-hybrid screen for proteins that interact with FtsZ (43). This study also showed that MciZ prevents FtsZ polymerization by blocking the GTPase activity required for polymerization (43). Given that MciZ is specifically expressed during sporulation, it was proposed that the physiological role of MciZ is to prevent inappropriate Z-ring formation during sporulation (Figure 4b). A fused variant of MciZ and truncated *B. subtilis* FtsZ allowed for the resolution of a crystal structure of this complex, which showed that MciZ interacts with the polymerization interface at the C terminus of FtsZ (10). We suggest that other small protein regulators that similarly block cell constriction in response to specific cellular signals will be discovered.

Regulation of Enzymatic Activity

The activities of many cytoplasmic enzymes are intricately tuned to suit cellular requirements. Regulatory mechanisms include end-product feedback inhibition, activation by small molecules, or posttranslational modifications such as phosphorylation. The binding of small proteins presents one more mechanism.

VcdP activator of citrate synthase.—An sRNA, VcdR, identified in a screen for altered synthesis of the *Vibrio cholerae* toxin (CTX), was unexpectedly long for a base pairing sRNA and was also shown to encode a 29–amino acid protein denoted VcdP, which was validated through tagging and Western blot analysis. As for two other dual-function sRNAs described thus far, AzuC–AzuR and SpfP–Spot 42, transcription of the VcdP–VcdR RNA is also repressed by CRP (112). To identify the mechanism of action of VcdP, the protein was tagged and used in a coimmunoprecipitation assay where it interacted with GltA, citrate synthase. Further characterization unveiled that VcdP activates GltA, in vivo as well as in vitro with synthetic VcdP, potentially mitigating NADH inhibitory activity on this critical enzyme.

Rpn inhibitors of DNA endonucleases.—The development of ribosome profiling, particularly in the presence of antibiotics that trap ribosomes on start or stop codons, allowed for the identification of small protein-coding genes in intergenic sequences and also revealed high levels of translation initiation internal to longer coding sequences, which could lead to the synthesis of small proteins (70). We have already discussed the ComS paradigm of a protein encoded out-of-frame to a larger protein. Examples of small proteins encoded in-frame are the Rpn_S proteins that correspond to the C-terminal end of Rpn_L proteins. The broadly distributed Rpn_L proteins, which have a PD-(D/E)XK phosphodiesterase domain, have DNA endonuclease activity and consequently cause DNA damage and toxicity. However, this toxic activity can be blocked by the corresponding Rpn_S derivatives, which can oligomerize with the longer Rpn_L versions (127).

A high degree of variability in the C terminus region present in both the Rpn_L and the Rpn_S proteins suggested pressure for rapid evolution, and Rpn_L can serve as a defense against phage infection. Although studies of the Rpn_L and Rpn_S proteins are still at an early stage,

we imagine that other examples of domains expressed separately as small proteins that regulate the larger protein remain to be found.

MODULATORS OF CELL DEATH

Finally, this overview of the function of small proteins would not be complete without a brief mention of proteins of ≤50 amino acids that have toxic effects on the cells that encode them or on cells into which they are introduced (Supplemental Table 2). Many of these toxins are a single transmembrane or amphipathic α-helices like many of the proteins described above; however, some poorly understood features of these proteins render them toxic.

Pore Formation in Endogenous Cells

Perhaps the most prevalent of the toxic small proteins are those that are part of toxin–antitoxin systems, specifically type I toxin systems where the antitoxin is an antisense sRNA that blocks the translation of the small toxin (reviewed in 29, 30, 54). In *E. coli*, like in many organisms, a surprising number of genes encode these proteins, whose sizes vary (e.g., 18 to 19 amino acids for the Ibs family, 26 amino acids for ShoB, 29 amino acids for TisB, 35 amino acids for the Ldr family, 41 amino acids for TimP, and 49 to 50 amino acids for the Hok family). Some of these proteins disrupt membranes by forming pores and causing a decline in cellular ATP levels, but exactly how these pores form and whether all these toxins act similarly are not known (reviewed in 29). The roles of these toxin–antitoxin systems also are still not fully understood. They may play a role in allowing bacterial cells to persist in stressful environments or to undergo abortive infection to block the spread of phage infection (reviewed in 29, 54). What is clear is that expression of the toxins is extensively regulated (reviewed in 69). Transcription of some toxins is induced in response to specific environmental conditions. For example, TisB is expressed as part of the SOS response when cells are exposed to DNA damage. There also is increasing evidence of posttranscriptional regulation in addition to the antisense RNAs, including but not limited to extensive RNA secondary structures and ribosome standby sites, where ribosomes are poised to translate the toxic proteins.

Disruption of Heterologous Cells

Two broad classes of small toxic bacterial proteins are released into other cells. The first of these are phage-encoded holins. These proteins vary in size and sequence but all are hydrophobic and are inserted into the bacterial membrane to bring about membrane permeabilization during phage infection (reviewed in 115). The second class is composed of phenol-soluble modulins (PSMs), which also vary in size but are amphipathic α-helices (reviewed in 20). PSMs are secreted by bacteria such as *Staphyloccocus aureus* whereupon they insert into host (eukaryotic) membranes and cause lysis. The eukaryotic defenses against bacteria in turn include proteins such as cysteine-rich cationic defensin proteins of 18 to 45 amino acids and a broad family of antimicrobial peptides, which share properties with the small proteins discussed here. Given the potential applications to combat bacterial infections, the numerous classes of cell-killing small proteins warrant further identification and study.

OUTLOOK

This review has provided an overview of the diverse functions that small proteins of ≤50 amino acids can have in the cell. In the process, we have described a variety of approaches used to identify the small proteins, ranging from conservation to ribosome profiling and unexpected density in a structure. We have also provided examples of small proteins encoded in intergenic regions as well as in intragenic regions, either in-frame or out-of-frame with the larger encoded protein. Additionally, we have discussed some approaches used to characterize the proteins along with features such as important proline or charged residues critical for some functions. However, our summary is not complete, as we have not been able to cover all small proteins, especially since the lists of identified small proteins keep expanding. What new functions remain to be found? We are hopeful that the elucidation of functions will become easier with increasing general insights about small proteins and how to characterize them. At the same time, our mechanistic understanding of the functions is still limited but will expand with further genetic, structural, and biophysical analyses. Additionally, more remains to be learned about how the small proteins themselves are regulated: When are they expressed, are they modified, and how are they degraded? Finally, how do small proteins evolve and how can they be exploited? In sum, all these unanswered questions make exploration of the vast world of small proteins and their impacts on regulation in all organisms so exciting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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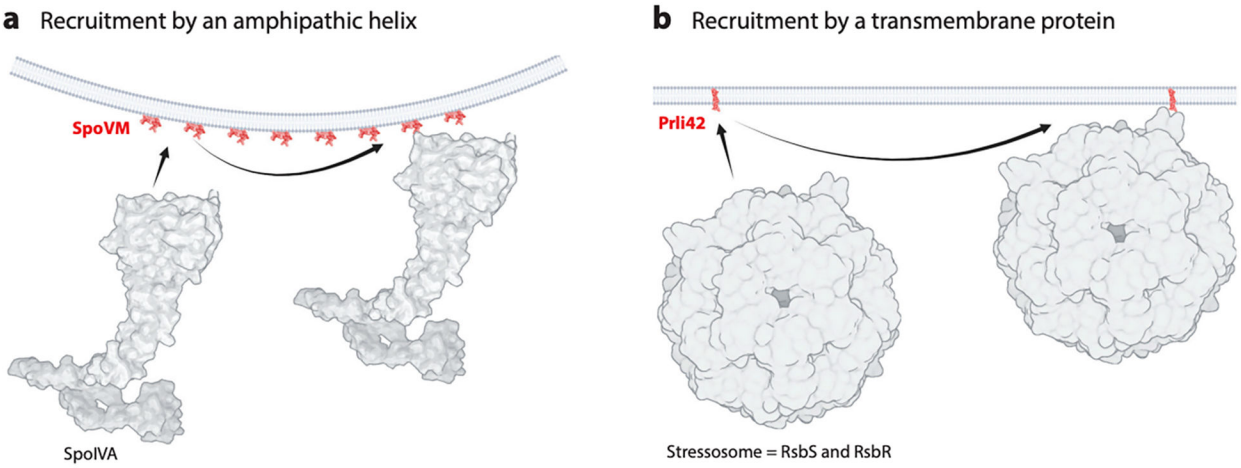

Figure 1.

Small proteins recruit larger proteins to membranes. (*a*) In *Bacillus subtilis*, SpoVM recruits SpoIVA to the membrane during the start of sporulation. The SpoVM structure (PDB ID: 2MVH) is from Reference 36 and the SpoIVA structure (P35149) is from AlphaFold (53, 111). (*b*) In *Listeria monocytogenes*, Prli42 recruits the stressosome to the membrane during stress. The Prli42 (A0A7X0Y229) structure is from AlphaFold (53, 111) and the stressosome (PDB ID: 6QCM) is from Reference 118. All small proteins are shown in red, and all large proteins are shown in gray. Interactions between small and large proteins are predicted, and while the illustrated structures are static, small proteins might induce conformational changes. Additionally, the oligomerization state of small proteins and number bound to larger proteins may be different than predicted. Abbreviation: PDB ID, Protein Data Bank identifier. Figure adapted from images created with Biorender.com.

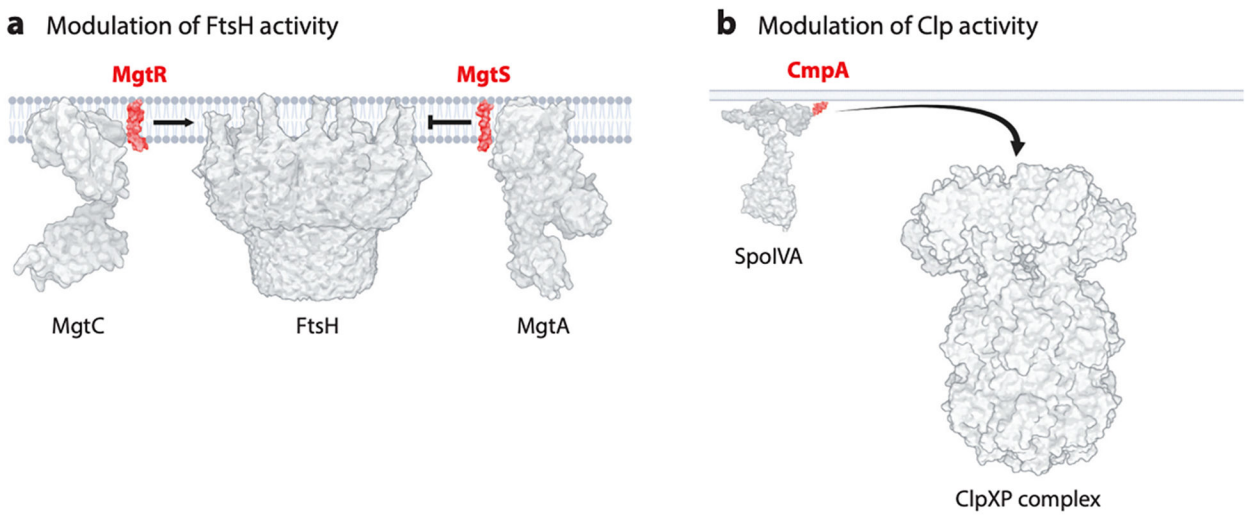

Figure 2.

Small proteins regulate the degradation of larger proteins. (*a*) *Salmonella enterica* MgtR promotes the degradation of MgtC via FtsH, and *Escherichia coli* MgtS prevents degradation of MgtA. The FtsH structure (PDB ID: 7WI3) is from Reference 81 and the MgtR structure (PDB ID: 2MC7) is from Reference 51. The structures of MgtS (A0A5W9F6R6), MgtA (A0A7U0S7U7), and MgtC (A0A241VD96) are from AlphaFold (53, 111). (*b*) *Bacillus subtilis* CmpA acts as an adaptor for ClpXP to degrade erroneously formed SpoIVA on the spore cortex. The ClpXP structure (PDB ID: 6VFS) is from Reference 88, the CmpA structure (A0A6M4JD97) is from AlphaFold (53, 111), and SpoIVA is as described in Figure 1. Proteins are colored as in Figure 1 with the same caveats. Abbreviation: PDB ID, Protein Data Bank identifier. Figure adapted from images created with Biorender.com.

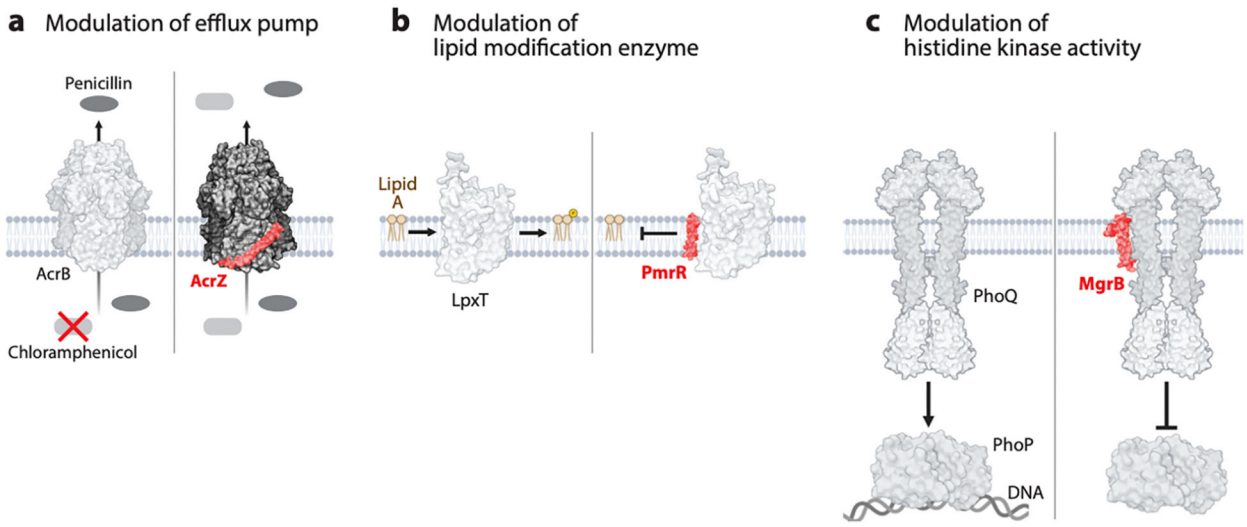

Figure 3.

Small proteins regulate the activities of membrane proteins. (*a*) *Escherichia coli* AcrZ binds to the AcrB multidrug efflux pump. In doing so, AcrZ induces a structural change in AcrB that promotes the efflux of specific antibiotics. The AcrB structure without AcrZ (PDB ID: 1IWG) is from Reference 74, and the AcrB structure with AcrZ (PDB ID: 4CDI) is from Reference 26. (*b*) *Salmonella enterica* LpxT, an enzyme that modifies lipid A, is inhibited by PmrR and thus provides additional diversity to the lipopolysaccharide pool. The structures of LpxT (P76445) and PmrR (G3MTW7) are from AlphaFold (53, 111). (*c*) *E. coli* PhoQ, a sensor histidine kinase, recognizes low magnesium levels and activates the cognate response regulator, PhoP, to illicit an appropriate response. MgrB, a small protein whose expression is regulated by PhoP, inhibits PhoQ, creating a negative feedback loop for this two-component system. The PhoP structure (PDB ID: 2PL1) is from Reference 7, and the structures of PhoQ (P23837) and MgrB (P64512) are from AlphaFold (53, 111). Proteins are colored as in Figure 1 with the same caveats, except for AcrB-ArcZ, for which the structure has been solved. Abbreviation: PDB ID, Protein Data Bank identifier. Figure adapted from images created with Biorender.com.

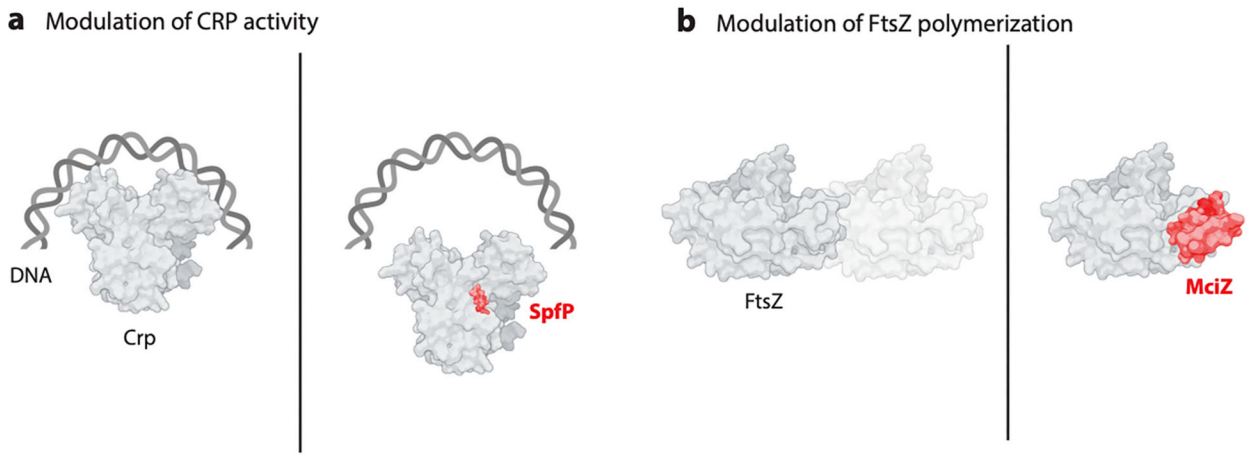

Figure 4.

Small proteins regulate the activities of cytoplasmic proteins. (a) *Escherichia coli* SpfP blocks the activity of the transcriptional regulator, CRP. The CRP structure (PDB ID: 3RYP) is unpublished and SpfP is from Colabfold (71). (*b*) During sporulation in *Bacillus subtilis*, FtsZ is inhibited by MciZ to prevent sporadic assembly of a division septum and to ensure faithful asymmetric cell division that results in the production of a spore (43). The FtsZ structure (PDB ID: 2VAM) is from Reference 77 and the MciZ structure (A0A6M3ZDR2) is from AlphaFold (53, 111). Proteins are colored as in Figure 1 with the same caveats. Abbreviations: CRP, cyclic AMP receptor protein; PDB ID, Protein Data Bank identifier. Figure adapted from images created with Biorender.com.