Regulated Proteolysis in Bacteria: *Caulobacter*

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Abstract

Protein degradation is essential for all living things. Bacteria use energy-dependent proteases to control protein destruction in a highly specific manner. Recognition of substrates is determined by the inherent specificity of the proteases and through adaptor proteins that alter the spectrum of substrates. In the α-proteobacterium *Caulobacter crescentus*, regulated protein degradation is required for stress responses, developmental transitions, and cell cycle progression. In this review, we describe recent progress in our understanding of the regulated and stress-responsive protein degradation pathways in *Caulobacter*. We discuss how organization of highly specific adaptors into functional hierarchies drives destruction of proteins during the bacterial cell cycle. Because all cells must balance the need for degradation of many true substrates with the toxic consequences of nonspecific protein destruction, principles found in one system likely generalize to others.

INTRODUCTION

Energy-dependent AAA$^+$ (ATPases associated with cellular activities) proteases use the energy provided by the consumption of ATP to power recognition, unfolding, and degradation of target proteins. Although there are a number of AAA$^+$ proteases in *Caulobacter*, the two most characterized are the Lon protease (44, 63, 76, 131) and the Clp family of enzymes (11, 20, 49, 62, 84, 115, 120). These are the main proteases discussed in this review. Like most bacteria, *Caulobacter* contains other AAA$^+$ proteases such as HslUV and FtsH; however, their characterization has been limited (4, 37). Both Lon and Clp proteases consist of ATP hydrolyzing domains and hydrolysis active peptidase domains, but Lon encodes both functions on the same polypeptide, whereas the Clp family separates these functions into distinct unfoldases or ATPases (ClpX or ClpA) and peptidases (ClpP) that assemble to form either ClpXP or ClpAP proteases (**Figure 1**, and see 93 for a recent overview). Importantly, owing to their chambered architecture, the peptidases themselves are normally restricted in their access to substrate, digesting only small peptides. Degradation of larger proteins requires initial recognition by the ATPase, unraveling of the

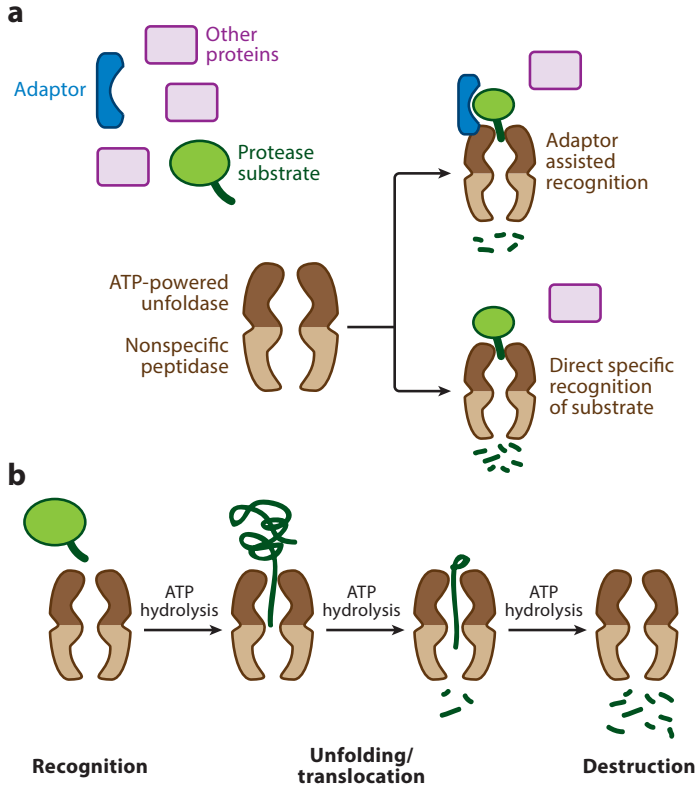


Figure 1

(*a*) Energy-dependent AAA$^+$ proteases must discriminate true targets from a large background of other nondegraded proteins. AAA$^+$ proteases are composed of an ATP-dependent unfoldase and a nonspecific peptidase chamber. In vivo, specificity is principally determined by the unfoldases, which recognize substrates directly or through auxiliary proteins, known as adaptors, that alter specificity. (*b*) Although these proteases differ in sequence and specificity, their core function is conserved. The unfoldase recognizes a substrate and uses cycles of ATP hydrolysis to power the unfolding of this protein. This unfolded polypeptide is concurrently translocated through a central pore to a peptidase chamber, where the target is destroyed.

substrate, and translocation of the unfolded polypeptide through a central pore that leads to the proteolytic chamber of the peptidase. The detailed mechanistic transactions for this unthreading and translocation have been elucidated through elegant single-molecule and solution biochemical approaches (26, 94, 112). From these studies, it seems that once committed these proteases operate with common principles for any given polypeptide substrate. Therefore, the limiting step for degradation in living cells is the initial recognition and engagement of the protein targets.

Different proteases have their own specificity for certain substrates, with some recognition determinants better defined than others. A particularly well understood example is that of the ClpXP protease and ssrA peptide recognition. The ssrA peptide is attached to nascent polypeptides via the *trans*-translation pathway that tags failed translation products and rescues stalled ribosomes (70). Therefore, any endogenous polypeptide with an ssrA tag must be a product of incomplete translation and should be immediately destroyed. ClpXP recognizes ssrA-tagged proteins with such stringency that a single point mutation in the tag greatly reduces degradation (35, 39, 47). By contrast, the Lon protease appears to recognize clusters of exposed hydrophobic residues within a given polypeptide (51), a general indicator of poor protein folding, but with little other sequence specificity. Auxiliary factors called adaptors aid in generating specificity or altering substrate choice for these proteases as required in different bacteria or pathways (for a recent overview, see 6). For example, in *Escherichia coli* the stationary phase sigma factor RpoS is degraded by the ClpXP protease in the presence of the RssB adaptor only (9, 100, 132), and, as is described in detail below, adaptors play a central role in driving regulated protein degradation during the *Caulobacter* cell cycle (64, 74). The combination of adaptors and inherent protease specificity provides for rapid yet selective protein degradation in both stress and normal growth conditions (**Figure 1**).

STRESS AND DAMAGE RESPONSES

Bacteria rely on AAA^+ proteases to properly respond to stressful conditions. Misfolded proteins generated during proteotoxic stress (such as heat, oxidative conditions, amino acid misincorporation, etc.) can be toxic to cells and are often eliminated by the Lon protease in bacteria and eukaryotic organelles (10, 43, 50). In *Caulobacter*, Lon plays a particularly intriguing role in degrading the replication initiator DnaA during proteotoxic stress, which leads to an arrest of cell cycle progression in toxic conditions (**Figure 2*a***) (63). Lon alone is unable to robustly degrade DnaA in vitro, but addition of an unfolded polypeptide (which is readily degraded by Lon alone) can allosterically activate Lon. This activated Lon can now rapidly degrade DnaA (63). Given that other known Lon substrates, such as CcrM and SciP (44, 131), can also stimulate DnaA degradation (63), this effect is not likely limited to a single stress condition. Indeed, allosteric stimulation of other Lon orthologs by protein substrates has been previously observed (52, 107, 126–128), supporting a conserved role for Lon activation.

Cells lacking Lon show fitness defects under a number of growth conditions, such as during stationary phase growth, where nutrient depletion causes cellular stress (76). DnaA degradation is one critical function of Lon, but it is clear that Lon is responsible for other crucial functions as cells lacking Lon show phenotypes distinct from overabundance of DnaA (76). Interestingly, cells lacking ClpA are also defective during extended growth in the stationary phase, suggesting that ClpAP plays a crucial role during nutrient stress conditions (78a). In *Caulobacter*, ClpAP degrades the flagella regulator FliF (49) and the cytoskeletal proteins FtsZ and FtsA (130), and also serves as a redundant protease for DnaA (J. Liu, L. Francis, P. Chien, unpublished data). Because ClpAP can degrade misfolded or aggregated proteins (30), it is tempting to speculate that defects in *Caulobacter* due to loss of ClpA may also be due in part to the failure of a proper response to proteotoxic stresses.

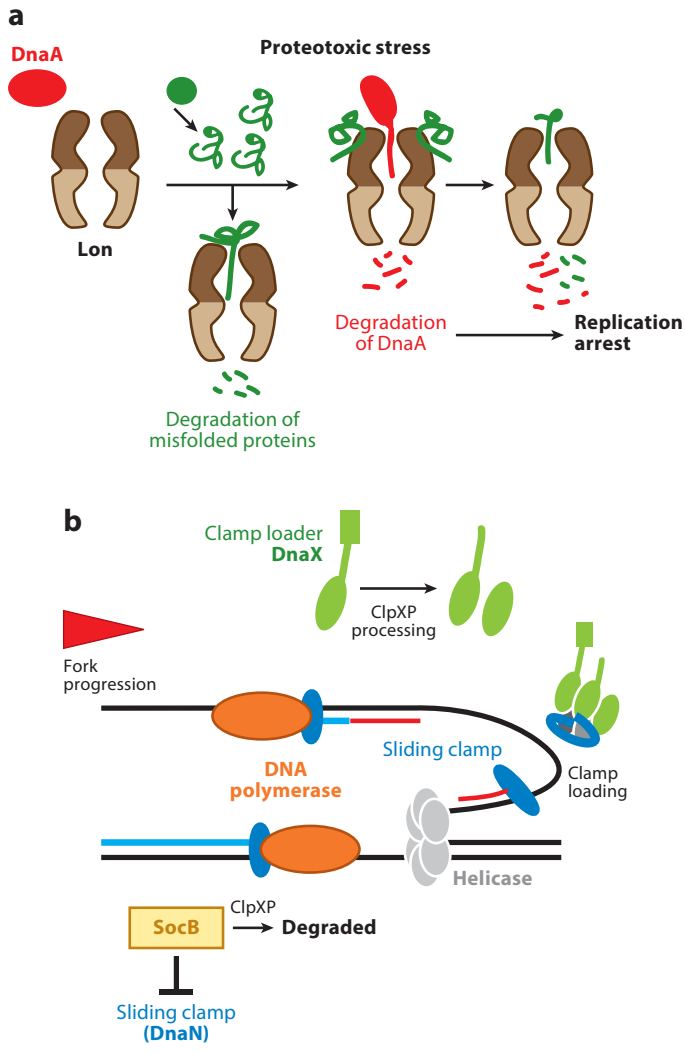


Figure 2

In *Caulobacter*, AAA$^+$ proteases contribute to normal growth and stress responses. (*a*) In *Caulobacter*, the Lon protease rapidly degrades DnaA (*red*) during proteotoxic stress conditions, resulting in cell cycle arrest. This stress also causes protein misfolding (*green circles to squiggly lines*). Reconstitution experiments support a model in which misfolded proteins (*green squiggly lines*) that are normally Lon substrates can allosterically activate the Lon protease to degrade DnaA. This protective mechanism ensures that cells wait until damage has been repaired before continuing with growth. (*b*) Chromosomal DNA replication requires sliding clamps that hold the polymerase to the template DNA. These clamps are loaded by an energy-dependent clamp loader, which is a complex comprising several proteins, including the ATP hydrolyzing subunit DnaX (*green*). In *Caulobacter*, DnaX is processed by ClpXP to generate a shortened form that is required for normal growth, and altering these processing dynamics reduces tolerance to DNA damage. ClpXP also degrades the SocB toxin, a sliding clamp inhibitor, which is upregulated during DNA damage. The upregulation of SocB seems to be the primary cause of cell death upon loss of ClpXP.

In contrast to Lon and ClpA, ClpX and ClpP are essential in *Caulobacter* (62). However, deletion of the *socB* toxin gene can suppress this essentiality (1). A satisfying explanation for this result came from the elegant demonstration that the SocA antitoxin promotes SocB toxin degradation by the ClpXP protease (**Figure 2*b***). SocB binds the replication sliding clamp DnaN and arrests replication but levels of this toxin are kept low through SocA-dependent degradation of SocB by ClpXP. Thus, one immediate consequence of ClpX loss is the stabilization of SocB which leads to replication arrest and cell death (1). SocB is also highly upregulated during DNA damaging conditions, suggesting it may play a physiological role in these conditions (86). Interestingly, the clamp loader subunit DnaX is also a ClpXP substrate, but in this case, partial processing of full-length DnaX to a shorter form is required for normal growth (11, 124) (**Figure 2*b***). Strains constitutively expressing a nondegradable full-length DnaX and a truncation mimicking the shorter form are viable but fail to mount a robust DNA damage response (124). Taken together with the fact that LexA, the principal regulator of the SOS response, was also identified as a candidate ClpP substrate (11), it appears that ClpXP plays an important role in managing DNA damage in *Caulobacter*. Because destruction of a sliding clamp toxin (1) and processing of the sliding clamp loader are both essential (124), it is particularly tempting to consider that ClpXP may play a central role in balancing clamp dynamics and activity during normal or stress conditions.

Finally, stressful conditions also arise when normal processes are overtaxed. For example, ribosome stalling on damaged or nonstop mRNAs results in a loss of translation capacity, and failure to rescue these ribosomes results in cell death (36, 66). During such conditions, most bacteria, including *Caulobacter* (69), use the specialized transfer messenger RNA (tmRNA) to cotranslationally append the ssrA peptide tag that encodes its own stop codon to clear stalled ribosomes (70). The ssrA peptide also targets the tagged polypeptide for degradation (47, 70). The SspB adaptor augments this process by enhancing delivery of tagged proteins to ClpXP (78). In *Caulobacter*, mutants lacking tmRNA show delays in replication initiation that cannot be complemented by a nondegradable ssrA tag (67, 68), supporting a need for ssrA-dependent degradation during normal growth. SspB also influences the extracellular stress response in *E. coli* by enhancing degradation of RseA, a negative regulator of this response (38). *Caulobacter* also has a functional SspB adaptor system (21, 77), but it is yet unknown whether physiological substrates outside of ssrA-tagged proteins exist in this bacteria.

CELL CYCLE PROGRESSION

A defining feature of *Caulobacter* growth is a robust cell cycle in which coordination of replication is tightly coupled to an obligate developmental transition (see 23 for a recent overview). Like in the eukaryotic cell cycle, progression of the *Caulobacter* cell cycle relies on oscillating levels of many proteins (see **Table 1**). As shown in **Figure 3*a***, nonreplicative motile swarmer (SW) cells develop into nonmotile replication-competent stalked (ST) cells. This is followed by an asymmetric cell division to generate a new daughter SW cell and the original mother ST cell. The ST cell immediately initiates replication and undergoes another round of growth and cell division, whereas the SW cell must first transition again into an ST cell. During this SW-to-ST transition, also called the G1-to-S transition, owing to the tight coupling between developmental state and DNA replication state, the levels of many proteins change dramatically (48).

Because steady-state protein levels are determined by the balance of protein synthesis and degradation, cell cycle–dependent regulation of either synthesis or degradation is sufficient to generate these changes (**Figure 3**). In *Caulobacter*, cyclic changes in synthesis partnered with constitutive degradation can in some cases support these fluctuating levels, such as is suggested for CcrM (131). In other cases, oscillating protein levels can be driven principally by regulated

Table 1 A set of cell cycle–dependent protease substrates in *Caulobacter*[a]

Substrate	Function	Protease responsible	References
CtrA	Replication initiation inhibitor and transcriptional regulator[b,c]	ClpXP	21, 29, 115
TacA	Transcriptional regulator[b,c]	ClpXP	11, 64
CC3144	Unknown function[b]	ClpXP	64
PdeA	Cyclic di-GMP phosphodiesterase[b,c]	ClpXP	3, 74
McpA	Transmembrane chemoreceptor[b,c]	ClpXP	74, 120
McpB	Cytoplasmic chemoreceptor	ClpXP	99
CpdR	Single-domain response regulator/adaptor[b]	ClpXP	59, 74
GdhZ	NAD-dependent glutamate dehydrogenase	ClpXP	8
KidO	NAD(H)-binding oxidoreductase homolog[c]	ClpXP	103
TipF	Flagellar regulator	ClpXP	28
NstA	Negative switch for Topo IV decatenation activity	ClpXP	90
MopJ	Single-domain PAS (Per-Arnt-Sim) protein	ClpXP	109
FliF	MS ring protein	ClpAP	49
FtsZ	Cell division cytoskeletal protein[b,c]	ClpAP/ClpXP	71, 130
FtsA	Cell division protein[b,c]	ClpXP/ClpAP	81, 130
FtsQ	Cell division protein[c]	Unknown	81
DnaA	DNA replication initiator[b]	Lon	24, 63
CcrM	DNA methyltransferase[b]	Lon	63, 131
SciP	Small CtrA inhibitory protein[b,c]	Lon	44, 45
GcrA	Cofactor for sigma70[c]	Unknown	24, 53

[a]This table includes proteins that are degraded selectively during the cell cycle and cell cycle–regulated substrates whose levels in vivo are protease dependent.
[b]Substrates validated by in vitro reconstitution experiments.
[c]Substrates that are degraded in a cell cycle–dependent manner when expressed constitutively.

proteolysis, where protein degradation rates change during cell cycle progression (see **Table 1**). Monitoring cell cycle–dependent protein levels when candidate substrates are expressed constitutively allows us to discriminate between these cases. For example, although CtrA is normally expressed differentially during the cell cycle (102), protein levels still oscillate when CtrA is expressed constitutively during the cell cycle (29), suggesting that changes in degradation alone are sufficient to modulate protein levels. Genetic studies implicate the Clp or Lon proteases in many cases in which changes in degradation are sufficient to drive changes in protein levels (44, 49, 62, 131). However, levels of ClpX, ClpP, and Lon do not change during the cell cycle (62, 131) (**Figure 4**). Therefore, there must be more complex controls governing the stability of cell cycle–dependent protease substrates.

General Mechanisms of Cell Cycle Proteolytic Control

From a general perspective, controlled proteolysis during the cell cycle can arise through either cell cycle–dependent inhibition or activation (**Figure 3**). Activation is discussed in more detail below, but it is worth mentioning that controlled inhibition during the cell cycle can drive oscillating levels of proteins. For example, the transcriptional regulator SciP is degraded by the Lon protease in a cell cycle–dependent manner, and its degradation is strongly inhibited by DNA binding (44). SciP forms a complex with CtrA to bind at specific sites (45, 118) and binding of CtrA to DNA is

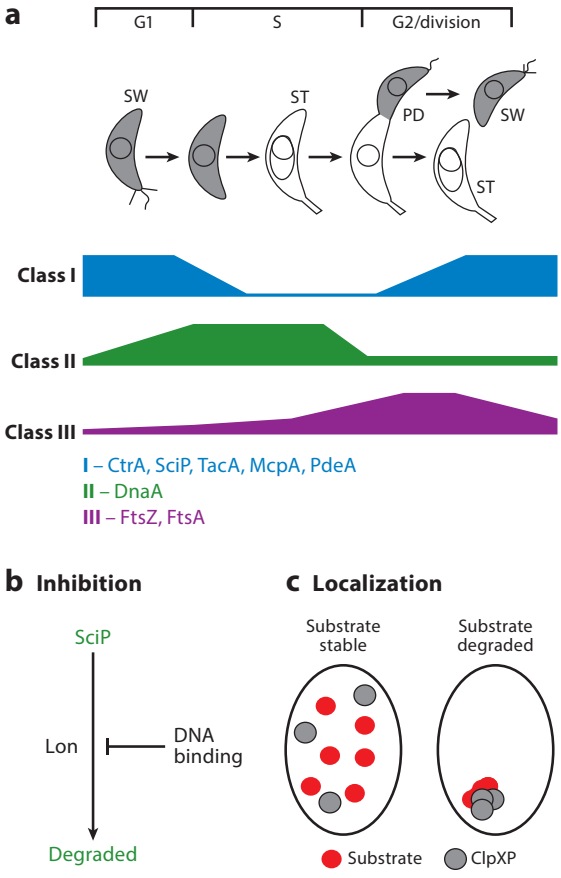
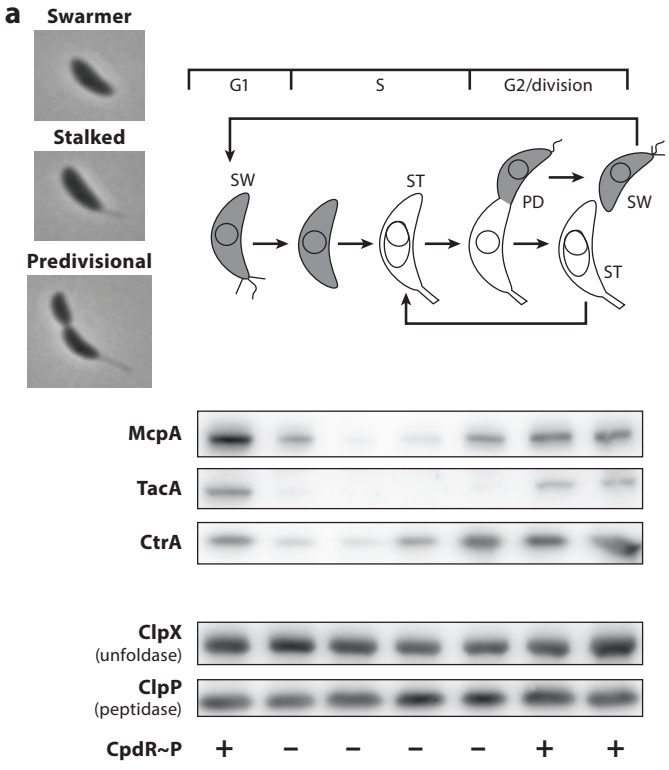


Figure 3

(*a*) Levels of many proteins oscillate during the *Caulobacter* cell cycle. One source of control is that different classes of proteins can be degraded at different times during the cell cycle. Class I proteins are lost during the SW-to-ST (G1-S) transition, Class II proteins are more abundant (more stable) in ST cells, and Class III proteins are preferentially reduced in SW cells. Examples of each class are shown. See **Table 1** for a more complete listing of proteins and proteases. There are several models that describe how proteins are selectively degraded during the cell cycle, including (*b*) cell cycle–dependent inhibition of SciP (small CtrA inhibitory protein). Binding to DNA inhibits degradation of SciP by Lon protease. (*c*) Changes in localization of protease and substrate lead to substrate degradation. Abbreviations: PD, predivisional cell; ST, stalked cell; SW, swarmer cell.

regulated through cell cycle–dependent phosphorylation (102). A parsimonious model to explain these results shows that SciP degradation is simply controlled through its DNA binding state, in which cell cycle–dependent changes in DNA binding protect this protein from degradation by Lon (**Figure 3*b***) (44). Examples of this type of inhibitory control have been seen with other AAA$^+$ protease/substrate systems (80, 85, 97, 101, 114).

Another regulatory mechanism that controls proteolysis is spatial compartmentalization within a cell to avoid unwanted protein degradation (**Figure 3*c***). These examples are well characterized in eukaryotes, where the lysosome is a dedicated organelle that removes the bulk proteins delivered to it. Such spatial compartmentalization was also proposed for the removal of the master regulator CtrA in *Caulobacter*. The assumption was that during the SW-to-ST transition, ClpXP would



b

Substrate / Adaptor	McpA	TacA	CtrA
CpdR	+	+	+
RcdA	–	+	+
PopA	–	–	+

Figure 4

(*a*) Levels of ClpXP substrates change during cell cycle progression, but levels of ClpXP remain constant. Purified swarmer (SW) cells are released into fresh media to initiate synchronized growth. Aliquots taken during synchronized growth are probed with antibodies against the McpA, TacA, CtrA (substrates), ClpX, and ClpP (protease components) (adapted with permission from 64). Cell cycle–dependent phosphorylation of CpdR (58) is shown as +/−. (*b*) Cell cycle–dependent degradation of McpA relies only on CpdR, whereas TacA requires both CpdR and RcdA. CtrA additionally requires PopA for cell cycle–regulated degradation. – Indicates dispensable and + indicates requirement of adaptor for cell cycle-dependent degradation of substrate. Abbreviations: PD, predivisional cell; ST, stalked cell; SW, swarmer cell.

localize to the nascent stalked pole upon dephosphorylation of the CpdR protein, which was thought to be a localization factor (58). At the same time, the proteins RcdA and PopA are recruited to this same pole and promote localization of the substrate CtrA (33, 84). The model arising from these observations is that a net increase in the effective concentration of substrate and protease at the pole promotes CtrA recognition by ClpXP (**Figure 3*c***). In support of this model, CtrA degradation is lost in cells lacking CpdR, RcdA, or PopA (33, 58, 84). CpdR is needed for degradation of another ClpXP substrate, McpA, but neither RcdA nor PopA is needed for McpA turnover (33, 58, 84). Taken together, these data point to a situation in which CpdR controls ClpXP localization, which is needed for regulated degradation of all ClpXP substrates, whereas RcdA/PopA specifically controls CtrA localization.

More recent studies suggested that localization of substrate and protease might not be essential for degradation. For example, mutants of RcdA that fail to localize to stalked poles and also fail to localize CtrA to stalked poles do not exhibit changes in cell cycle–dependent degradation of CtrA (119). Furthermore, localization of the ClpXP protease is not essential for all its activity in vivo, as some ClpXP substrates, such as FtsZ, are degraded in SW cells when ClpXP is delocalized (130). Because many proteins are degraded specifically during the G1-to-S transition, localization alone seems insufficient to explain the degradation of all these substrates (**Figure 4**). Recent observations reconstituting protein degradation with purified protein components support a model in which CpdR, RcdA, and PopA act as biochemical adaptors that coordinate the delivery of a range of substrates, including CtrA, directly to ClpXP protease for destruction during the *Caulobacter* cell cycle (64, 74, 115) (**Figure 4*b***). More precise roles for each of these adaptors are described below.

CpdR-Dependent Degradation

CpdR is a single-domain response regulator that was originally identified as a factor needed for the cell cycle–dependent degradation of CtrA (58). Cell cycle–dependent CpdR activity is controlled by phosphorylation mediated by the CckA-ChpT kinase cascade (12, 58, 59, 115). Phosphorylation inactivates CpdR, whereas overexpression of a nonphosphorylatable CpdR (CpdRD51A) results in prolific degradation of CtrA and other ClpXP-dependent substrates (3, 12, 58). Interestingly, the same CckA-ChpT kinase cascade also phosphorylates and activates CtrA (12, 60, 61). Because of this convergence, CckA turns on CtrA activity by preventing CtrA degradation (through CpdR phosphorylation) and by activating CtrA directly. Like many histidine kinases, CckA can also act as a phosphatase (19). Therefore, dephosphorylation through CckA-ChpT turns off CtrA activity by inactivating the transcription factor and inducing degradation by CpdR. Control of CckA activity requires additional localized proteins (5, 34, 57, 105, 121, 122), and it was recently shown that CckA phosphatase activity is stimulated by cyclic di-GMP (cdG) (79), a point that is more completely addressed below.

Most intriguingly, CpdR is required for degradation of all known ClpXP substrates that are specifically destroyed during the SW-to-ST transition (3, 11, 58, 103) (see **Table 1**), whereas RcdA and PopA are required for only a subset of these substrates. For example, McpA degradation requires CpdR but not RcdA or PopA (33, 58, 84). Similarly, the cdG phosphodiesterase PdeA is degraded during the SW-to-ST transition, and its degradation is dependent on only CpdR and ClpXP (3). Biochemical experiments showed that PdeA is not degraded by ClpXP alone, but the addition of CpdR is sufficient to stimulate PdeA degradation (3). Consistent with the in vivo observations, phosphorylation of CpdR blocked PdeA degradation in vitro (3). Structural dissection of PdeA showed that it contains an N-terminal domain required for CpdR-dependent degradation and a C-terminal ClpXP recognition motif (106), suggesting that CpdR may work as an adaptor to selectively deliver proteins to ClpXP.

Adaptors for ClpXP have been best characterized based on models from *E. coli*. For example, the SspB adaptor binds ssrA-tagged proteins and delivers them to ClpXP (**Figure 5*a***) (31, 78). Similar to the *E. coli* protein, the *Caulobacter* SspB has two important domains, a substrate domain that binds the ssrA peptide with high affinity ($K_D \sim 200$ nM) (20, 77) and an unstructured tethering motif at the extreme C terminus that binds the N-terminal domain of ClpX with weaker affinity ($K_D \sim 20$ μM) (22). This scaffold allows for the robust tethering of a cargo substrate, although not gripping so tightly that substrate translocation is hindered. On the basis of this model, it was unclear how CpdR worked as an adaptor as CpdR does not strongly interact with the substrate PdeA on its own (74).

During the initial characterization of CpdR as a stimulatory factor for PdeA, bacterial two-hybrid experiments suggested that the two directly interact (3); however, purified CpdR and PdeA failed to bind (74). This discordance was resolved when bacterial two-hybrid experiments performed in reporter cells lacking ClpX failed to show an interaction between CpdR and PdeA, suggesting that ClpX is required for CpdR and PdeA to be in the close proximity needed for two-hybrid complementation (74). This led to a model in which CpdR acts as an adaptor by priming ClpX to generate a CpdR-ClpX state that is capable of binding PdeA and other substrates (**Figure 5*b***). As seen with other ClpXP adaptor systems, the N-terminal domain of ClpX is essential for CpdR binding. Finally, it was shown that the phosphorylation of CpdR blocked its ability to interact with ClpX, linking cell cycle–dependent phosphorylation to CpdR-dependent proteolysis. Thus, CpdR is directly responsible for facilitating delivery of one class of substrates that includes PdeA and the chemoreceptor McpA (**Figure 5*b***) (74).

Recent studies suggest the existence of similar priming mechanisms for protein degradation in other bacteria. For example, the YjbH adaptor enhances ClpXP degradation of Spx, a transcriptional regulator in *Bacillus subtilis* (40). However, YjbH does not directly interact with ClpX (17) but binds to the C-terminal region of Spx to induce a conformational change that reveals a degron for ClpX recognition (18). Similarly, the RssB adaptor promotes degradation of RpoS in *E. coli* by binding RpoS and promoting ClpX recognition, but RssB alone appears to bind poorly to ClpX (54, 116, 132). In these cases, priming of the substrate induces the ability to be recognized by the protease, whereas in the case of CpdR, priming of the protease induces recognition of the substrates.

RcdA-Dependent Degradation

RcdA was initially discovered as necessary for the polar localization and cell cycle–dependent degradation of CtrA in *Caulobacter* (84). However, other studies suggested that RcdA-mediated localization of CtrA to the stalked pole might not be critical for CtrA degradation (119). Therefore, it was thought that RcdA might be playing an additional role in CtrA degradation together with or independent of its localization function. Initially, RcdA was dismissed as an adaptor, as purified RcdA did not stimulate ClpXP-mediated degradation of CtrA in vitro (21). More recent work shows that RcdA does act as an adaptor but binds only to a CpdR-primed ClpXP protease (**Figure 5*c***) (64). RcdA directly binds a number of substrates, e.g., the developmental transcription factor TacA, and delivers them to CpdR-primed ClpXP proteases for degradation (64) (**Table 1**). In this regard, RcdA function is reminiscent of that of canonical adaptors, but instead of tethering directly to the ClpXP protease, as seen with SspB (13, 22, 31, 125), RcdA tethers to only a CpdR-primed ClpX (64). RcdA expression peaks during SW-to-ST transition (84), which further ensures that RcdA accumulates when it is needed (84). Therefore, RcdA acts as an adaptor to deliver a second class of substrates in a CpdR-dependent fashion (**Figure 5*c***).

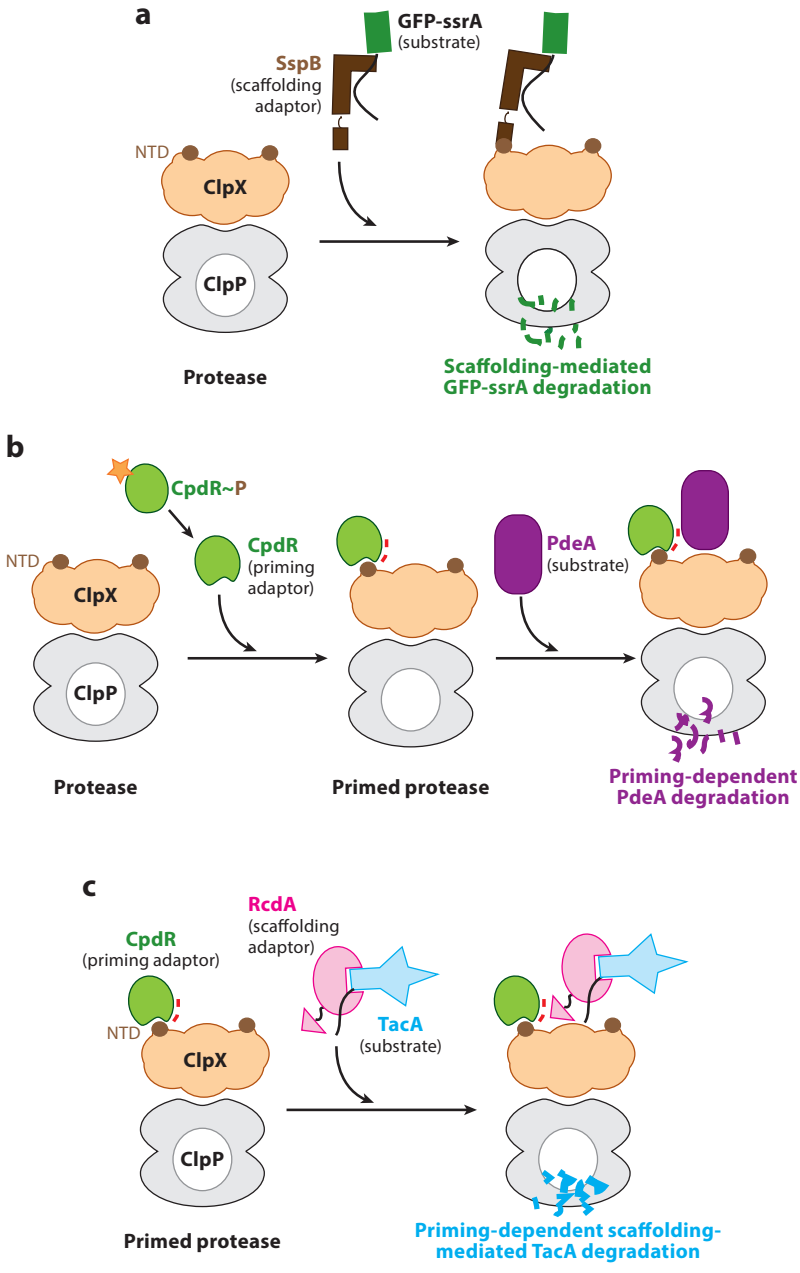


Figure 5

(*a*) Canonical scaffolding adaptors, such as SspB, bind strongly to their substrates and tether them directly to the ClpXP protease. (*b*) CpdR binds directly to the N-terminal domain (NTD) of ClpX, facilitating the recognition of protease substrates (PdeA, McpA). Phosphorylation of CpdR prevents binding to ClpX. CpdR does not seem to directly bind its cargo substrates with any detectable affinity (74). (*c*) RcdA directly binds substrates such as TacA, facilitating their degradation by ClpXP protease. Here, RcdA acts as a scaffolding adaptor delivering the substrate to only a protease that is first primed by CpdR (64). Modified with permission from Reference 64.

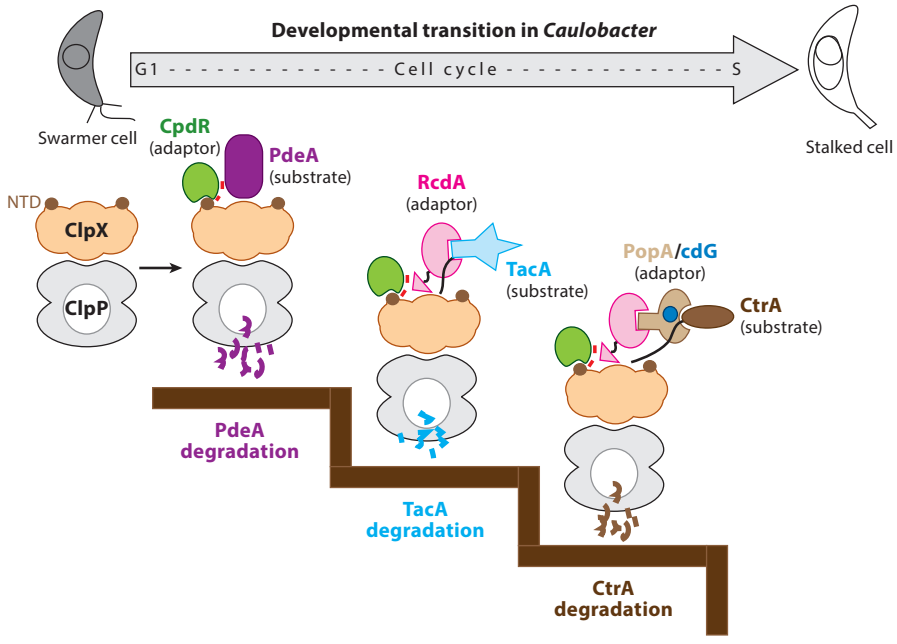


Figure 6

An adaptor hierarchy regulates degradation during the cell cycle. During the developmental transition in *Caulobacter* that overlaps with its cell cycle, the adaptor CpdR binds the N-terminal domain (NTD) of ClpX ATPase, priming (*red dashed line*) the protease to recruit substrates such as PdeA for degradation. The primed protease then recruits the scaffolding adaptor RcdA to degrade a range of substrates, including TacA. The second messenger [cyclic di-GMP (cdG)]-dependent adaptor PopA binds the adaptor RcdA to deliver substrate CtrA to the ClpXP protease (modified with permission from Reference 64).

PopA-Dependent Degradation

PopA (paralog of PleD) is a cdG-binding effector protein essential for cell cycle–dependent CtrA degradation (33). PopA mutants deficient in cdG binding do not sustain cell cycle–dependent degradation of CtrA (33). On the basis of bacterial two-hybrid experiments, PopA binds directly to RcdA even in the absence of cdG binding (33), and in vitro pull-down experiments confirm this result (115). By contrast, PopA binds CtrA in a cdG-dependent manner (115). Domain analysis of PopA suggests that binding of cdG induces dimerization of PopA via its C-terminal GGDEF domains, whereas the N-terminal receiver domains are responsible for additional interactions with proteins like RcdA (95). The working model shows that PopA serves as an adaptor between RcdA and CtrA, promoting degradation of a third class of substrates in a CpdR-dependent manner (**Figure 6**) (64).

Given that cell cycle–dependent degradation of other ClpXP substrates, such as KidO and GdhZ (8, 103), rely on CpdR, RcdA, and PopA, it appears likely that PopA serves as an adaptor to RcdA for these substrates as well. Activity of adaptors can be regulated by the binding of anti-adaptor proteins that have been shown to competitively inhibit the adaptor binding to its substrate (7, 14). In this regard, PopA can also act as a competitive anti-adaptor for RcdA-dependent substrates such as TacA, where loss of PopA results in more rapid degradation of TacA, demonstrating how a single protein can be both an adaptor and anti-adaptor (64). This leads to the possibility that other anti-adaptors, such as the Ira family proteins that normally repress RssB-dependent RpoS degradation (7, 14), could act as adaptors for as yet unknown substrates.

It is worth mentioning that PopA requires cdG binding in order to deliver CtrA (33, 64) and that CpdR degrades PdeA, a cdG-hydrolyzing phosphodiesterase (3, 74). Levels of cdG oscillate during the *Caulobacter* cell cycle (2) and PdeA contributes to this control (2). Recently, the CckA kinase was shown to switch from a kinase state to a phosphatase state upon cdG binding (79), directly linking cdG to activation of CpdR (which turns on when dephosphorylated) and the resulting cascade of proteolysis. Thus, in SW cells when CpdR is phosphorylated, high levels of PdeA keep cdG levels low, maintaining CckA in a kinase state, which keeps CpdR phosphorylated. If a fraction of CpdR is activated, then the resulting degradation of PdeA could cause a local upshift in cdG that further activates even more CpdR through cdG-dependent CckA phosphatase activity. Activation of CpdR leads to recruitment of RcdA and PopA (activated now by cdG), which together deliver CtrA for degradation and free the origin for replication initiation. By coupling fluctuating second messenger pools to an irreversible process (protein degradation), the cell ensures a unidirectional and robust G1-to-S transition (2, 29, 64, 79).

Conservation and Impact of the CpdR-RcdA-PopA Adaptor Hierarchy

CpdR, RcdA, and PopA form an adaptor hierarchy wherein adaptors activate proteases to facilitate binding of additional adaptors that can in turn recruit more adaptors, with each level responsible for degradation of different classes of substrates (**Figure 6**) (64, 74). Interestingly, CpdR and RcdA are present in all known α-proteobacteria (15); however, PopA is poorly conserved, being found only in *Caulobacter* and closely related stalked bacteria (95). For example, in the plant symbiont *Sinorhizobium meliloti*, there are two orthologs of CpdR, but only one (CpdR1) causes physiological defects when either deleted or overactivated (73). CpdR1 appears to play a role in controlling CtrA stability, which is particularly important in the endoreduplication process during symbiosis (98, 110). Less is known about the role of RcdA in *S. meliloti*, but depletion of RcdA increases CtrA levels (98), supporting its role in controlling CtrA stability similar to that seen in *Caulobacter*. Interestingly, RcdA appears to be essential in *S. meliloti* and in *Agrobacterium tumefaciens* (27, 98). A tempting speculation is that CpdR and RcdA represent a more broadly conserved ancestral adaptor system found throughout α-proteobacteria. In this light, the inclusion of PopA in *Caulobacter* allows cells to link cdG levels with CtrA destruction, timing this process to cell cycle events. The absence of PopA in other bacteria in which CtrA is degraded in a CpdR/RcdA-dependent manner leads one to ask what the equivalent for PopA is in these cases and how this adaptor hierarchy might impact physiology in other bacteria.

Adaptor-Regulated Proteolysis in Other Systems

ClpXP adaptors were first characterized in *E. coli* and have been recently reviewed (6, 72). Here, we briefly describe other systems in which adaptor-mediated protein degradation appears to play important physiological roles.

The gram-positive bacterium *B. subtilis* passes through many developmental stages, such as competence, sporulation, and contact-dependent differentiation, during its normal life cycle (32, 42, 55, 65). During exponential growth, the MecA adaptor maintains low levels of the transcriptional factor ComK by promoting ComK destruction through the ClpCP protease (96, 123). When cells reach an appropriate density, the anti-adaptor ComS is synthesized in response to quorum sensing. The anti-adaptor ComS then binds MecA, stabilizing ComK, which induces competence-related genes (**Figure 7*a***) (92, 123). Quality control during sporulation was also recently shown to be under adaptor-mediated control. Here, the CmpA protein stimulates destruction of the coat assembly protein SpoIVA by ClpXP in cells with envelope assembly defects, causing lysis of these cells that would fail to produce robust spores (**Figure 7*b***) (117). An adaptor for Lon has

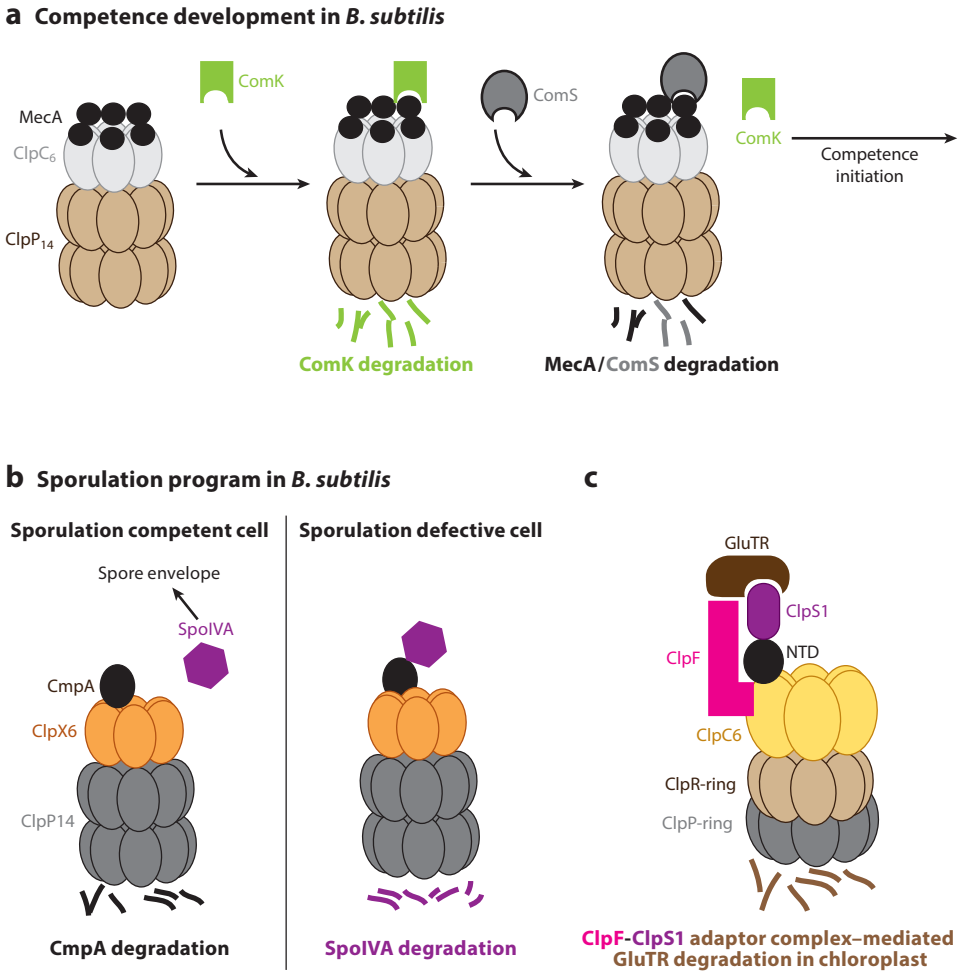


Figure 7

Adaptors in other systems: (*a*) Competence development in *Bacillus subtilis*. The binding of a MecA adaptor to the ClpC ATPase activates the ClpCP protease. Once activated, the MecA adaptor binds and facilitates ComK degradation during exponential growth. When the cells reach a higher density, the anti-adaptor ComS competitively inhibits ComK degradation, thus stabilizing ComK for expression of competence-related genes. (*b*) Fidelity of sporulation program in *B. subtilis*. In a sporulation-competent cell, the adaptor CmpA is degraded, inhibiting degradation of the coat protein SpoIVA and leading to the completion of the sporulation program (117). In a sporulation-defective cell, the adaptor CmpA facilitates degradation of SpoIVA, ultimately resulting in the lysis of the cell. (*c*) ClpF-ClpS1–mediated GluTR degradation in chloroplast. ClpF and ClpS1 together form a multiprotein adaptor complex to deliver substrate GluTR to the ClpCRP protease for degradation in chloroplasts (91). Abbreviation: NTD, N-terminal domain.

been shown to play an important role during differentiation into SW cells in *B. subtilis*. The SmiA adaptor limits accumulation of the flagellar biosynthesis regulator SwrA in liquid medium through Lon-mediated proteolysis. Upon contact with solid surfaces, SwrA is stabilized, which turns on flagellar genes and increases motility (89). Given the fact that Clp proteases also affect motility development (87), there appears to be a significant link between proteolysis and cell dispersion. How the adaptors are themselves regulated in these latter two examples is an intriguing mystery.

Adaptor-mediated protein degradation also affects growth of cyanobacteria. NblA was identified as a proteolytic adaptor to facilitate degradation of the phycobilisomes, the light harvesting complexes, in response to limited nutrient conditions (25, 113). Reconstituting this pathway in vitro sheds more light on the mode of action of NblA, which may also be involved in the disassembly of the large phycobilisome complexes prior to their degradation. Finally, adaptor-dependent protein degradation is found in eukaryotic organelles of bacterial origin. Most recently, a putative adaptor complex comprising the ClpF and ClpS1 proteins was found to stimulate degradation of GluTR, a key enzyme in tetrapyrrole synthesis in chloroplasts (**Figure 7*c***) (91). The identification and characterization of new adaptors such as these will shape our understanding of proteolytic control throughout biology.

Challenges of Protease Adaptor/Substrate Discovery

There is a central difficulty in discovering new AAA$^+$ protease adaptors and their substrates. In order to identify a candidate protein as an adaptor, one must know which substrate's degradation is affected. In order to validate that a substrate is degraded, one must know the adaptor needed for promoting its degradation. This circular challenge is one major reason why defining the adaptor hierarchy of the *Caulobacter* cell cycle required a combination of genetic, cell biology, and biochemical approaches.

Genetic experiments initially pointed to a need for RcdA during the cell cycle–dependent degradation of CtrA in vivo (84). However, RcdA alone did not stimulate degradation of CtrA in vitro (21). CpdR is necessary for CtrA degradation in vivo (12, 58), but CpdR alone is insufficient to stimulate CtrA degradation in vitro (115). In fact, CtrA degradation by ClpXP alone in vitro is sufficiently rapid to account for its in vivo dynamics (21), but the observation that additional regulators can inhibit CtrA degradation (44) suggested a need for a stimulatory factor. The findings that CpdR, but not RcdA or PopA, was needed for PdeA degradation in vivo and that CpdR alone could stimulate PdeA degradation by ClpXP in vitro (3, 106) were key results that led to the current understanding of the CpdR/RcdA/PopA adaptor hierarchy (64, 74).

How do we then identify new adaptors or substrates? For the case of ClpX, the unique N-terminal domain is the binding site for all known adaptors and some substrates (11, 20, 74). Therefore, proteins that interact with this domain would include as yet unknown adaptors and substrates that rely directly on this domain. Identifying new adaptors will likely require a combination of genetic, cell biology, and biochemical studies. In an ideal case, the genetics would point to the necessity of a particular factor for degradation of a substrate in vivo, whereas biochemical reconstitution experiments would inform on the sufficiency of that factor in vitro. Recent advances in quantitative proteomics and high-throughput genetics will likely be key in identifying and characterizing new adaptor/substrate pairs.

Importance of Energy-Dependent Protein Degradation Across Bacteria

A final consideration of the AAA$^+$ proteases described in this review is the need for these proteases in different bacterial species. In *E. coli*, neither the Clp family nor Lon family proteases are essential (82, 83). Neither Clp nor Lon proteases are essential in *B. subtilis*, although loss of ClpX results in pleotropic growth defects (41, 88, 108, 111). By contrast, both ClpX and ClpP are essential in *Caulobacter* (62). As mentioned previously, the accumulation of the replication clamp inhibitor SocB is likely the immediate cause of cell death upon loss of ClpXP (1). However, even when *socB* is deleted, cells completely lacking ClpX or ClpP are still very sick (1) (R.H. Vass & P. Chien, unpublished data), supporting a critical role for ClpXP activity beyond preventing SocB toxin buildup.

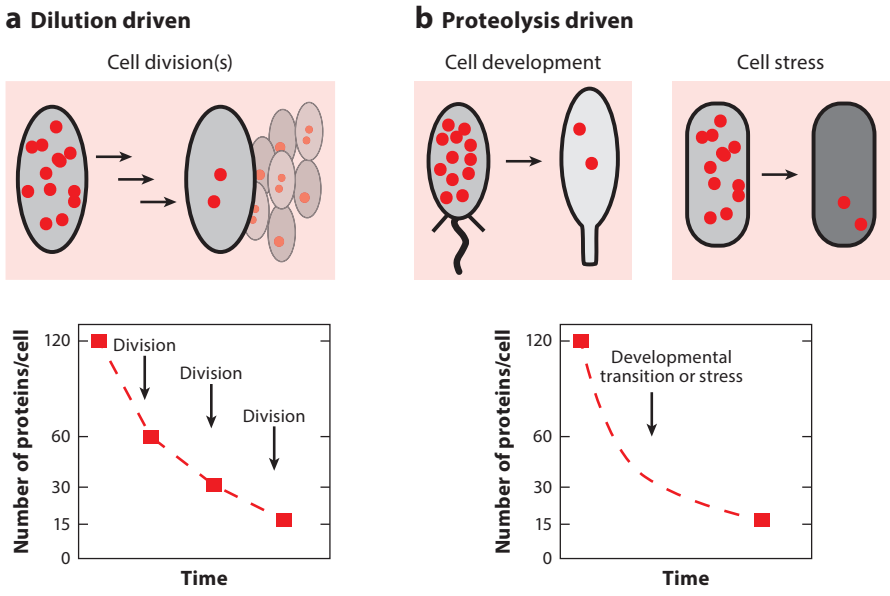


Figure 8

Changes in protein numbers in different cellular conditions illustrate the need for regulated protein degradation in the absence of cell division. (*a*) Rapidly dividing cells can easily reduce protein levels by shutting off protein synthesis and diluting the protein pool through multiple cell divisions. (*b*) By contrast, cells undergoing a developmental transition or stress response must change proteins levels in the absence of cell division. Rapid, regulated protein degradation likely plays a particularly important role during these conditions.

Indeed, protease-trapping experiments identify hundreds of candidate substrates in *Caulobacter*, including many essential proteins (11).

This observation raises the question of why different bacterial species have different protease needs. A simple rationale results from considering the speed of cell division and the role of cell differentiation in various species (**Figure 8**). The amount of protein per cell is governed by synthesis and loss. In the absence of synthesis, the minimum half-life of a protein is determined by the division time of the cell. Therefore, if cell division is sufficiently fast, then sufficient loss of a particular protein can be served simply by shutting off synthesis without the need for rapid protein degradation. For example, *E. coli* cells divide into equivalent daughter cells every 20 minutes in rich media. Therefore, a level of protein of 120 copies/cell is reduced to 15 copies/cell in 1 hour if protein production is halted, an order of magnitude change without the need for proteolysis. By contrast, every *Caulobacter* SW cell must differentiate into a stalked cell prior to cell division, during which time dramatic changes in protein levels occur in the absence of cell division (3, 8, 11, 29, 48, 103) (**Table 1**). Thus, it is perhaps unsurprising that loss of energy-dependent proteases generally have stronger phenotypic consequences in *Caulobacter* than in *E. coli*.

An extension of this reasoning suggests that many bacteria that undergo developmental programs without cell division have a greater need for proteolysis than bacteria that undergo only clonal division. For example, ClpX is essential in *S. meliloti* (73), which dramatically alters its replication capacity and morphology during execution of its developmental program during symbiosis. *Brucella abortus* relies on CpdR-dependent ClpXP activity to successfully replicate during macrophage infection, illustrating the need for proteolysis during the developmental transition to a virulent state for pathogenic bacteria (129). By this logic, the developmental transition to a

biofilm state for many bacteria would rely heavily on regulated protein degradation. Similarly, bacteria that respond to stresses that occur at timescales faster than cell division would also rely on the presence and activity of energy-dependent proteases to manage the dynamics of these responses. For example, DnaA degrades rapidly during immediate starvation in *Caulobacter* (46, 75, 76), an excellent mechanism to pause growth in nutrient-limiting conditions. Finally, slow-growing bacteria may depend on proteolysis even more because of the reduced dilution through cell division.

Perspective

Protein degradation is an essential process for replication and growth of *Caulobacter*. Because proteolysis is irreversible, cells must execute this process only when needed. This need could be for general protein quality control, upon stress or damaging conditions, during developmental transitions, or during cell cycle progression. Although much has been discovered about how protein degradation is controlled in *Caulobacter*, there are many outstanding questions for both immediate and future consideration.

The specific roles of AAA^+ proteases, such as Lon proteases or Clp proteases, during stress responses have been derived mainly from studies in other model bacteria. However, substrates for these proteases are not necessarily conserved in *Caulobacter* or vice versa, even though all bacteria must respond to similar stresses. Therefore, understanding how degradation of different substrates by different proteases in different bacteria occurs in response to the same stress will assuredly yield general insight into microbial stress responses.

Binding of the adaptor CpdR to ClpX primes the protease for recruitment of substrates or additional adaptors. How does the adaptor CpdR perform this function? RcdA and CpdR are conserved in many α-proteobacteria, but PopA is not (15, 95). What does RcdA/CpdR do in other bacteria? Are there proteins equivalent to PopA in function that serve to further adapt adaptors in other bacteria? Is there evidence for adaptor hierarchies in other bacteria during important cellular transitions?

Adaptors assemble on the protease to facilitate substrate delivery. How do these adaptors shield themselves from degradation by the protease? For *Caulobacter*, the CpdR adaptor is itself degraded in a ClpXP-dependent manner (59, 74), but whether degradation has any biological significance or whether adaptor degradation must be managed more broadly is currently unclear.

Finally, growing evidence indicates that energy-dependent degradation of key proteins by AAA^+ proteases is crucial for virulence in many pathogens (16, 56, 104, 129). Understanding how proteases can maintain their specificity through adaptors, stimulators, or other control is critical to developing new antibiotics or therapies to target regulated protein degradation.

SUMMARY POINTS

1. Proteolytic control is critical when *Caulobacter* deals with stress/damaging environment or during normal cell cycle progression.
2. Different proteases (Lon, ClpAP, and ClpXP) are responsible for managing different stress responses.
3. Many proteins are degraded during development and cell cycle progression.
4. A hierarchical assembly of adaptors coordinates destruction of key regulators specifically during the G1-to-S transition in *Caulobacter*.
5. Adaptor hierarchies may orchestrate proteolysis in other bacteria and organeller systems.

6. Protein degradation is particularly critical in bacteria that undergo rapid development transitions or that must respond to environmental insults at timescales shorter than cell division.
7. Energy-dependent proteases are excellent, emerging targets for the development of new antibiotics.

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