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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 will likely generalize to others.

Keywords

ClpAP; ClpXP; Lon; adaptor; cell cycle; AAA+ protease

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 will be the main proteases discussed in this review, but like most bacteria, *Caulobacter* contain other AAA+ proteases such as HslUV and FtsH, but 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, while 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, due 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 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 the ClpXP protease recognizes ssrA-tagged proteins in a highly specific manner with mutation of single residues within the tag resulting in a substantial loss of recognition (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 *E. coli* the stationary phase sigma factor RpoS is degraded by the ClpXP protease only in the presence of the RssB adaptor (9; 100; 132) and as will be 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 that leads to an arrest of cell cycle progression in toxic conditions (Figure 2) (63). Lon alone is incapable of robust DnaA degradation *in vitro*, but recognition of an unfolded polypeptide substrate results in allosteric activation of Lon and rapid degradation of 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 stationary phase, suggesting that ClpAP plays a crucial role during nutrient stress conditions (unpublished data- JL and PC). In *Caulobacter*, ClpAP degrades the flagella regulator FliF (49), the cytoskeletal proteins FtsZ and FtsA (130), and also serves as a redundant protease for DnaA (unpublished data- JL, LF and PC). Because ClpAP can degrade misfolded/

aggregated proteins (30), it is tempting to speculate that defects in *Caulobacter* due to loss of ClpA may also be due in part to a failure of responding properly to proteotoxic stresses.

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)(1). SocB binds the replication sliding clamp DnaN and arrests replication (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). 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*. Since 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 co-translationally 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 (67; 68), supporting a need for ribosome rescue and 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 have a functional SspB adaptor system (21; 77) but it is as yet unknown if 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 where 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, nonreplicative motile swarmer cells (SW) develop into nonmotile replication competent stalked cells (ST). 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/cell division, while the SW cell must first transition again into a ST cell. During this SW-ST transition, also called the G1-S transition, due 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 suggested with CcrM (131). In other cases oscillating protein levels can be driven principally by regulated proteolysis, where changes in 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 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 of these cases where changes in degradation are sufficient to drive changes in protein levels (44; 49; 62; 131). However, levels of ClpX, ClpP or Lon do not change during the cell cycle (62; 131). 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 will be 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 regulated through cell cycle-dependent phosphorylation (102). A parsimonious model to explain these results is that SciP degradation is simply controlled through its DNA binding state, where cell cycle-dependent changes in DNA binding protect this protein from degradation by Lon (Figure 3) (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). These examples are well characterized in eukaryotes where the lysosome is a dedicated organelle that removes bulk proteins that are 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-ST transition dephosphorylation of CpdR activates its ability to drive the localization of the ClpXP protease to the nascent stalked pole (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). 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 are needed for McpA turnover (Figure 4) (33; 58; 84). Taken together, these data pointed to a situation where CpdR controls ClpXP localization which is needed for regulated degradation of all ClpXP substrates, while 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 which 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 swarmer cells when ClpXP is delocalized (130). Finally, recent observations reconstituting protein degradation with purified protein components support a model where 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 *Caulobacter* cell cycle (64; 74; 115). 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 while 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 will be more completely addressed later.

Most intriguingly, CpdR is required for degradation of all known ClpXP substrates that are specifically destroyed during the SW-ST transition (3; 11; 58; 103) (see Table 1), while RcdA and PopA are required for only a subset of these substrates. For example, McpA degradation requires CpdR, but not RcdA nor PopA (33; 58; 84). Similarly, the cdG phosphodiesterase PdeA is degraded during the SW-ST transition dependent only on CpdR and ClpXP (3). Biochemical experiments showed that PdeA is not degraded by ClpXP alone, but 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 contained an N-terminal domain required for CpdR-dependent degradation and 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) (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 a weaker affinity ($K_D \sim 20$ μ M) (22). This scaffold allows for the robust tethering of a cargo substrate, while not gripping so tightly that substrate translocation is hindered. Based on 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 where CpdR acts as an adaptor by priming ClpX in order to generate a CpdR-ClpX state that is capable of binding PdeA and other substrates (Figure 5). 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 responsible directly for facilitating delivery of one class of substrates including PdeA and the chemoreceptor McpA (Figure 5) (74).

Recent studies indicate 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-terminus region of Spx to induce a conformational change which 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, while 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) (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 canonical adaptors, but instead of tethering directly to the ClpXP protease, as seen with SspB (13; 22; 31; 125), RcdA tethers only to a CpdR-primed ClpX (64). RcdA

expression peaks during SW-ST transition 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).

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). Based on 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, while the N-terminal receiver domains are responsible for additional interactions with proteins like RcdA (95). The working model is 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 will serve as an adaptor to RcdA for these substrates as well. PopA can also act as a competitive anti-adaptor for RcdA-dependent substrates such as 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 which 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 swarmer 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 frees 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-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 very highly related bacteria

(95). For example, in the plant symbiont *Sinorhizobium meliloti*, there are two orthologs of CpdR, but only one (CpdR1) exhibits 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 where 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 where adaptor mediated protein degradation appear to play important physiological roles.

The gram-positive bacterium *Bacillus subtilis* passes through many development stages during its normal life cycle such as competence, sporulation and contact-dependent differentiation (32; 42; 55; 65). During exponential growth, the MecA adaptor maintains low levels of the transcriptional factor ComK by promoting its destruction by the ClpCP protease (96; 123). When cells enter a higher-density state, the anti-adaptor ComS binds MecA, stabilizing ComK to induce expression of competence-related genes (Figure 7) (92; 123). Quality control during sporulation was also shown to be under adaptor-mediated control with the CmpA protein stimulating destruction of the coat assembly protein SpoIVA by ClpXP in unfit cells (Figure 7) (117). An adaptor for Lon has been shown to play an important role during differentiation into swarmer 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* will shed 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 comprised of the ClpF and ClpS1 proteins was found to stimulate degradation of GluTR, a key enzyme in tetrapyrrole synthesis in

chloroplasts (Figure 7) (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 the substrate whose 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 was 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 finding 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) was a key result 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 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*, while 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 adaptors/substrates 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 pleiotropic 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 incredibly sick (1) (unpublished data - RHV and PC), supporting a critical role for ClpXP activity beyond preventing SocB toxin buildup. Indeed, protease-trapping experiments identify hundreds of candidate substrates in *Caulobacter*, including many essential proteins (11).

This observation begs 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, levels of a protein present at 120 copies / cell will be reduced to 15 copies / cell in one hour if protein production is halted, an order of magnitude change without the need for proteolysis. By contrast, every *Caulobacter* swarmer 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). Therefore 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 would suggest that many bacteria that undergo developmental programs without cell division would have a need for proteolysis more than bacteria that only undergo clonal division. 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 is degraded 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 due to reduction of dilution through cell division.

Perspective

Protein degradation is an essential process for replication and growth of *Caulobacter*. Since 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. While much has been discovered about how protein degradation is controlled in *Caulobacter*, there are many outstanding questions for both immediate and future considerations, including:

The specific roles of AAA+ proteases like Lon or Clp proteases during stress responses have been mainly derived from studies in other model bacteria. However, substrates for these proteases are not necessarily conserved in *Caulobacter*, nor 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 do 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.

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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.

Figure 2.

In *Caulobacter*, AAA+ proteases contribute to normal growth and stress responses. A. 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 comprised of several proteins including the ATP hydrolyzing subunit DnaX. In *Caulobacter*, DnaX is processed by ClpXP to form 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. B. Proteotoxic stress results in protein misfolding. In *Caulobacter*, the Lon protease rapidly degrades DnaA during stress conditions, resulting in cell cycle arrest. Reconstitution experiments support a model where buildup of misfolded proteins 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.

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-ST (G1-S) transition, Class II proteins are more abundant (more stable) in ST cells, while Class III proteins are preferentially reduced in SW cells. Examples of each class are shown. See Table I for a more complete listing of proteins and proteases. There are several models for how proteins are selectively degraded during the cell cycle, including cell cycle dependent inhibition (B), changes in localization (C), or cell cycle dependent activation.

Figure 4.

Levels of ClpXP substrates change during cell cycle progression, but levels of ClpXP remain constant. Purified swarmer cells are released into fresh media to initiate synchronized growth. Aliquots taken during synchronized growth are probed with antibodies against the chemoreceptor McpA, transcription factors TacA and CtrA, ClpX, and ClpP (adapted from (64)). Cell cycle-dependent phosphorylation of CpdR (58) is shown as +/- . Degradation of McpA relies only on CpdR, while TacA requires both CpdR and RcdA. CtrA additionally requires PopA for cell cycle regulated degradation.

Figure 5.

Dephosphorylation of CpdR primes ClpXP for substrate recognition. **A.** CpdR binds directly to the N-terminal domain (NTD) of ClpX, facilitating the recognition of protease substrates (PdeA, McpA, etc). Phosphorylation of CpdR prevents binding to ClpX. Importantly, CpdR does not seem to directly bind its cargo substrates with any detectable affinity (74). **B.** RcdA directly binds substrates such as TacA facilitating their degradation by ClpXP protease. Here RcdA acts as a scaffolding adaptor delivering the substrate only to a protease that is first primed by CpdR. **C.** By contrast, canonical scaffolding adaptors, such as SspB, bind strongly to their cargo and tether substrates directly to the ClpXP protease.

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 (marked by pink 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) dependent adaptor PopA binds the adaptor RcdA to deliver substrate CtrA to ClpXP protease.

Figure 7.

Adaptors in other system: **A.** Competence development in *B. subtilis*. The binding of MecA adaptor to the ClpC ATPase activates the ClpCP protease. Once activated, the adaptor MecA binds and facilitates ComK degradation during exponential growth of *B. subtilis*. As the cells reaches to 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 leading to the initiation 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 ClpCRP protease for degradation in chloroplast (91).

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 of 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.

Table 1

A set of cell cycle-dependent protease substrates in *Caulobacter*. This table includes proteins that are degraded selectively during the cell cycle and cell cycle regulated substrates whose levels *in vivo* are protease dependent.

Substrate	Function	Protease responsible	References
CtrA	Replication initiation inhibitor and transcriptional regulator ^{**}	ClpXP	(21; 29; 115)
TacA	Transcriptional regulator ^{**}	ClpXP	(11; 64)
CC3144	Unknown function [*]	ClpXP	(64)
PdeA	Cyclic di-GMP phosphodiesterase ^{**}	ClpXP	(3; 74)
McpA	Transmembrane chemoreceptor ^{**}	ClpXP	(74; 120)
McpB	Cytoplasmic chemoreceptor	ClpXP	(99)
CpdR	Single-domain response regulator/adaptor [*]	ClpXP	(59; 74)
GdhZ	NAD-dependent glutamate dehydrogenase	ClpXP	(8)
KidO	NAD(H)-binding oxidoreductase homolog [#]	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)
FlhF	MS ring protein	ClpAP	(49)
FtsZ	Cell division cytoskeletal protein ^{**}	ClpAP/ ClpXP	(71; 130)
FtsA	Cell division protein ^{**}	ClpXP/ ClpAP	(81; 130)
FtsQ	Cell division protein [#]	unknown	(81)
DnaA	DNA replication initiator [*]	Lon	(24; 63)
CerM	DNA-methyltransferase [*]	Lon	(63; 131)
SciP	Small CtrA inhibitory protein ^{**}	Lon	(44; 45)
GcrA	Co-factor for sigma70 [#]	unknown	(24; 53)

^{*} indicates substrates validated by *in vitro* reconstitution experiments.

[#] indicates substrates that are degraded in cell cycle-dependent manner when expressed constitutively.