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Cell cycle-dependent adaptor complex for ClpXP-mediated proteolysis directly integrates phosphorylation and second messenger signals

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The cell-division cycle of *Caulobacter crescentus* depends on periodic activation and deactivation of the essential response regulator CtrA. Although CtrA is critical for transcription during some parts of the cell cycle, its activity must be eliminated before chromosome replication because CtrA also blocks the initiation of DNA replication. CtrA activity is down-regulated both by dephosphorylation and by proteolysis, mediated by the ubiquitous ATP-dependent protease ClpXP. Here we demonstrate that proteins needed for rapid CtrA proteolysis in vivo form a phosphorylation-dependent and cyclic diguanylate (cdG)-dependent adaptor complex that accelerates CtrA degradation in vitro by ClpXP. The adaptor complex includes CpdR, a single-domain response regulator; PopA, a cdG-binding protein; and RcdA, a protein whose activity cannot be predicted. When CpdR is unphosphorylated and when PopA is bound to cdG, they work together with RcdA in an all-or-none manner to reduce the K_m of CtrA proteolysis 10-fold. We further identified a set of amino acids in the receiver domain of CtrA that modulate its adaptor-mediated degradation in vitro and in vivo. Complex formation between PopA and CtrA depends on these amino acids, which reside on alpha-helix 1 of the CtrA receiver domain, and on cdG binding by PopA. These results reveal that each accessory factor plays an essential biochemical role in the regulated proteolysis of CtrA and demonstrate, to our knowledge, the first example of a multiprotein, cdG-dependent proteolytic adaptor.

The alpha-proteobacterium *Caulobacter crescentus* is a powerful model organism for understanding cell polarity, differentiation, and cell-cycle regulation in bacteria (1). Each *Caulobacter* cell division is asymmetric, yielding a motile swarmer cell (SW) and a sessile stalked cell (ST). The stalked cell immediately initiates chromosome replication and enters a new division cycle, whereas the swarmer cell must first differentiate into a stalked cell before undergoing cell division (Fig. 1). An elaborate network of two-component signaling proteins orchestrates the *Caulobacter* cycle of division and development (2). The essential DNA-binding response regulator CtrA is at the center of this regulatory scheme, activating or repressing the transcription of >100 genes that are required for cell division, motility, DNA methylation, and other processes (3). Because CtrA also inhibits the initiation of chromosome replication (4, 5), its activity must be temporarily eliminated at the G1–S cell-cycle transition for DNA replication and the subsequent cell division to occur. Inactivation of CtrA is achieved through two redundant mechanisms: dephosphorylation (6) and proteolysis by the protease ClpXP (7).

ClpXP is a highly conserved ATP-dependent protease crucial for protein quality control and in regulated degradation of key substrates to implement developmental switches and adapt to external cues (8). The barrel-shaped oligomer of ClpP contains hydrolysis active sites on its inner surface. To access these sites, substrates are recognized, unfolded, and translocated into the ClpP chamber by a ring-shaped hexamer of the ATPase ClpX.

Although ClpX alone recognizes some substrates, the degradation of other substrates requires or is enhanced by adaptor proteins (9). For example, the *Escherichia coli* protein SspB is an adaptor for the degradation of substrates with a C-terminal ssrA peptide (10), which arise during failed translation events (11). SspB binds to ClpX and to the ssrA peptide, increasing the local effective substrate concentration through tethering (12, 13). Adaptors themselves can be tightly regulated; for example, the response regulator RssB acts as an adaptor for the rapid ClpXP-mediated proteolysis of the stationary phase sigma factor RpoS during exponential growth in *E. coli* (14). In stationary phase or in response to specific stresses, up-regulation of antiadaptor proteins that block RssB from binding RpoS drives a surge in RpoS levels (15, 16).

In the *Caulobacter* cell cycle, CtrA proteolysis is spatially and temporally restricted such that it occurs only during the G1–S transition, either during the development of a motile swarmer cell into a sessile stalked cell, or in the stalked compartment of the late predivisional cell (Fig. 1; ref. 17). In addition to ClpX and ClpP, which are present throughout the cell cycle (7), the accessory proteins RcdA, CpdR, and PopA, and the small molecule cyclic di-GMP (cdG), are required for the rapid degradation of CtrA in vivo (18–20).

RcdA is conserved in some alpha-proteobacteria, and its transcription is regulated directly by CtrA in *Caulobacter* (18). RcdA exists as a dimer, with each monomer comprising a three-helix

Significance

Controlled degradation of specific proteins is used by all organisms to change cell behavior in response to internal or external cues. Because ATP-dependent proteases, such as ClpXP, have a broad range of targets, accessory proteins called adaptors are often necessary for selective substrate proteolysis. Here we show that three proteins work together as a multicomponent adaptor to stimulate the degradation of a key regulatory protein, CtrA, in *Caulobacter crescentus*. The adaptor is only functional when one of the components, CpdR, is unphosphorylated and when another component, PopA, is bound to the signaling molecule cyclic diguanylate. These features ensure that CtrA is only proteolyzed during a specific window in the *Caulobacter* cell-division cycle.

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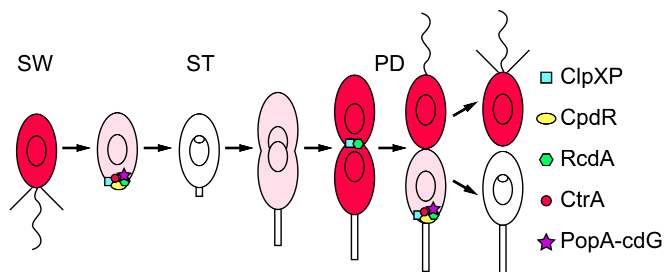

Fig. 1. CtrA dynamics during the *Caulobacter* cell cycle. SW, swarmer cell; ST, stalked cell; PD, predivisional cell. Straight lines represent pili. Wavy lines represent flagella. Filled circles denote nonreplicating chromosomes. Theta structures denote replicating chromosomes. Red and pink shading represent CtrA protein.

bundle connected by disordered loops and a disordered C-terminal peptide necessary for regulated CtrA proteolysis (21). CpdR is a single-domain response regulator that is phosphorylated by the histidine kinase CckA and the histidine phosphotransferase ChpT, which also act on CtrA (22). However, CpdR must be unphosphorylated to promote CtrA degradation (19). This signaling logic dictates that when CtrA is phosphorylated and active, it will also be stabilized due to the phosphorylation of CpdR. Before S phase, when both are dephosphorylated, the activity and stability of CtrA are concomitantly reduced (6, 19). PopA is composed of two tandem receiver domains and a C-terminal diguanylate cyclase (DGC) domain. The DGC domain of PopA is inactive, but cdG binding at an allosteric site is required for PopA to stimulate CtrA proteolysis in vivo (20). PopA is thought to link CtrA proteolysis to cellular cdG levels, which transiently increase during swarmer cell differentiation (23, 24).

During CtrA degradation, ClpXP, the accessory proteins, and CtrA all accumulate at the stalked pole of the predivisional cell, or at the nascent stalked pole of the developing swarmer cell (18–20, 25), leading to a model in which the accessory proteins function in CtrA proteolysis merely by ensuring that the protease and substrate are concentrated in the same region of the cell (9, 18, 19). However, some mutations in RcdA that prevent CtrA accumulation at the pole do not inhibit the degradation of CtrA during swarmer cell differentiation, suggesting a dispensable role for localization in regulated CtrA proteolysis (21). Despite this elaborate system for regulating CtrA proteolysis in vivo, ClpXP can degrade CtrA in vitro without any additional factors with rates theoretically rapid enough to permit clearing of CtrA before S phase (26). The apparent discrepancy between cellular and in vitro conditions has raised several questions about the precise functions of the accessory factors in CtrA degradation.

Here, we used a bioinformatics approach to identify specific residues in the CtrA receiver domain that are needed for rapid CtrA proteolysis in vivo, but did not affect degradation by ClpXP alone in vitro. Immunoprecipitation experiments show that these residues are needed for the interaction of CtrA with PopA and RcdA in vivo, leading us to reconstitute the entire CtrA proteolytic system in vitro. We find that CpdR, RcdA, PopA, and cdG work together to accelerate CtrA degradation in vitro. Addition of these accessory factors reduces the K_m of the proteolytic reaction 10-fold, while leaving v_{max} unchanged, consistent with the accessory factors collectively acting as an adaptor for CtrA degradation. Strikingly, this adaptor complex is essential for degradation when CtrA is poorly recognized by ClpXP alone, such as when CtrA is bound to DNA (27). We dissect the adaptor complex to show that RcdA binds PopA constitutively, but PopA binding to CtrA is cdG-dependent. Together, our results reveal the mechanistic framework for how an adaptor complex responds to phosphorylation cues and second messengers to promote robust degradation of an essential cell-cycle regulator, reconciling in vitro and cellular observations of CtrA degradation.

Results

Identification of Degradation-Enhancing Amino Acids in the CtrA Receiver Domain. CtrA contains two internal signals that determine its pattern of cell cycle-dependent proteolysis. One determinant consists of two alanine residues at the C terminus, which are required for direct recognition by ClpX (17, 26, 28), and the other signal resides somewhere in the first 56 amino acids of the CtrA receiver domain (25). We reasoned that amino acids in CtrA that are important for degradation would be coconserved with *rcdA* and *cpdR*, which are found in ∼1/3 of sequenced α-proteobacteria (29). We therefore aligned CtrA protein sequences from genomes in 26 different genera of the α-proteobacteria (Fig. S1; ref. 30) and identified residues in the first half of the CtrA receiver domain that are highly conserved in genomes containing homologs of *rcdA* and *cpdR*, but that are divergent in bacteria that lack these genes. We then mapped these residues onto a model of the CtrA receiver domain based on the structure of the response regulator Spo0F (Protein Data Bank ID code 1NAT; ref. 31). Six of the residues we identified are predicted to lie on the exposed surface of the first alpha-helix (α1) of CtrA (S10, A11, Q14, K21, S22, and E23), whereas two of the residues are predicted to be partially or completely buried in the interior of the protein (F25 and G40; Fig. 2*A*).

Based on this analysis, we examined the in vivo degradation pattern of a chimeric protein containing segments of CtrA and CzcR, a nondegradable CtrA ortholog (25). CtrA–RD+15, which contains the receiver domain and the last 15 amino acids of CtrA, but lacks the DNA-binding domain, has the same cell-cycle pattern of abundance as the full-length CtrA protein (Fig. S2*B*). Whereas CtrA–RD+15 is degraded rapidly in developing swarmer cells, the CzcR receiver domain fused to the CtrA C terminus (CzcR–RD+15) is not specifically degraded at this time (Fig. S2*B*; ref. 25). We sought to confer regulated degradation upon CzcR–RD+15 by replacing residues 9–31 with the corresponding sequences from CtrA, which includes the α1 and β2 secondary structural elements (Fig. S2*A* and Fig. 2*A*). The resulting chimeric protein α1-RD+15 was degraded during swarmer cell differentiation, similar to full-length, wild-type

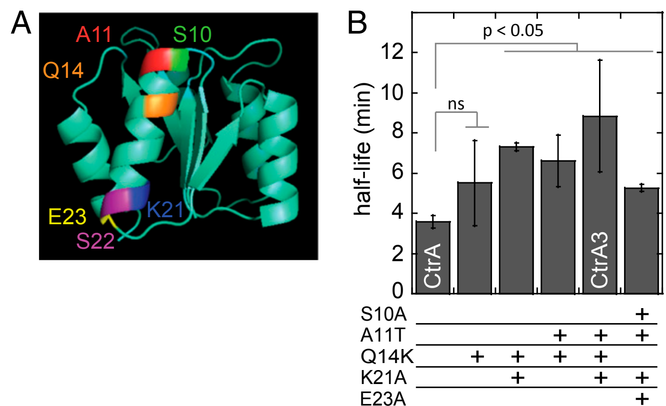

Fig. 2. Residues in α1 of the CtrA receiver domain are needed for efficient proteolysis. (*A*) Model of the CtrA receiver domain highlighting surface-exposed residues that co-occur in α-proteobacterial genomes with *rcdA* and *cpdR*. (*B*) Half-lives of the indicated CtrA–RD+15 variants measured during swarmer cell development. Error bars represent SDs. Each construct was compared with CtrA–RD+15 with no amino acid substitutions (Student's *t* test, two-tailed, nonpaired data). ns, not significantly different from wild-type.

CtrA present in the same strain (Fig. S2*B*). This result indicates that a determinant in residues 9–31 of CtrA, along with the CtrA C terminus, can confer regulated degradation on the otherwise stable CzcR receiver domain. Interestingly, although CzcR is not degraded, it only differs from CtrA at three of the residues on α1 that are coconserved with *rcdA* and *cpdR*. Thus, we predict that these positions in CtrA (A11, Q14, and K21) are important for its cell cycle-regulated proteolysis.

CtrA Residues Co-Occurring with RcdA and CpdR Are Important for Rapid Proteolysis and Interactions with Accessory Factors in Vivo. To assess the contribution of the residues identified above, we made single and multiple amino acid substitutions within CtrA–RD+15, changing each residue to the most common residue occurring at the same position in CtrA homologs from bacteria lacking *rcdA* and *cpdR*. Mutation of all six residues yielded a poorly soluble protein that formed single, randomly placed fluorescent aggregates in vivo (Fig. S3), distinct from the discrete polar foci seen with wild-type CtrA fusions (25). By contrast, mutating a subset of these residues produced well-behaved proteins both in vivo and in vitro, so we focused on the characterization of these less-modified variants.

We made the indicated combinations of mutations within CtrA–RD+15 and measured their degradation during swarmer cell development (Fig. 2*B*). In these experiments, wild-type CtrA–RD+15 was degraded with a half-life of 3.6 ± 0.3 min. CtrA variants with one or two amino acid substitutions in A11, Q14, or K21 showed moderate degradation defects, and the variant containing three substitutions—A11T, Q14K, and K21A (CtrA3–RD+15)—was degraded the most slowly, with a half-life of 8.5 ± 2.6 min. A variant with four substitutions—S10A, A11T, K21A, and E23A—had a shorter half-life than CtrA3–RD+15, underscoring the importance of the Q14 residue for cell cycle-regulated degradation (Fig. 2*B*). To verify that these residues are also important in the context of the full-length substrate, we placed the A11T, Q14K, and K21A mutations in CtrA to create CtrA3. Consistent with the results using the truncated reporters (Fig. 2*B*), when CtrA3 was expressed as the only variant of CtrA in the cell, it was degraded more slowly than wild-type CtrA (Fig. S4).

Coimmunoprecipitation (co-IP) experiments have shown that CtrA associates directly or indirectly with ClpX and RcdA in vivo (18) and that CpdR associates with ClpXP (19). Because the amino acid substitutions in CtrA3 impair proteolysis, we examined interactions with CtrA variants, ClpX, RcdA, and PopA in vivo. CtrA– or CtrA3–FLAG was expressed from the *xylX* locus in a strain lacking the native copy of *ctrA*. In a control strain, the unrelated protein Hfq–FLAG was expressed from the *hfq* locus (32). The FLAG-tagged proteins were immunoprecipitated from cell lysates, and the lysates and immunoprecipitates were analyzed by Western blotting.

We confirmed that RcdA and ClpX coprecipitate with wild-type CtrA, and demonstrated, to our knowledge, for the first time that PopA associates directly or indirectly with CtrA in vivo (Fig. 3). As expected, Hfq–FLAG failed to interact with any of the tested proteins. When CtrA3–FLAG was precipitated, less RcdA and PopA were bound, even though the levels of CtrA3–FLAG, RcdA, and PopA in the cell lysate were comparable to those in the lysate containing wild-type CtrA–FLAG. In contrast, CtrA3 efficiently coprecipitated ClpX, suggesting that the amino acid substitutions in CtrA3 affect accessory-specific protein–protein interactions (Fig. 3). Because CpdR is known to precipitate with ClpX (19), we would expect CpdR to be coprecipitated as well, but visualization of CpdR by Western blotting was too inconsistent to observe this interaction.

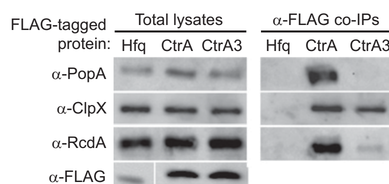

Fig. 3. Conserved residues in the CtrA receiver domain are necessary for robust interactions with proteolytic accessory factors in vivo. Hfq, CtrA, and CtrA3 were fused at their C termini to the 3xFLAG epitope and expressed in *Caulobacter* strains lacking the untagged version of Hfq or CtrA. After formaldehyde cross-linking, cells were lysed and immunoprecipitated with anti-FLAG antibodies. Total cell lysates and coimmunoprecipitates (co-IPs) from equal numbers of cells were analyzed by SDS/PAGE and Western blotting.

CtrA3 Is Impaired Specifically in Adaptor-Mediated Proteolysis by ClpXP. We purified the full-length CtrA and CtrA3 proteins to compare their degradation by ClpXP in vitro. Consistent with the co-IP assays above, CtrA3 was degraded at the same rate as CtrA by ClpXP alone (Fig. 4*A* and Fig. S5*A*). Because CtrA3 was specifically impaired in interactions with RcdA and PopA in vivo, we hypothesized that CtrA and CtrA3 may be degraded at different rates by ClpXP in the presence of the entire suite of accessory factors (i.e., CpdR, RcdA, PopA, and cdG). Similar to previous reports (26), 1 μM CtrA was degraded with a half-life of 8.8 ± 1.8 min by ClpXP alone. Interestingly, the addition of RcdA, CpdR, PopA and cdG reduced the CtrA half-life to 3.8 ± 0.4 min. Degradation of CtrA3 was also moderately enhanced by addition of the accessory factors (half-life 5.4 ± 0.6 min; Fig. 4*A* and Fig. S5*A*). Consistent with in vivo results (33), CpdR was degraded slowly in these assays, whereas RcdA and PopA were stable (Fig. S6).

Because the rapid degradation of CtrA by ClpXP alone made the stimulatory effect of the accessory proteins difficult to assess, we took advantage of conditions in which CtrA proteolysis is inhibited to measure wild-type and mutant CtrA degradation. DNA fragments containing CtrA-binding sites, such as a 50-bp fragment of the *pilA* promoter (P*pilA*), reduce the rate of CtrA degradation in vitro by ClpXP alone (27). The protective effect of CtrA binding sites is amplified in the presence of the protein SciP (27), which acts as a transcriptional coregulator for a subset of genes in the CtrA regulon (34, 35). CtrA maintains some ability to bind its specific DNA sites, even when unphosphorylated (4). Based on known binding constants for CtrA to DNA (36), we chose concentrations of CtrA and P*pilA* such that we expect ∼90% of the initial pool of CtrA molecules to be bound to DNA. Because the addition of SciP increases binding of CtrA to DNA by more than fivefold (34, 35), we infer that in our conditions, almost all of CtrA is in the DNA-bound form.

In the presence of SciP and a P*pilA* DNA fragment (5 μM each), ClpXP degraded CtrA and CtrA3 (2 μM) with similar rates (half-lives 175 ± 21 min and 168 ± 5 min, respectively), but substantially more slowly than in reactions lacking SciP and DNA (Fig. 4*B*; compare with Fig. 4*A*). Importantly, the addition of CpdR, RcdA, PopA, and cdG shortened the half-life of CtrA by 4.7-fold to 37 ± 2 min, whereas the enhancement of CtrA3 degradation was more modest (half-life 52 ± 7 min), supporting a role for residues in the α1 helix of CtrA in interacting with one or more of the proteolytic accessory proteins (Fig. 4*B* and Fig. S5*B*). These results, to our knowledge, demonstrate for the first time that the accessory factors important for cell cycle-regulated CtrA proteolysis in vivo play a direct biochemical role in stimulating CtrA degradation.

Accessory Factors Work in an All-or-None Manner to Reduce the K_m of ClpXP for CtrA. Next, we used the substrate GFP–CtrA–RD+15 in a fluorescence-based proteolysis assay to examine the effects of omitting single accessory factors. GFP–CtrA–RD+15 was degraded by ClpXP in an ATP-dependent manner (Fig. 4*C*). The addition of all accessory factors accelerated CtrA degradation, but omission of any single factor returned the rate of CtrA

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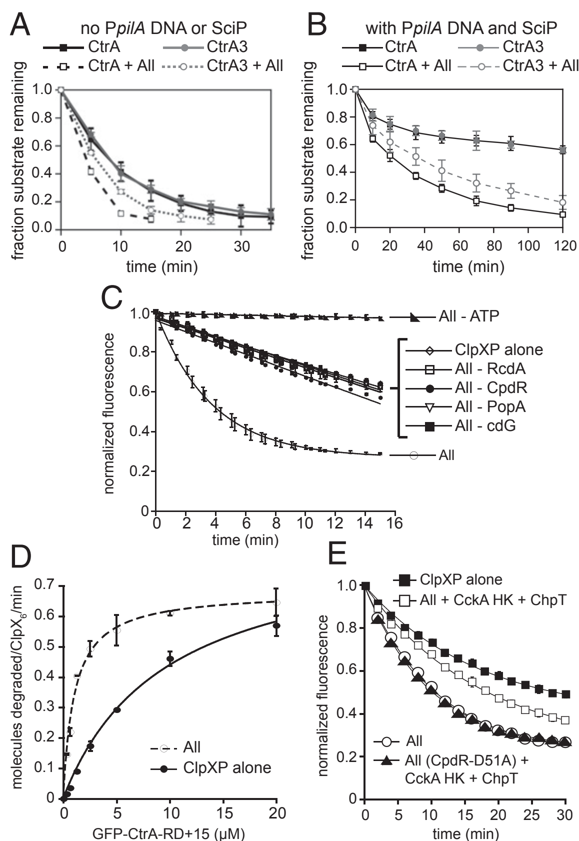

Fig. 4. Accessory proteins and cdG stimulate CtrA proteolysis in vitro. (*A*) In vitro degradation of CtrA or CtrA3 visualized by SDS/PAGE. Initial substrate concentration was 1 μM CtrA or CtrA3. Each reaction included 0.3 μM $ClpX_6$, 0.6 μM $ClpP_{14}$ and an ATP regeneration system. Reactions labeled All also included 20 μM cdG, 1 μM CpdR, 1 μM RcdA, and 1 μM PopA. (*B*) Degradation of CtrA and CtrA3 in the presence of SciP and P*pilA*. Initial substrate concentration was 2.0 μM CtrA or CtrA3. Each reaction included 0.25 μM $ClpX_6$, 0.5 μM $ClpP_{14}$, 5 μM P*pilA* DNA, 5 μM SciP, and an ATP regeneration system. Reactions labeled All also contained 20 μM cdG, 1 μM CpdR, 1 μM RcdA, and 1 μM PopA. Quantification of substrate band intensity was plotted over time as the fraction of substrate remaining. (*C*) Degradation of GFP–CtrA–RD+15 was monitored by following loss of fluorescence. Reactions contain 2 μM substrate, 0.4 μM $ClpX_6$, 0.8 μM $ClpP_{14}$, and an ATP regeneration system (except reactions where ATP was omitted). Reactions labeled All also include 1 μM RcdA, 2 μM CpdR, 1 μM PopA, and 20 μM cdG. Single components were omitted from All reactions as indicated. (*D*) The GFP–CtrA–RD+15 concentration was varied as indicated both in the absence (ClpXP alone) and presence (All) of accessory factors as in *C*. Data were fit to the Michaelis–Menten equation. (*E*) Unphosphorylated CpdR is needed for maximal stimulation of GFP–CtrA–RD+15 degradation. Phosphorylation of CpdR (*SI Methods*) reduces accessory-enhanced degradation of GFP–CtrA–RD+15, whereas a nonphosphorylatable CpdR variant (CpdR–D51A) was resistant to this inactivation. Error bars represent SDs.

proteolysis to the "unstimulated" rate seen with ClpXP alone (Fig. 4*C*). These results indicate that the accessory factors must work in an all-or-none fashion to accelerate CtrA degradation.

Fitting initial degradation rates to Michaelis–Menten kinetics, we found that ClpXP alone degraded GFP–CtrA–RD+15 with a v_{max} of 0.68 ± 0.04 molecules per $ClpX_6$ per min and a K_m of 9.9 ± 2.1 μM (Fig. 4*D*). Addition of CpdR, RcdA, PopA, and cdG reduced the K_m nearly 10-fold to 1.1 ± 0.2 μM, but stimulated v_{max} only minimally to 0.87 ± 0.1 molecules per $ClpX_6$ per min. The reduction in K_m without a significant impact on enzyme turnover is consistent with the accessory proteins functioning as a proteolytic adaptor, binding both the substrate and protease to effectively increase local concentration of the target substrate.

We favor an adaptor model for the function of the accessory factors, where specific tethering of CtrA to the ClpXP complex drives rapid degradation, but it is possible that one or more of the adaptor components generally stimulate ClpXP activity. In support of our model, degradation of an unrelated ClpXP substrate, GFP appended with a C-terminal ssrA tag (26, 37), was not affected by addition of the accessory factors (Fig. S7), arguing against a general stimulatory role that affects all ClpXP substrates.

Phosphorylation and cdG Levels Control CtrA Degradation Through the Adaptor Complex. Rapid CtrA degradation during swarmer cell differentiation is triggered by two events, the dephosphorylation of CpdR and an increase in cdG levels, that occur before S phase (19, 24, 38, 39). As shown above, degradation of GFP–CtrA–RD+15 was enhanced by addition of all accessory factors. Phosphorylation of CpdR by addition of CckA and ChpT resulted in an intermediate degradation rate, presumably because a fraction of the CpdR was inactive due to phosphorylation (Fig. 4*E*; ref. 22). However, because CtrA is also a target of CckA and ChpT, the inhibition of proteolysis could have been caused by changing the phosphorylation state of GFP–CtrA–RD+15. Importantly, substituting the unphosphorylatable protein CpdR–D51A for CpdR resulted in enhanced degradation, even in the presence of CckA and ChpT (Fig. 4*E*), indicating that phosphorylation of CpdR can modulate CtrA proteolysis in vitro in the presence of all of the accessory factors.

Next, we sought to dissect the binary interactions that comprise this multiprotein adaptor complex. In particular, bacterial two-hybrid (BACTH) studies had demonstrated a direct interaction between PopA and RcdA and between CpdR and ClpX (20, 39). Surprisingly, no studies have demonstrated a direct interaction between CtrA and any component of the proteolytic machinery. However, it is assumed that ClpX must interact with CtrA, because ClpXP alone degrades CtrA.

We performed coaffinity purification assays using His_6-tagged CtrA variants to identify a member of the proteolytic complex that directly recognizes CtrA. His_6–CtrA3 contains the A11T, Q14K, and K21A substitutions, whereas His_6–CtrA–DD replaces the two alanines at the C terminus of CtrA with aspartic acids, resulting in a nondegradable protein (17). We used the unrelated His_6-tagged EnvZ histidine kinase domain (EnvZ HK) as a negative control bait protein. PopA bound directly to His_6–CtrA and His_6–CtrA–DD, but only in the presence of cdG. In contrast, His_6–CtrA3 bound PopA poorly with background levels similar to His_6–EnvZ HK, even when cdG was included (Fig. 5*A*). Using His_6–PopA or –EnvZ HK as bait, we confirmed that RcdA and PopA interact directly (20), but this interaction was independent of cdG (Fig. 5*B*). Together, our results show how an adaptor complex crucial for CtrA degradation during the cell cycle can directly respond to both phosphorylation and second messenger cues.

Discussion

A major paradox in the study of *Caulobacter* cell-cycle regulation has been that CtrA proteolysis is dependent on additional regulatory factors in vivo, but CtrA can be degraded by ClpXP alone in vitro. Here we demonstrate that the accessory proteins RcdA, CpdR, and PopA work together in vitro to accelerate CtrA degradation by ClpXP. Both unphosphorylated CpdR and cdG-bound PopA are needed for maximal stimulation of CtrA proteolysis, providing a direct biochemical link to the two cellular events that trigger CtrA degradation in vivo: the dephosphorylation of CpdR (19, 22) and a transient increase in cellular levels of cdG (24, 39). The fact that each of the accessory factors is needed to stimulate CtrA proteolysis in vitro indicates that each one plays a direct biochemical role in the reaction, rather than functioning as localization factors that only bring the substrate and protease together in a cellular context, as has been proposed (9, 18, 19).

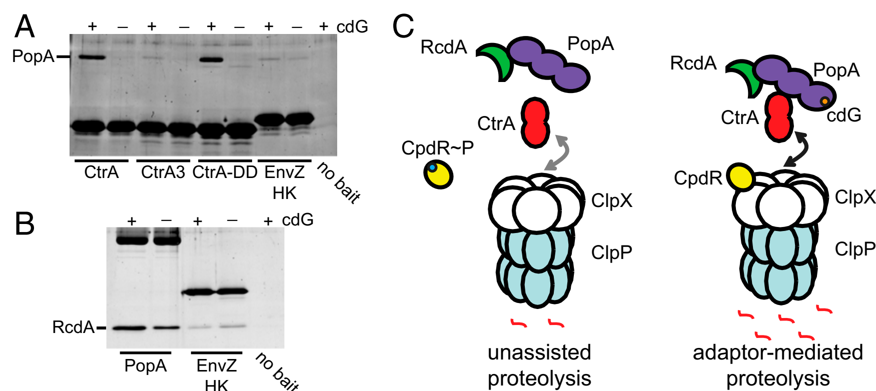

Fig. 5. Direct interactions between CtrA, PopA, and RcdA support a cdG-dependent adaptor model for stimulation of CtrA proteolysis. (*A*) His_6-tagged bait proteins were incubated with Ni-NTA resin, PopA, and 20 μM cdG where indicated. Stable complexes were eluted and analyzed by SDS/PAGE and Lumitein staining. (*B*) The indicated His_6-tagged bait proteins (PopA and EnvZ HK) were incubated with Ni-NTA resin, RcdA, and 20 μM cdG where indicated. Protein complexes were visualized as in *A*. (*C*) Models of unassisted and adaptor-mediated CtrA proteolysis.

A swarmer cell contains ~9,000 copies of CtrA, corresponding to a concentration of ~30 μM (40). CtrA proteolysis by ClpXP alone could proceed rapidly at first, but it would slow when CtrA levels fall below the unassisted K_m of ~10 μM, as measured here. The adaptor activity of the accessory factors could sustain a high rate of proteolysis, even after most of the CtrA has been degraded, ensuring its complete clearance. Given that CtrA binds the *Caulobacter* origin of replication with nanomolar affinity (36), even trace amounts of active CtrA could interfere with replication initiation, suggesting a need for complete proteolysis. In support of this model, a CtrA variant that is unable to strongly bind PopA (Figs. 3 and 5) is only moderately impaired with regard to adaptor-enhanced proteolysis (Figs. 2 and 4), suggesting that even weak substrate binding to the adaptor complex—such as would be seen at low CtrA concentrations—is sufficient for robust degradation by the adaptor-assisted ClpXP protease.

When degradation is unimpeded, the accessory factors have only a modest (approximately twofold) effect on the half-life of CtrA. However, when CtrA degradation is slowed, such as when it is bound to DNA and SciP, the addition of the accessory factors reduces the half-life of CtrA almost fivefold. Thus, accessory factors play a more crucial role when CtrA degradation by ClpXP is inhibited. Our results suggest a possible route for this inhibition under cellular conditions, in which at least a fraction of the CtrA molecules are bound to specific sites on the chromosome. It remains to be determined whether ClpXP and the accessory factors merely compete with DNA and SciP for binding to CtrA, or whether the proteolytic complex can actively remove CtrA from DNA–SciP complexes.

The specific architecture of the proteolytic complex remains undetermined, but, based on demonstrated pairwise interactions among the components, we can propose a model of adaptor-mediated CtrA proteolysis (Fig. 5*C*). CpdR binds directly to ClpX in BACTH assays (20), and studies in *Caulobacter* indicated that only unphosphorylated CpdR stimulates CtrA proteolysis (20). BACTH assays also found that PopA interacts directly with RcdA (21). Using purified proteins, we show here that the PopA–RcdA interaction is independent of cdG, whereas a direct interaction between PopA and CtrA requires cdG. In addition, our work reveals α1 of the CtrA receiver domain as a crucial point of interaction with PopA. Our results are most consistent with a model in which a complex of RcdA/PopA·cdG/CtrA is recruited to ClpX in a manner dependent on unphosphorylated CpdR. Alternatively, the binding of PopA could change the conformation of CtrA to make its C-terminal degradation signal more accessible to ClpX. Although we did not directly address protein–protein interactions of CpdR in this study, prior BACTH assays and the fact that CpdR acts alone to accelerate ClpXP-mediated degradation of PdeA (39) strongly suggest that CpdR can bind ClpX independently of RcdA or PopA. Additional work, based on the framework established here, is needed to further define the structure of the complex.

The first α-helix of a response regulator receiver domain is critical for proper interactions with its cognate histidine kinase or phosphotransferase (41–43). In this work, we identified residues within α1 of the CtrA receiver domain that are needed for binding PopA to facilitate CtrA degradation. It is possible that the CtrA phosphotransferase ChpT could compete with PopA–CtrA binding and inhibit CtrA proteolysis. It is also possible that CtrA3, which is deficient in PopA binding, might be phosphorylated at a lower level than wild-type CtrA. Additional work, including quantification of cellular ChpT and PopA levels, is needed to test these hypotheses. However, our observation that either CtrA3 or CtrA3–FLAG can sustain growth indicates that CtrA3 is not completely deficient in phosphorylation by ChpT.

Finally, we note that, although *cpdR*, *rcdA*, and *popA* are necessary for regulated CtrA proteolysis in *Caulobacter*, obvious *popA* orthologs are found in only a few α-proteobacteria closely related to *C. crescentus.* When fused to the C-terminal degradation signal for *Caulobacter* CtrA, the receiver domains of CtrA homologs from some α-proteobacteria are competent to specify cell cycle-regulated proteolysis in *Caulobacter* (25). It is unknown whether these CtrA homologs are degraded in a regulated manner in their native hosts, but if they are, then the process must not require a strict PopA ortholog. Given that PopA is a paralog of PleD, a DGC found in many α-proteobacteria, it is possible that PleD plays a dual role in other bacteria, facilitating cdG production as well as enhancing CtrA degradation. Nonetheless, future studies will determine whether CtrA undergoes regulated proteolysis in α-proteobacteria other than *Caulobacter*, and, if so, which component(s) of the machinery characterized here are needed.

Methods

Strains and plasmids used in this study are listed in Table S1. Additional methods may be found in *SI Methods*.

Gel-Based Proteolysis Assays. Reactions were initiated by adding the substrate to prewarmed mixtures containing the indicated concentrations of protease and accessory factors, along with an ATP regeneration system (0.9 μg/mL creatine kinase, 4 mM ATP, 16 mM creatine phosphate). Reactions were conducted at 30 °C in PD buffer [25 mM Hepes–KOH, pH 7.6, 5 mM KCl, 5 mM $MgCl_2$, 15 mM NaCl, 10% (vol/vol) glycerol] with a final concentration of 100 mM KCl. Samples were removed at the indicated times into tubes containing SDS/PAGE sample buffer and frozen in liquid

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nitrogen. Samples were analyzed by SDS/PAGE and staining with Lumitein fluorescent protein dye (Biotium). Proteins were visualized using a Bio-Rad Gel Doc XL, and band intensities were quantified with Imagelab software (Version 4.0).

Fluorescence-Based Proteolysis Assays. Degradation of GFP–CtrA–RD+15 was monitored as the loss of fluorescence over time as in ref. 27, with modifications described in *SI Methods*.

In Vivo Protein Stability Assays. Half-lives of CtrA–RD+15 variants were measured during swarmer cell development as in ref. 25, with modifications described in *SI Methods*.

Co-IP of Proteins from *Caulobacter* Lysates. KR2973, KR3178, and KR3179 were grown in 60 mL of peptone–yeast extract medium with 0.03% xylose to OD_{660} = 0.3 and harvested by centrifugation. Cells were resuspended in 1 mL of co-IP lysis buffer [20 mM Hepes–KOH, pH 7.5, 100 mM NaCl, 20% (vol/vol) glycerol] supplemented with 0.37% formaldehyde. After 1 h at room temperature, cross-linking was quenched by the addition of glycine to a concentration of 0.125 M. Cells were washed and resuspended in co-IP lysis buffer containing 0.5% dodecyl maltoside, treated with 1 mg/mL lysozyme and 40 units of Benzonase nuclease for 1 h on ice, and sonicated to achieve lysis. Lysates were cleared by centrifugation and incubated overnight at 4 °C with 40 μL of anti-FLAG M2 resin (Sigma-Aldrich). The resin was washed with co-IP buffer, and precipitated proteins were released by boiling in the presence of SDS/PAGE sample buffer. Equal volumes of coimmunoprecipitates, and cell lysates from equal numbers of cells, were analyzed by SDS/PAGE and Western blotting. Membranes were probed with the following primary antibodies: RcdA (18), ClpX, PopA (20), or anti-FLAG (Sigma-Aldrich). Chemiluminescent signals were visualized with Western Lightning (Perkin-Elmer) using a Bio-Rad Gel Doc XL.

Coaffinity Purification. His_6–CtrA, His_6–CtrA3, His_6–CtrA–DD, His_6–PopA, and His_6–EnvZ–HK (0.5 mL at 1.8 μM protein) were incubated with 20 μL of Ni–nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen) at 4 °C for 1 h in PD buffer/10 mM imidazole/30 mM KCl. After washing with 0.5 mL of PD buffer/10 mM imidazole, resin was resuspended using 0.25 mL of PD buffer/25 mM imidazole/30 mM KCl containing 0.6 μM prey protein. Assays included 20 μM cdG where indicated. After incubating 1 h, the beads were washed with PD buffer/25 mM imidazole supplemented with 20 μM cdG, if it was present in the binding stage. Bound proteins were eluted with PD buffer/500 mM imidazole. Eluted proteins were analyzed by SDS/PAGE and Lumitein staining as described above.

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