Title

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A novel adapter mechanism regulates the *Caulobacter* cell cycle by promoting the degradation of the transcriptional regulator CtrA.

By

Stephen Carl Smith

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University of California, Berkeley

Committee in charge:
Associate Professor Kathleen R. Ryan
Associate Professor Arash Komeili
Associate Professor Andreas Martin

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Abstract

A novel adapter mechanism regulates the *Caulobacter* cell cycle by promoting the degradation of the transcriptional regulator CtrA.

By

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Doctor of Philosophy in Microbiology
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Caulobacter crescentus is a powerful model organism for understanding cellular differentiation, cell polarity and cell cycle regulation in bacteria. An elaborate network of two-component signaling proteins works to orchestrate the developmental program that characterizes the *Caulobacter* cell cycle. The essential DNA-binding response regulator CtrA is at the center of this regulatory scheme and acts to control the transcription of >100 genes that are required for cell cycle progression, motility, DNA methylation, morphology and other processes. Because CtrA also inhibits chromosome replication at specific stages of the *Caulobacter* cell cycle, its activity must be temporarily eliminated in order for DNA replication to occur. Inactivation of CtrA is achieved though dephosphorylation and regulated degradation by the broadly conserved energy-dependent protease ClpXP.

In this dissertation, I analyze the roles of three proteins that are required for CtrA degradation in living cells. These are a single domain response regulator CpdR, a protein with no predicted function, RcdA, and a cyclic diguanylate (cdG)-binding protein, PopA. Structure-directed mutagenesis of RcdA was used to probe RcdA function. Results from these studies undermine the prevailing model for RcdA function, which suggest that RcdA does not participate directly in delivering CtrA to ClpXP, but instead acts simply as a localization factor increasing the concentration of CtrA at the cell pole where the protease is located. Additionally, I reconstituted the regulated proteolytic reaction *in vitro* and probed the role of all three accessory proteins and the small molecule cdG in promoting CtrA degradation. Although ClpXP alone is known to degrade CtrA *in vitro*, I observed a dramatic acceleration of proteolysis in the presence of the accessory proteins and cdG. This accelerated proteolysis was characterized by a nearly 10-fold reduction in the K_M of the reaction, which is consistent with predictions for an adaptor mediated mechanism. I began to characterize protein-protein interactions within the proteolytic complex using *in vivo* and *in vitro* techniques. These experiments demonstrate that CtrA interacts directly with PopA in a cdG-dependent fashion. CtrA also interacts directly with RcdA and with ClpX. The CtrA-PopA(cdG) and CtrA-RcdA interactions are weakened or abolished by mutations in the receiver domain of CtrA that slow its proteolysis *in vivo*.

We propose a mechanism in which CtrA forms a ternary complex with PopA and RcdA in response to rising cdG concentrations in the cell. In this complex, PopA and RcdA act as a multi-protein adaptor complex to enhance the delivery of CtrA to the catalytic pore of ClpX. CpdR is required for accelerated CtrA proteolysis, but its precise role is still unknown. The accessory proteins were able to stimulate CtrA degradation even in the presence of a DNA fragment containing a CtrA binding site, which is known to inhibit CtrA proteolysis. Future work will determine if the accessory factors prevent the formation of inhibitory CtrA-DNA complexes or actively disassemble them. This dissertation alters the concept of proteolytic adaptors to

include multi-protein complexes and expands the range of mechanisms by which proteolytic adaptors are controlled to include direct regulation by the small molecule cdG.

Dedicaton

I dedicate this dissertation to my wife Audrey Gavino Parangan-Smith, and our unborn child (title: to be determined).

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Chapter 1: Cell cycle and developmental regulation by two-component signaling proteins in *Caulobacter crescentus*

Abstract

The intricate cell division and developmental cycle of the alpha-proteobacterium *Caulobacter crescentus* has been studied for four decades. During that time, elegant genetic screens and comprehensive post-genomic methods have uncovered a branched network of two-component signaling proteins that orchestrates *Caulobacter* cell cycle progression and morphological development. In addition to yielding the first and most detailed picture of bacterial cell cycle control, *Caulobacter* studies have revealed novel ways in which two-component proteins generate cellular outputs, interact with each other, and are themselves regulated.

Introduction

Both two-component signaling and other regulatory mechanisms play important roles in the *Caulobacter* life cycle, as reviewed in Curtis, *et al.* (2010). Here we present a two-component centric view of the cell cycle, focused on the activities, locations, and regulation of two-component proteins. We will first describe key properties of the *Caulobacter* cell cycle and morphological development and explain how changes in the activity of a central response regulator, CtrA, drive cell cycle progression. We will then work our way upstream to the branched network of two-component proteins that regulates CtrA activity in time and space. We will also discuss the intimate linkage between the two-component network and the second messenger cyclic diguanylate (c-d-GMP), which is a key mediator of developmental processes. We will then describe two-component systems that function directly in the synthesis of polar organelles. Finally, we will briefly discuss two-component signaling proteins that are involved in selected *Caulobacter* environmental responses, because they exemplify unusual modes of action for histidine kinases or response regulators. In all cases, we will point out unanswered questions and areas of active research.

The *Caulobacter* genome encodes 62 histidine kinases and 44 response regulators. Of these 106 two-component proteins, 60 are encoded as "orphans" without a cognate histidine kinase or response regulator in the same operon (Nierman et al, 2001; Skerker et al, 2005). In an attempt to delete each two-component gene individually, nine were found to be essential for viability under standard laboratory conditions (Table 1), and 39 in all contribute to some aspect of growth, cell cycle progression, or morphology (Skerker et al, 2005). A previously unknown essential two-component system composed of the histidine kinase CenK and the DNA-binding response regulator CenR was uncovered in this comprehensive genomic approach. CenK and CenR are necessary for cell envelope integrity, as mutants depleted of these proteins are extensively blebbed, and cells overexpressing a phosphomimetic form of CenR become misshapen and lyse (Skerker et al, 2005). The transcriptional targets of CenKR are as yet unknown, and connections between this two-component system and the cell cycle are still to be determined.

Table 1. Essential two-component signaling proteins in *Caulobacter crescentus*.

Gene	Name	Type	Function	Reference
CC_0530	*cenK*	HK	Cell envelope biogenesis and integrity	(Skerker et al., 2005)
CC_1078	*cckA*	HK	DNA replication, cell division	(Jacobs et al., 1999)
CC_1743	*ntrX*	RR	(not essential on minimal M2G medium)	(Skerker et al., 2005)
CC_2931	*petR*	RR		(Skerker et al., 2005)
CC_2932		HK	Putative kinase for PetR	(Skerker et al., 2005)
CC_3035	*ctrA*	RR	DNA replication, cell division	(Quon et al., 1996)
CC_2463	*divK*	RR	DNA replication, cell division	(Hecht et al., 1995)
CC_3484	*divL*	HK	DNA replication, cell division	(Wu et al., 1999)
CC_3743	*cenR*	RR	Cell envelope biogenesis and integrity	(Skerker et al., 2005)

Gene numbers refer to the sequence of *Caulobacter crescentus* CB15 (Nierman, et al., 2001). Protein type is either histidine kinase (HK) or response regulator (RR). Each gene is essential for growth in rich (PYE) medium (Skerker, et al., 2005).

The Caulobacter cycle of cell division and morphogenesis

A detailed description of the *Caulobacter* cell cycle is available in Curtis, *et al.* (2010). *Caulobacter* cells growing exponentially in rich medium have a generation time of ~90 min, and the populations are dimorphic. The motile swarmer cell has a single flagellum and multiple pili at one pole. The nonmotile stalked cell lacks pili, and the polar flagellum is replaced by the stalk, a thin extension of the cell envelope. These distinct cell types are generated by asymmetric division of a polarized predivisional cell, in which one pole possesses the stalk and the other pole the flagellum and pili (Fig. 1A).

Swarmer and stalked cells not only have different shapes, they also occupy different phases of the cell cycle (Degnen & Newton, 1972). Unlike bacteria that can initiate more than one round of DNA replication per cell division, *Caulobacter* practices once-and-only-once chromosome replication (Marczynski, 1999). The stalked progeny is born into S-phase; after division it can immediately begin a new cycle of chromosome replication and cell division. As the stalked cell grows, it builds a flagellum at the pole opposite the stalk, creating an asymmetric predivisional cell. In contrast, swarmer cells are born into a pre-replication G1-phase, unable to initiate chromosome replication. When the swarmer cell enters S-phase, it also differentiates into a stalked cell by shedding the flagellum and building a stalk at the same pole (Fig. 1A). In wild-type, well-fed cultures of *Caulobacter*, morphological changes at the cell pole are coupled to cell cycle progression, but this linkage can be broken by mutations in regulatory genes (Hottes et al, 2005; Sommer & Newton, 1989).

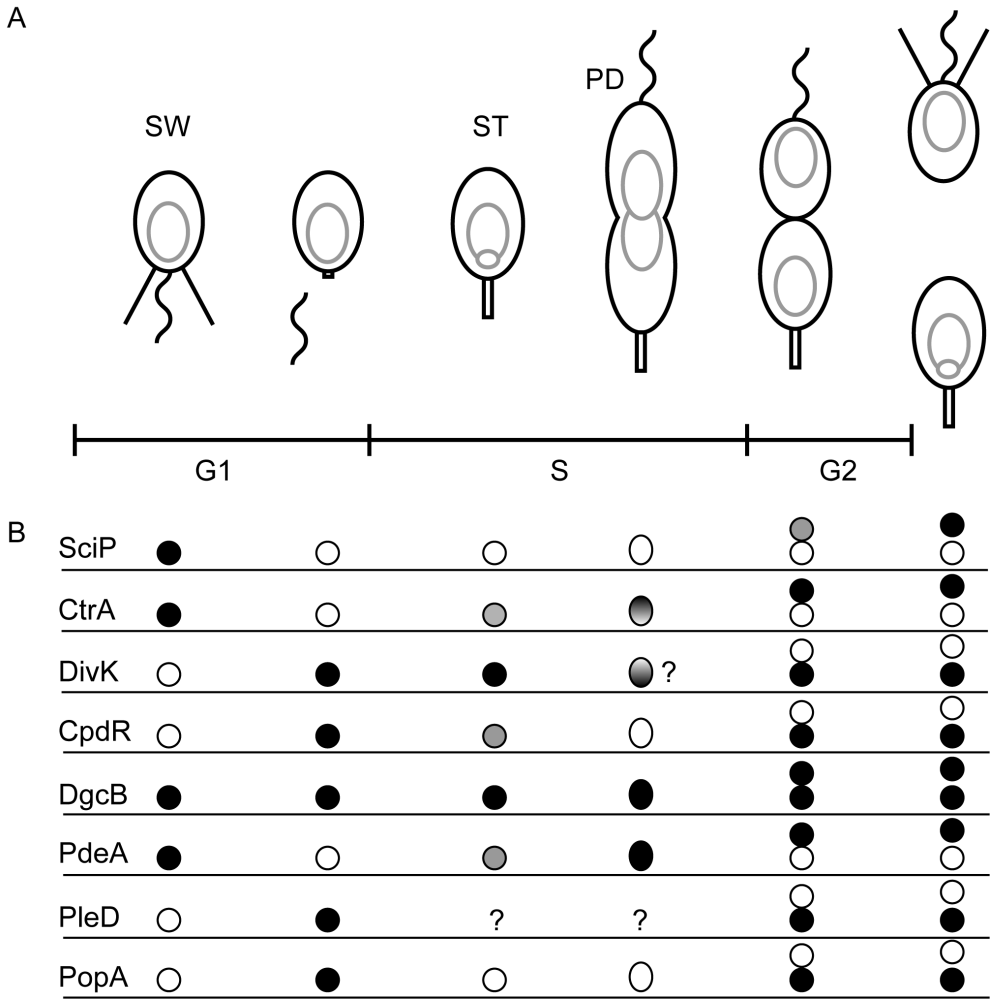


Fig. 1. The abundance of regulatory proteins fluctuate over the course of the Caulobacter cell cycle. (A) Schematic diagram of the *Caulobacter crescentus* cell cycle. Wavy lines indicate the flagellum and straight lines indicate pili. The rectangular extension at the bottom of the cell denotes the stalk. Gray interior circles indicate the chromosome in successive stages of replication and segregation. Swarmer (SW), stalked (ST) and predivisional (PD) cells are indicated. (B) Activities of indicated proteins at successive stages during the cell cycle. Black circles/ovals indicate high activity, gray circles/ovals indicate intermediate activity, and white circles/ovals indicate low activity. Ovals containing gradient shading indicate that a gradient of CtrA activity has been observed, and a gradient of DivK activity is postulated to exist (denoted by a question mark). The direction of the gradient corresponds to the poles of the cell in panel A. SciP is thought to be active whenever it is present in the cell. CtrA and DivK are active when present and phosphorylated. CpdR is active when present and unphosphorylated. DgcB is always present and thought to be constitutively active. PdeA is thought to be active when present in the cell. PleD's DGC activity is low in swarmer cells, increases during the SW-ST transition, and is high again in newly born stalked cells. However, PleD's activity level in late stalked and early predivisional cells is unknown, indicated by question marks. PopA is active at promoting CtrA degradation at the stalked pole when c-di-GMP levels are high. PopA may have other activities, but these are not represented in the diagram. See text for references.

Oscillations in the activity of a key response regulator are necessary for orderly cell cycle progression

The essential response regulator CtrA is a critical hub connecting *Caulobacter* cell cycle progression with the development of distinct cell types (Quon et al, 1996). CtrA is a member of the OmpR family of transcriptional regulators that activates or represses the transcription of 26% of the cell cycle-regulated genes in *Caulobacter*, including direct regulation of 95 genes (Laub et al, 2002; Laub et al, 2000). Through its transcriptional targets, CtrA promotes critical events that occur late in the cell cycle, such as DNA methylation, flagellum and pilus biogenesis, and cell division, and several CtrA targets are themselves essential for viability. However, CtrA also inhibits cell cycle progression by binding to 5 sites within the *Caulobacter* origin of replication (*Cori*) (Quon et al, 1998). If CtrA is constitutively active, *Caulobacter* cells arrest in the G1-phase of the cell cycle with one chromosome (Domian et al, 1997). Therefore, CtrA activity must be suppressed in any cell that is preparing to initiate chromosome replication. Conversely, cells harboring conditional loss-of-function mutations in *ctrA* accumulate multiple chromosomes without dividing (Domian et al, 1997). Because of CtrA's dual function, redundant mechanisms ensure that CtrA activity is eliminated at the G1-S transition and restored later in the cell cycle to drive polar morphogenesis and division.

CtrA activity oscillates as a result of cell cycle-regulated phosphorylation and proteolysis. CtrA is rapidly degraded during swarmer-to-stalked cell (SW-ST) differentiation and in the stalked compartment of the predivisional cell (Domian et al, 1997). Regulated proteolysis by the ATP-dependent ClpXP protease at these times ensures that newly born stalked cells and cells proceeding through the SW-ST transition will be able to initiate chromosome replication (Jenal & Fuchs, 1998). Swarmer cells, which are born containing active CtrA, must wait until CtrA is eliminated at the SW-ST transition before beginning chromosome replication. CtrA regulates its own transcription, and later in the cell cycle, CtrA is replenished by a positive transcriptional feedback loop (Domian et al, 1999). *ctrA* transcription also depends on the methylation state of the *ctrA* promoter, thus linking CtrA synthesis to the progress of chromosome replication (Reisenauer & Shapiro, 2002). If CtrA is artificially stabilized by mutation, so that the protein is present throughout the cell cycle, CtrA~P levels still oscillate (Domian et al, 1997). CtrA phosphorylation is high in swarmer and predivisional cells and nearly undetectable in stalked cells. This variation in the level of CtrA~P is sufficient to allow chromosome replication to occur, and cells with stable CtrA protein have a near-normal life cycle.

CtrA function at cell cycle-regulated promoters and at the origin of replication

CtrA's mechanism of action at *Cori* and at promoters has been studied extensively. CtrA binds to the consensus sequence TTAA-N7-TTAA (Quon et al, 1998), in which each TTAA half-site binds a CtrA monomer (Siam & Marczynski, 2000). Phosphorylation stimulates CtrA binding to full sites, but not if one of the corresponding half-sites is altered by mutation, indicating that phosphorylation affects protein-protein contacts between CtrA molecules rather than the interaction between a single molecule and DNA (Siam & Marczynski, 2000).

Cori contains 5 full CtrA binding sites [a]-[e] (Quon et al, 1998). The cloned replication origin supports the replication of *Cori* plasmids, and plasmid replication occurs with the same cell cycle timing as chromosome replication (Marczynski & Shapiro, 1992). When individual CtrA binding sites in *Cori* plasmids are mutated, plasmid copy number increases, supporting the model that CtrA binding at the origin represses DNA replication (Bastedo & Marczynski, 2009; Quon et al, 1998). Chromatin immunoprecipitation experiments performed on synchronized *Caulobacter* populations indicate that CtrA occupancy of native *Cori* sites is high in swarmer

cells and drops sharply during the first 20 minutes of the cell cycle, coincident with CtrA proteolysis and the onset of DNA replication (Taylor et al, 2011).

CtrA~P binding to *Cori* sites likely blocks several steps necessary for the initiation of chromosome replication. First, the [a] and [b] sites overlap a strong promoter (*hemE* Ps), which directs transcription away from the *Cori* region. Transcription from this promoter is required for *Cori* plasmid replication (Marczynski et al, 1995), and CtrA binding represses its transcription (Quon et al, 1998). Second, the [c] site overlaps a binding site for the essential replication initiation protein DnaA, and the [e] site lies 4 bp from another DnaA binding site (Marczynski & Shapiro, 1992; Taylor et al, 2011). DnaA has relatively weak affinity for its binding sites in *Cori*, and in footprinting assays, DnaA can be displaced from the DNA by CtrA (Taylor et al, 2011). Finally, binding site [c] also overlaps a binding site for integration host factor (IHF) (Siam et al, 2003), which in *E. coli* facilitates the melting of origin sequences by DnaA (Hwang & Kornberg, 1992). CtrA dephosphorylation and proteolysis at the G1-S transition, combined with increased expression of DnaA and IHF, simultaneously permit binding of DnaA and IHF to *Cori* sites and derepress transcription from *hemE* Ps, allowing chromosome replication to commence (Domian et al, 1997; Gober & Shapiro, 1992; Jacobs et al, 2003; Zweiger & Shapiro, 1994).

Despite the strong effect that CtrA~P has on blocking the initiation of DNA replication (Domian et al, 1997), *Caulobacter* can survive under lab conditions without over-replicating its chromosome even when all five CtrA binding sites in *Cori* are mutated (Bastedo & Marczynski, 2009). This result contrasts with findings using *Cori* plasmids, where loss of CtrA binding sites increases copy number (Bastedo & Marczynski, 2009). A partial explanation for this phenomenon is that DnaA activity oscillates in *Caulobacter* cells independently of CtrA (Jonas et al, 2011). DnaA activity is apparently low during the predivisional phase, because mutants lacking CtrA activity or CtrA binding sites do not reinitiate replication late within the same cell cycle (Jonas et al, 2011). Lack of CtrA activity has little effect on the timing of replication initiation in stalked cells, but in swarmer cells, CtrA delays the onset of DNA replication, so that the swarmer cell cycle is longer than the stalked cycle (Jonas et al, 2011). CtrA binding sites in *Cori* become essential for viability when *Caulobacter* is exposed to a combination of stresses, nutrient upshift and exposure to low levels of antibiotics (Bastedo & Marczynski, 2009). Thus, CtrA may have evolved to help generate non-identical progeny and to integrate environmental information into the control of chromosome replication.

Transcriptional regulation by CtrA is similarly complex, in that CtrA activates some promoters and represses others, and not all activated promoters fire at the same time in the cell cycle. This difference in cell cycle timing has been partially attributed to the strength of CtrA~P binding to individual promoters. For example, CtrA has 10-to-20-fold greater affinity for the promoter of *fliQ*, a class II flagellar gene which is activated early in the predivisional cell, than it does for the *ccrM* promoter, which is activated very late in the cell cycle, when more CtrA~P has accumulated (Reisenauer et al, 1999). However, the strength of promoter binding does not entirely account for the transcription patterns of CtrA-regulated genes. While *fliQ* is transcribed in early predivisional cells, it is not transcribed in swarmer cells, which contain comparable levels of activated CtrA (Jacobs et al, 2003; Ouimet & Marczynski, 2000). It was proposed that a swarmer cell-specific repressor of transcription could function at CtrA-activated promoters that are *not* expressed in swarmer cells (Ouimet & Marczynski, 2000), and such a regulator has recently been identified.

SciP, a small protein with homology to helix-turn-helix proteins, is present in the swarmer cell where it binds to CtrA, repressing the transcription of a large group of genes that

CtrA normally activates late in the cell cycle. Genes normally repressed by CtrA are not affected by SciP (Gora et al, 2010; Tan et al, 2010). Electrophoretic mobility shift experiments demonstrated that SciP forms a complex with CtrA and DNA which prevents RNA polymerase from associating with the promoter of a CtrA-activated gene, *fliF*. To prevent SciP from inappropriately blocking the transcription of late cell cycle genes, SciP levels are strictly controlled. CtrA activates *sciP* transcription late in the cell cycle, but SciP protein is only abundant in swarmer cells, suggesting that its synthesis is post-transcriptionally regulated. In addition, SciP is very rapidly degraded at the swarmer-to-stalked cell transition, though the protease responsible has not yet been identified. The ability of SciP to inhibit CtrA-dependent transcription through direct contact without affecting CtrA phosphorylation or blocking DNA binding represents a novel way of regulating the activity of a DNA-binding response regulator (Gora et al, 2010).

Spatiotemporal regulation of CtrA activity by an extended, branched, two-component signaling network

CtrA phosphorylation on aspartate 51 (D51) is required for viability, and phosphorylation is accomplished by a phosphorelay comprised of the hybrid histidine kinase CckA and the histidine phosphotransferase ChpT. CckA contains two transmembrane spans which anchor the protein in the cytoplasmic membrane. The cytoplasmic portion of CckA consists of a sensory region containing a predicted PAS (Per-Arnt-Sim) domain, a dimerization and histidine phosphotransfer domain (DHp), a catalytic domain (CA), and a receiver domain. A temperature-sensitive (ts) *cckA* mutant contains drastically less CtrA~P *in vivo*, and the transcriptional profiles of *ctrA* and *cckA* ts mutants are very highly correlated (Jacobs et al, 2003; Jacobs et al, 1999). The phosphorelay was reconstituted *in vitro* after the discovery of ChpT, which shuttles the phosphoryl group between the receiver domains of CckA and CtrA (Biondi et al, 2006a).

In groundbreaking experiments, CckA was shown to be the first two-component signaling protein located at a particular site within a bacterial cell (Jacobs et al, 1999). CckA undergoes cell cycle-regulated changes in localization: it is delocalized in swarmer cells, but accumulates at the pole opposite the stalk (the swarmer or flagellar pole) during S-phase. A smaller focus of CckA is sometimes seen at the stalked pole of the predivisional cell. Shortly before cell division, CckA is released from the flagellar pole and delocalized around the swarmer compartment. Immediately after cell division, CckA is delocalized in the stalked progeny (Angelastro et al, 2010; Jacobs et al, 1999). These findings were intriguing, because CckA's localization at the swarmer pole coincides loosely with periods of high CckA~P and CtrA~P in the cell (Jacobs et al, 2003). Studies described below have further addressed the mechanism and consequences of CckA localization at the swarmer pole (Iniesta et al, 2010a; Iniesta et al, 2010b; Tsokos et al, 2011).

The CckA-ChpT phosphorelay was identified by a pioneering technique called phosphotransfer profiling (Biondi et al, 2006a). In a profiling experiment, one purified histidine kinase or phosphotransferase is used to phosphorylate multiple receiver domains in parallel. Histidine kinases are sometimes promiscuous *in vitro* and can phosphorylate response regulators other than their *in vivo* cognates. However, examining phosphotransfer after very short incubation times reveals the preferred partner(s) of a histidine kinase or phosphotransferase from among all the possible receiver domains encoded in an organism's genome (Laub et al, 2007). Such an experiment showed that ChpT could act as a phosphodonor for either CtrA or a single-domain response regulator, CpdR.

This bifurcated phosphorelay not only regulates CtrA phosphorylation, but CtrA stability as well (Biondi et al, 2006a; Iniesta et al, 2006). The unphosphorylated form of CpdR is required to promote CtrA degradation during swarmer cell differentiation and in the stalked compartment of the predivisional cell (see below). This signaling logic dictates that when CckA is active as a kinase, phosphate flows onto both CtrA and CpdR, and CtrA is both activated and stabilized (Fig. 2A). However, phosphate can also flow backward from CtrA and CpdR via ChpT to the CckA receiver domain, where CckA phosphatase activity stimulates hydrolysis (Chen et al, 2009). Thus, when CckA is in phosphatase mode, CtrA is both deactivated and destabilized (fig. 2B). *In vivo*, a version of CckA harboring kinase activity but lacking phosphatase activity (K^+P^-) causes only a mild cell cycle phenotype (Chen et al, 2009). This result suggests that the autophosphatase activities of CtrA (Chen et al, 2011) and CpdR are sufficient for their dephosphorylation, or that unknown phosphatases act on CtrA and CpdR to sharpen the G1-S cell cycle transition.

Since CckA activity determines the cellular level of CtrA~P, the next question is how CckA toggles between kinase and phosphatase modes. Although the mechanisms are not completely clear, recent studies have revealed that two other essential two-component proteins, DivL and DivK, modulate CckA activity.

DivL is a histidine kinase homolog composed of an N-terminal transmembrane anchor, a long domain of unknown function, and conserved DHp and CA domains. DivL is unusual because it contains a tyrosine residue (Y550) at the site normally occupied by the conserved, phosphorylatable histidine. Cells depleted of DivL or harboring a ts mutation in *divL* are filamentous and contain more than one chromosome, resembling *ctrA* ts mutants (Reisinger et al, 2007; Sciochetti et al, 2005; Wu et al, 1999). These phenotypes suggested that DivL could play a role in activating CtrA. DivL was at first thought to phosphorylate CtrA directly, but the DivL catalytic domain and Y550 residue are dispensable for viability, and mutants lacking these features of DivL have relatively mild phenotypes (Reisinger et al, 2007). Recent studies show that DivL functions instead by a novel mechanism, stimulating CckA kinase activity. *divL* ts mutants contain lower levels of CtrA~P, CpdR~P, and CckA~P, and DivL is also required for CckA localization at the swarmer pole (Iniesta et al, 2010a; Tsokos et al, 2011). DivL and CckA can be co-immunoprecipitated from *Caulobacter* lysates, indicating that they exist in a complex *in vivo* (Iniesta et al, 2010a). The current model encompassing these results is that DivL stimulates CckA kinase activity by contacting CckA directly (Fig. 2A).

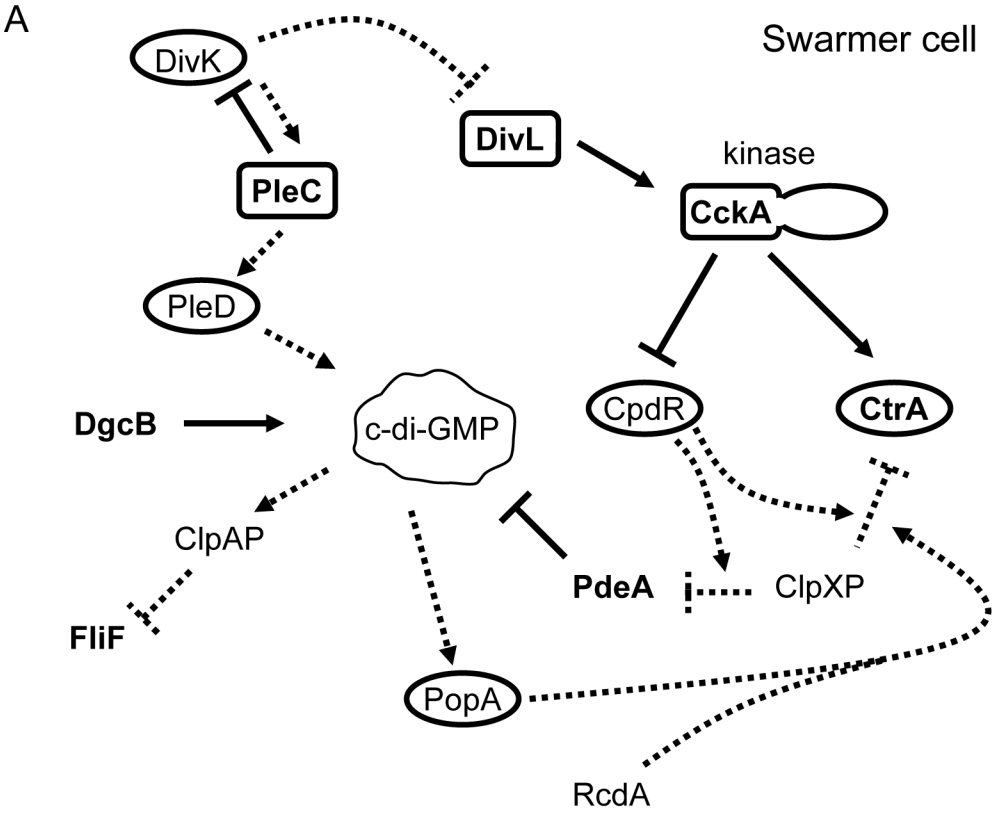
DivK is a single-domain response regulator whose phosphorylation on D53 is required for *Caulobacter* viability (Hecht et al, 1995; Lam et al, 2003). At the nonpermissive temperature, a cold-sensitive (cs) *divK* mutant forms long filaments arrested in the G1 phase of the cell cycle, with each cell containing one chromosome (Hecht et al, 1995; Hung & Shapiro, 2002). This phenotype is strikingly similar to cells overexpressing a constitutively active form of CtrA. In fact, *divKcs* cells at the nonpermissive temperature contain increased levels of CtrA~P and CckA~P, indicating that CckA kinase activity is upregulated when DivK is compromised (Biondi et al, 2006a; Tsokos et al, 2011).

When DivK was used as the bait in a yeast two-hybrid screen, one of the interacting proteins recovered was DivL (Ohta & Newton, 2003). The meaning of this interaction is now becoming clear. DivK~P, but not unphosphorylated DivK, was shown by *in vitro* Förster resonance energy transfer (FRET) experiments to interact with the DHp and CA domains of DivL (Tsokos et al, 2011). Amino acid substitutions in DivK or DivL that decrease the strength of their interaction have similar phenotypic effects, namely increasing the levels of CckA~P and

blocking the initiation of chromosome replication. In contrast, an amino acid substitution in DivK that increases its interaction with DivL yields decreased levels of CckA~P and an increase in chromosome content. Thus, DivK~P appears to downregulate CckA kinase activity *in vivo* by binding to DivL (Fig. 2B).

The DivK-DivL and DivL-CckA interactions represent exciting new mechanisms by which one two-component signaling protein regulates the activity of another. In the case of DivL and CckA, their interaction is thought to be direct, but this remains to be verified using purified proteins. The co-immunoprecipitation of DivL and CckA could be mediated by unknown proteins in the *Caulobacter* lysate. If DivL does contact CckA directly, what is the nature of the interaction? We do not yet know if DivL and CckA form heterodimers or a higher-order assembly. Based on the locations of mutations that alter DivK-DivL binding, DivK~P is predicted to interact with DivL in much the same way as a receiver domain interacts with a histidine kinase during phosphotransfer (Tsokos et al, 2011). Because unphosphorylated DivK does not interact strongly with DivL, phosphorylation is predicted to cause a conformational change in DivK that permits better binding to DivL. With regard to DivK's effects on CckA, how does DivK~P binding alter DivL's ability to stimulate CckA kinase activity? DivK~P could compete with CckA for binding to DivL, disrupting the complex. Alternatively, it could cause a conformational change in DivL that prevents it from activating CckA without disrupting the DivL-CckA interaction.

The essential role of DivL is to (reversibly) stimulate CckA kinase activity, and this function can be performed by the N-terminal portion of DivL up to and including the DHp domain (Iniesta et al, 2010a; Reisinger et al, 2007). Since the CA domain is not essential, it is unclear if DivL autophosphorylates *in vivo*. However, mutants lacking the DivL CA domain are not phenotypically wild-type (Reisinger et al, 2007). DivL may autophosphorylate at an undetectable level *in vivo*, or the CA domain may function to stabilize the interaction between DivL and DivK~P (Tsokos et al, 2011). Point mutations designed to disrupt ATP binding or hydrolysis may help to clarify the function of the DivL CA domain.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Swarmer cell
DivK
DivL
kinase
PleC
CckA
PleD
DgcB
c-di-GMP
CpdR
CtrA
ClpAP
PdeA
ClpXP
FliF
PopA
RcdA

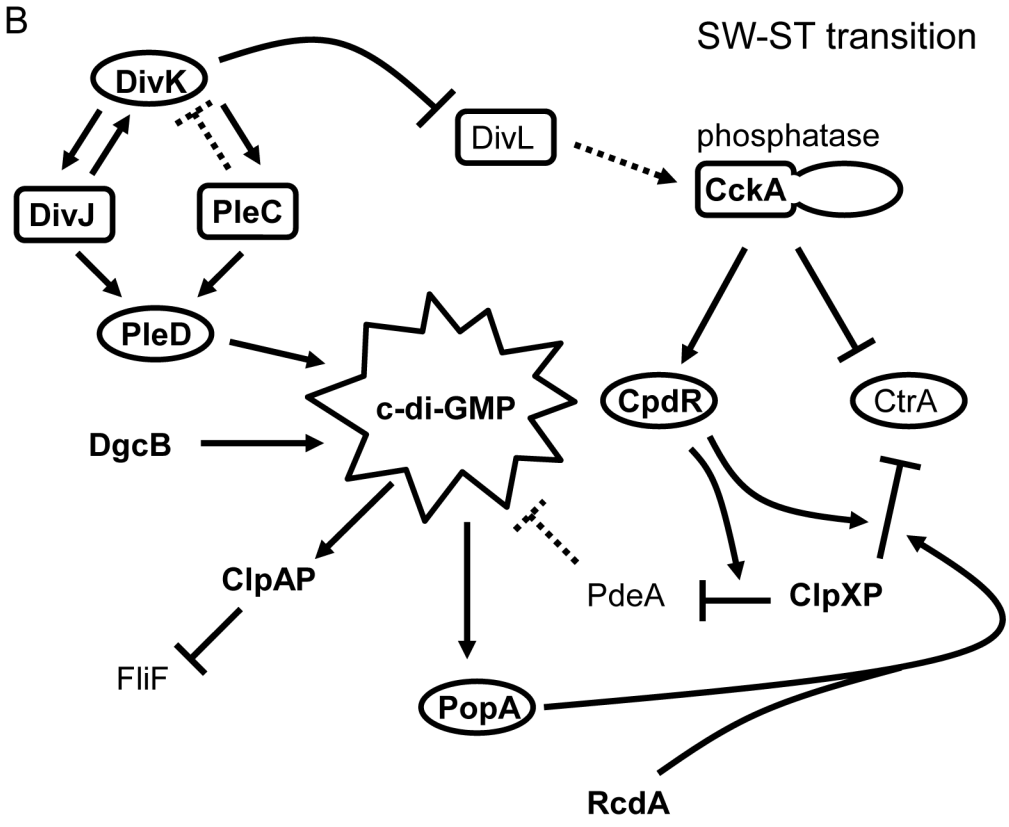
B
SW-ST transition
DivK
DivL
phosphatase
DivJ
PleC
CckA
PleD
c-di-GMP
CpdR
CtrA
DgcB
ClpAP
PdeA
ClpXP
FliF
PopA
RcdA

Fig. 2. Regulation of SW ST differntiation (A) Regulatory protein activities in the swarmer cell. (B) Regulatory protein activities during the swarmer-to-stalked cell (SW-ST) transition. Solid arrows and bars indicate processes that are occurring in that cell type, while dotted arrows and bars indicate processes that are not occurring. Bold type indicates components that are currently active, while regular type indicates components that are being proteolyzed or are present but inactive for the processes specified in the diagram. DivJ levels are low in swarmer cells (A), but DivJ is present and active at the SW-ST transition (B). In kinase mode (A), CckA phosphorylates both CpdR and CckA via ChpT, which activates CtrA and deactivates CpdR. In phosphatase mode (B), both CpdR and CtrA are dephosphorylated, which activates CpdR and deactivates CtrA. Arrows from CpdR denote stimulation of proteolysis by ClpXP. Bars from ClpXP and ClpAP indicate proteolysis of downstream components. Arrows from PopA and RcdA indicate stimulation of CtrA proteolysis. Arrows from DivJ and PleC to DivK or PleD denote phosphorylation, while the bar from PleC to DivK indicates dephosphorylation. Arrows from DivK to DivJ and PleC indicate allosteric stimulation of their kinase activities. PleC is thought to be active at both times, as a DivK phosphatase in panel A and a PleD kinase in panel B. The arrow from DivL to CckA denotes stimulation of CckA kinase activity via direct binding. The bar from DivK to DivL denotes inactivation of DivL by direct binding to DivK~P. Arrows from PleD and DgcB indicate diguanylate cyclase activity, while the bar from PdeA indicates c-di-GMP phosphodiesterase activity. In A, the cellular pool of c-di-GMP is smaller to indicate a lower concentration of this messenger in the swarmer cell. In B, the pool of c-di-GMP increases and is able to activate downstream proteins.

Polarly localized histidine kinases with opposite effects on DivK generate asymmetry

How does DivK~P downregulate CckA kinase activity via DivL specifically in the stalked progeny and not in the swarmer progeny? Two histidine kinases are known to regulate the phosphorylation state of DivK. DivJ contains five predicted transmembrane spans followed by DHp and CA domains. The PleC histidine kinase has two transmembrane spans flanking a periplasmic domain, followed by cytoplasmic DHp and CA domains. *In vitro*, both kinases can either phosphorylate or dephosphorylate DivK (Hecht et al, 1995; Wu et al, 1998), but *in vivo*, DivJ functions as a DivK kinase, while PleC functions as a DivK~P phosphatase (Matroule et al, 2004; Wheeler & Shapiro, 1999) (Fig. 2). Cellular asymmetry of DivK activation is accomplished by the differential localization of DivJ and PleC: DivJ is located at the stalked pole, while PleC resides at the swarmer pole (Fig. 3).

PleC is located at the flagellated pole of the swarmer cell. During development into a stalked cell, the flagellum is shed, stalk biogenesis begins, and PleC is replaced at the stalked pole by DivJ. During S-phase, a new focus of PleC appears at the pole opposite the stalk, and it remains there until after cell division. DivJ resides at the stalked pole for the remainder of the cell cycle, and the progeny stalked cell is born with this focus of DivJ (Wheeler & Shapiro, 1999). DivK diffuses throughout the predivisional cell, being phosphorylated at the stalked pole by DivJ and dephosphorylated at the swarmer pole by PleC (Jacobs et al, 2001; Matroule et al, 2004) (Fig. 3A). *Caulobacter* predivisional cells form a diffusion barrier between the stalked and swarmer compartments several minutes before daughter cell separation (Judd et al, 2003). Any DivK that is trapped in the stalked compartment becomes phosphorylated, while DivK trapped in the swarmer compartment becomes dephosphorylated (Lam et al, 2003; Matroule et al, 2004). As a result, CckA residing in the stalked compartment is downregulated by DivK~P, leading to CtrA dephosphorylation and proteolysis, while CckA and CtrA activity are preserved in the swarmer compartment (Domian et al, 1997; Jacobs et al, 2003). In this way, DivK acts as a cell division sensor that triggers different developmental programs in the two progeny (Fig. 3B).

A recent study provided evidence for a gradient of CtrA activity in the *Caulobacter* predivisional cell, even before cytoplasmic compartmentalization (Chen et al, 2011) (Fig. 1B). When division is inhibited by cephalexin or by FtsZ depletion, cells elongate for several hours without dividing. After a first round of DNA replication, each cell contains two chromosomes with an origin located at each pole. The nondividing *Caulobacter* cells then begin a new round of DNA replication, but in this case, only the origin located at the stalked pole fires, creating a cell with three chromosomes (Chen et al, 2011). Why are the two origins treated differently within the same contiguous cytoplasm? The authors hypothesized that while CckA located at the swarmer pole acts as a kinase to phosphorylate CtrA, CckA located at the stalked pole may predominantly act as a CtrA phosphatase. If the kinetics of phosphorylation and dephosphorylation are sufficiently fast compared to diffusion of CtrA through the cytoplasm, this arrangement can generate a roughly linear gradient of CtrA~P along the length of the cell (Chen et al, 2011). One test of the model was to repeat the experiment using cells whose CckA protein is specifically impaired in phosphatase activity (K+P-) (Chen et al, 2009), so that the stalked pole is no longer a sink for CtrA~P. In these cells, the second round of DNA replication was no longer biased toward the stalked pole, but occurred with equal frequency at the stalked and swarmer poles of division-inhibited cells (Chen et al, 2011). This indirect demonstration of a gradient of CtrA~P underscores the need for generalized methods to visualize the activated forms of response regulators *in vivo*.

Because DivK~P inhibits the kinase activity of CckA, the levels of DivK~P and CtrA~P are inversely related in swarmer and stalked cells. Surprisingly, both CtrA~P and DivK~P levels are high in predivisional cells (Jacobs et al, 2003; Jacobs et al, 2001) (Fig. 1B). How is it that CckA is not downregulated at this time in the cell cycle? Localization of CckA at the swarmer pole adjacent to PleC, the DivK~P phosphatase, is suggested to protect CckA from downregulation by DivK~P (Tsokos et al, 2011) (Fig. 3A). This model would account for the high level of CckA kinase activity in the face of high overall levels of DivK~P in predivisional cells. Phenotypes of *divK* and *pleC* mutants agree with the model, but it would be most convincing to examine CckA activity in cells where PleC was both present and able to dephosphorylate DivK~P, but mislocalized away from CckA. Below, we discuss the factors that mediate the polar localization of key histidine kinases. As this area of research advances, it will become easier to test the effects of protein mislocalization in *Caulobacter*.

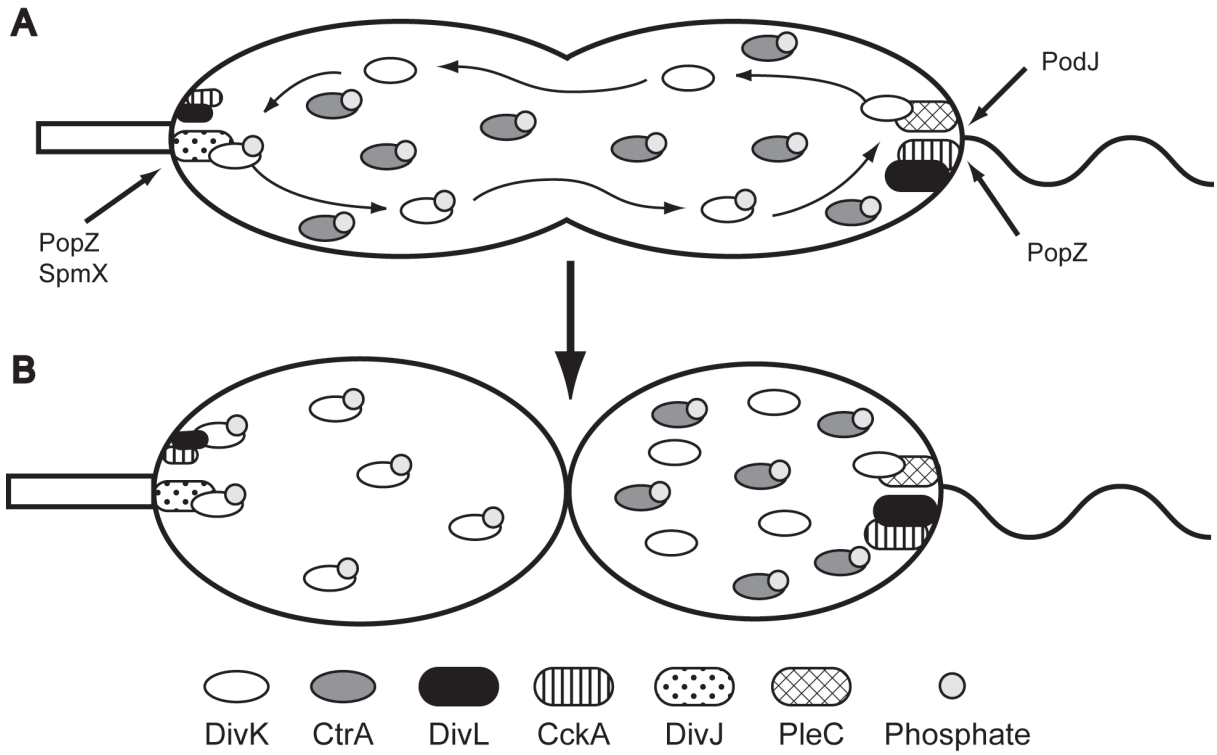

Fig. 3. Localized histidine kinases with opposing activities create an asymmetric predivisional cell and distinct cell identities after compartmentalization. (A) Arrows leading from PopZ, SpmX, and PodJ denote that these factors are required for the polar localization of the indicated histidine kinases. In the late predivisional cell (A), DivK diffuses throughout the cell (interior arrows), being phosphorylated by DivJ at the stalked pole and dephosphorylated by PleC at the flagellar pole. At the swarmer pole, colocalization of PleC with DivL and CckA keeps DivK~P levels low and maintains CckA kinase activity. At the stalked pole, the smaller foci of DivL and CckA are not shielded from DivK~P, so CckA at that location acts as a phosphatase. This arrangement results in a gradient of CtrA~P, with higher CtrA activity at the flagellar pole. After a barrier to cytoplasmic diffusion has been established between the two compartments of the predivisional cell (B), DivK becomes phosphorylated in the stalked compartment and dephosphorylated in the swarmer compartment. DivK~P in the stalked compartment triggers c-di-GMP accumulation and CtrA proteolysis, while CtrA remains present and active in the swarmer compartment.

Factors required for polar localization of signaling proteins

For this elegant mechanism to work, DivJ, PleC, CckA, and DivL must be located at the correct cell poles. Several studies have focused on the *cis* and *trans* factors required for localization of these histidine kinases, uncovering novel cellular organization proteins and links to other cell cycle processes. PleC localization to the pole opposite the stalk depends on PodJ, which occupies the pole before the arrival of PleC (Hinz et al, 2003; Viollier et al, 2002b)(Fig. 3A). In the absence of PodJ, PleC is delocalized or mislocalized to the stalked pole (Lawler et al, 2006). *podJ* mutants have defects in chemotaxis, flagellar release, and pilus biosynthesis, though these phenotypes are less severe than in cells lacking PleC, suggesting that localization is necessary for some, but not all functions of PleC (Wang et al, 1993). It is currently unknown how PodJ itself becomes localized at the flagellar pole, or if PodJ binds directly to PleC. During the SW-ST transition, PleC is released from the flagellar pole as it is remodeled into a stalked pole. PleC autophosphorylation is apparently required for its release from the pole, as the protein PleC-H610A lacking the conserved histidine residue is retained at the remodeled pole during differentiation (Viollier et al, 2002a).

DivJ localization requires SpmX (Fig. 3A), which contains a periplasmic muramidase domain followed by two transmembrane spans (Radhakrishnan et al, 2008). SpmX colocalizes with DivJ, and the two proteins can be immunoprecipitated together from *Caulobacter* lysates (Radhakrishnan et al, 2008). It is not clear if SpmX is active as a muramidase enzyme, but polar localization of SpmX depends on its muramidase domain (Radhakrishnan et al, 2008), suggesting that this domain may bind to a specific feature of stalked pole peptidoglycan. The entire SpmX protein is required for DivJ localization, so the interaction between them is most likely mediated by their respective transmembrane spanning domains (Radhakrishnan et al, 2008; Sciochetti et al, 2002).

DivL both stimulates CckA kinase activity and promotes CckA localization to the swarmer pole (Iniesta et al, 2010a). Deletion studies have identified regions of DivL and CckA that are needed for their localization to the swarmer pole. DivL requires its single transmembrane spanning domain and sequences at the extreme C-terminus (Sciochetti et al, 2005), while CckA requires its transmembrane spans, a PAS domain upstream of the DHp domain, and a loop within the CA domain for localization to the same pole (Angelastro et al, 2010; Jacobs et al, 1999). DivL and CckA only accumulate at the pole opposite the stalk after the initiation of DNA replication (Iniesta et al, 2010b).

Upstream of both CckA and DivJ localization is the polar organizing protein PopZ (Fig. 3A), a 19 kDa protein that self-associates to form a reticular network *in vitro* (Bowman et al, 2008). In *Caulobacter*, PopZ assembles in DNA-free zones at the cell poles and excludes ribosomes from these regions (Bowman et al, 2008; Ebersbach et al, 2008). PopZ is located at both poles of the predivisional cell; thus, both the swarmer and stalked progeny inherit a focus of PopZ at the "old" pole. After the initiation of DNA replication in each daughter cell, a new focus of PopZ forms at the opposite cell pole, yielding bipolar PopZ (Bowman et al, 2008; Ebersbach et al, 2008).

CckA and DivJ co-immunoprecipitate with PopZ, and both proteins are mislocalized in a *popZ* mutant (Ebersbach et al, 2008). In the case of CckA, these results suggest that DivL and CckA are recruited to the pole opposite the stalk by PopZ after the initiation of DNA replication. However, PopZ must not be sufficient to direct polar localization of DivL and CckA, because these proteins are only sometimes located at the stalked pole along with PopZ (Angelastro et al, 2010; Bowman et al, 2008; Ebersbach et al, 2008; Sciochetti et al, 2005). Both DivJ and SpmX

depend on PopZ for stalked-pole localization (Bowman et al, 2010; Ebersbach et al, 2008). However, additional players are likely involved in this process as well, since neither protein follows PopZ to the pole opposite the stalk in predivisional cells. Furthermore, since its periplasmic muramidase domain is sufficient to localize SpmX to the stalked pole (Radhakrishnan et al, 2008), it seems likely that another factor transmits the organizing effects of PopZ across the cytoplasmic membrane to SpmX.

Coordinated changes in two-component signaling and cyclic diguanylate link cell cycle progression and morphogenesis

Compartmentalization of the predivisional cell creates a sharp distinction between the biochemical environments in the two progeny, enabling immediate diversification of cell contents and functions. In contrast, swarmer cell differentiation, in which CtrA is deactivated, chromosome replication begins, and polar structures are dismantled and rebuilt, occurs without the benefit of a sharp structural transition. What triggers the onset of DNA replication and polar morphogenesis in the swarmer cell? Studies in *Caulobacter* have been among the first to uncover tight linkages between two-component signaling and the second messenger cyclic c-di-GMP. In the current model, changes in the activity of key histidine kinases CckA, DivJ, and PleC, are coordinated with an increase in the cellular level of c-di-GMP to trigger the G1-S transition and the remodeling of polar structures.

In the *Caulobacter* cell cycle, CtrA proteolysis is spatially and temporally restricted such that it occurs only during the SW-ST transition (Fig. 2B) and in the stalked compartment of the late predivisional cell (Domian et al, 1997) (Fig. 3B). Yet ClpXP, the protease that degrades CtrA, is present throughout the cell cycle (Jenal & Fuchs, 1998). Determining how the stability of this key regulator is controlled is central to understanding cell cycle progression and morphogenesis in *Caulobacter*. At one level, CtrA proteolysis is understood: it is triggered by the inhibition of CckA kinase activity. In the unphosphorylated state, the single-domain response regulator CpdR promotes the degradation of a number of proteins, including CtrA. The activities of both CpdR and CtrA are regulated by the CckA-ChpT phosphorelay (Biondi et al, 2006a). When CtrA is activated by phosphorylation, proteolysis of CtrA is simultaneously blocked by phosphorylation of CpdR (Iniesta et al, 2006). CpdR itself is degraded by ClpXP shortly after proteolysis of CtrA, allowing for re-accumulation of CtrA after the initiation of chromosome replication (Iniesta & Shapiro, 2008). However, if we look closer, CtrA proteolysis becomes more complex, involving an additional response regulator and c-di-GMP.

In addition to CpdR and ClpXP, regulated CtrA proteolysis requires RcdA, a protein of unknown function, and PopA, a c-di-GMP-binding response regulator (Duerig et al, 2009; McGrath et al, 2006) (Fig. 2). Prior to CtrA degradation, all of these proteins are localized, either to the nascent stalked pole at the SW-ST transition, or to the stalked pole of the predivisional cell (Duerig et al, 2009; Iniesta et al, 2006; McGrath et al, 2006; Ryan et al, 2002). A branched dependency pathway mediates their localization, such that the polar organizing protein PopZ is needed for CpdR localization (Bowman et al, 2010), and localization of ClpXP requires localized CpdR (Bowman et al, 2010; Iniesta et al, 2006). ClpX is one factor needed to localize RcdA, and CtrA localization is dependent on RcdA (McGrath et al, 2006; Taylor et al, 2009). The second factor required for RcdA localization is PopA, a response regulator that binds c-di-GMP (Duerig et al, 2009). Despite the existence of this elaborate scheme, it is not clear how polar localization of the protease and accessory factors affects the recognition and degradation of CtrA by ClpXP. For example, some mutations in *rcdA* that prevent polar accumulation of CtrA still yield wild-

type rates of CtrA proteolysis *in vivo* (Taylor et al, 2009). Thus, the accessory factors seem not to stimulate CtrA proteolysis simply by increasing the local substrate concentration at the cell pole.

Co-immunoprecipitation experiments indicate that CtrA, ClpXP, and RcdA form a complex *in vivo* (Duerig et al, 2009; McGrath et al, 2006), and CpdR also forms a complex with ClpXP (Iniesta et al, 2006). CpdR, RcdA or PopA could, singly or in concert, act as an adaptor that binds CtrA and physically delivers it to the protease at the appropriate time in the cell cycle (Schmidt et al, 2009). We would expect such an adaptor to be required for CtrA proteolysis *in vitro*, or to stimulate the rate of degradation, and to interact directly with the substrate and protease. Currently none of the accessory proteins has been shown to perform these functions. First, although PopA-RcdA, CpdR-ClpX, and CpdR-RcdA interactions have been observed in BACTH assays, none of these proteins gives a positive result for interaction with CtrA (Duerig et al, 2009). Second, CtrA can be degraded *in vitro* by purified ClpXP without any additional proteins, and adding RcdA does not accelerate the reaction (Chien et al, 2007). The effects of PopA, c-di-GMP, and CpdR on *in vitro* CtrA proteolysis have not yet been reported. Proteolysis of CtrA by ClpXP alone is extremely surprising, because CtrA is stabilized in mutants lacking any one of the accessory proteins. More work is required to elucidate the mechanism of regulated CtrA proteolysis, the roles of individual accessory proteins, and the relationship between ClpXP localization and its activity toward CtrA.

As noted above, one protein that stimulates CtrA proteolysis is PopA, which contains two tandem receiver domains followed by a GGDEF domain. GGDEF domains often have diguanylate cyclase activity, synthesizing c-di-GMP (Schirmer & Jenal, 2009). However, PopA acts as a sensor of c-di-GMP levels, binding this second messenger at an allosteric site (I-site) in the GGDEF domain (Duerig et al, 2009). The second receiver domain in PopA lacks an intact phosphoryl acceptor site, and mutational analysis showed that phosphorylation of the conserved aspartate residue in the first receiver domain is not required for PopA function. Instead, PopA is localized to the stalked pole and promotes CtrA degradation when c-di-GMP is bound to its I-site (Duerig et al, 2009), underscoring the fact that two-component proteins can have critical inputs other than phosphorylation (Fig. 2B).

The regulatory scheme for PopA suggests that cellular levels of c-di-GMP rise at the G1-S transition, when CtrA proteolysis is triggered (Fig. 1B). In fact, the overall c-di-GMP concentration does oscillate during the *Caulobacter* cell cycle, with highest levels occurring coincident with CtrA degradation and remodeling of the flagellar pole into a stalked pole (Paul et al, 2008). PopA binds c-di-GMP with a K_d of ~2 μM (Duerig et al, 2009), and mixed population of rapidly-growing *Caulobacter* was found to contain 1.1 μM c-di-GMP (Christen et al, 2005). In a different set of experiments using a FRET-based c-di-GMP biosensor, newly born stalked cells contained >500 nM c-di-GMP, while newly born swarmer cells transiently contained low levels of c-di-GMP, below 100 nM (Christen et al, 2010). Thus, although *in vivo* measurements of [c-di-GMP] vary slightly, it appears that PopA is poised to respond to physiological changes in c-di-GMP levels during the cell cycle and in different cell types.

Two-component proteins in *Caulobacter* are not only sensors of c-di-GMP, they also contribute to cellular levels of this second messenger, reflecting a tight interweaving of these signaling mechanisms in cell cycle regulation. One of these proteins is PleD, a paralog of PopA containing two tandem receiver domains followed by a GGDEF domain. Structural and biochemical studies using PleD were the first to establish the diguanylate cyclase (DGC) activity of GGDEF domains and provided critical insights into the catalytic mechanism of c-di-GMP production (Chan et al, 2004; Paul et al, 2004). When phosphorylated on aspartate 53 in the first

receiver domain, PleD dimerizes, creating a two-fold symmetric active site where two GTP molecules react to form one molecule of c-di-GMP (Paul et al, 2007; Wassmann et al, 2007). This activation mechanism provides a direct linkage between two-component signaling and c-di-GMP production (Fig. 2B).

pleD mutants are hypermotile, defective in stalk biogenesis, and delayed in the production of holdfast, an adhesive material located at the tip of the stalk, indicating that PleD is necessary for morphological changes during swarmer-to-stalked cell development (Aldridge et al, 2003; Hecht & Newton, 1995; Levi & Jenal, 2006). PleD activity is excluded from swarmer cells and is stimulated during development by histidine kinases that regulate its phosphorylation state (see below and Fig. 2) (Paul et al, 2008). During differentiation, c-di-GMP produced by PleD is necessary for the degradation of the flagellar MS-ring protein FliF by ClpAP, though the mechanism by which c-di-GMP affects this process is not yet understood (Aldridge & Jenal, 1999; Paul et al, 2004). On the other hand, CtrA degradation by ClpXP proceeds normally in strains lacking PleD (Aldridge & Jenal, 1999). This result suggested that other DGCs contribute to the cellular pool of c-di-GMP that activates PopA and promotes CtrA degradation.

Recently two proteins with opposing enzymatic activities were found to work with PleD in modulating c-di-GMP levels during the SW-ST transition (Abel et al, 2011). DgcB is a GGDEF protein with diguanylate cyclase activity that is present throughout the cell cycle, while PdeA is a swarmer cell-specific phosphodiesterase that hydrolyzes c-di-GMP to 5' phosphoguanylyl-(3'-5')-guanosine (pGpG) (Abel et al, 2011; Christen et al, 2005). Mutants lacking PdeA are defective in swarming and have an increased propensity to attach to surfaces, due to premature holdfast synthesis. These phenotypes can be rescued by *ΔdgcB*, but not by *ΔpleD*, indicating that unlike PleD, DgcB is active in the swarmer cell (Fig. 2A). PdeA maintains low c-di-GMP levels in the swarmer cell despite the presence of active DgcB, thus prolonging the motile G1-phase of the cell cycle. During swarmer cell differentiation, two events increase the levels of c-di-GMP: PleD activation and PdeA degradation by ClpXP (Fig. 2B). If both *pleD* and *dgcB* are deleted, or if PleD is absent and PdeA is stabilized, then c-di-GMP levels are not sufficient to activate PopA, and CtrA proteolysis is impaired (Abel et al, 2011).

Through PleD activation and PdeA proteolysis, the increase in [c-di-GMP] during the swarmer-to-stalked cell transition is linked to the extended phosphorelay that also controls CtrA activation and stability. CpdR, the same response regulator that is necessary for CtrA degradation, interacts with PdeA and ClpX in BACTH assays, and its unphosphorylated form is required for PdeA proteolysis *in vivo* and *in vitro* (Abel et al, 2011). This recent discovery is the first example of a single-domain response regulator acting as an adaptor protein to trigger proteolysis in a cell cycle-regulated manner. In this way, CpdR indirectly stimulates CtrA proteolysis via degradation of PdeA and the subsequent rise in c-di-GMP levels (Fig. 2B). However, it appears that CpdR also performs a second role in CtrA proteolysis, because deletion of *pdeA* cannot overcome the CtrA degradation defect of a *ΔcpdR* mutant (Abel et al, 2011).

The essential single-domain response regulator DivK is a central node in coordinating cell cycle progression via CtrA elimination and polar morphogenesis triggered by increased c-di-GMP levels. Dephosphorylation of CpdR is stimulated when DivK~P downregulates the CckA/ChpT phosphorelay (Biondi et al, 2006a). DivK also stimulates PleD phosphorylation by allosterically modulating the activities of DivJ and PleC (Paul et al, 2008). *divJ* and *pleC* mutants both contain less PleD~P than a wild-type strain, suggesting that both kinases contribute to PleD phosphorylation (Aldridge et al, 2003). DivJ readily phosphorylates PleD *in vitro*, but phosphotransfer from PleC to PleD is inefficient unless DivK is added to the reaction (Paul et al,

2008; Paul et al, 2004). DivK also stimulates DivJ autophosphorylation and subsequent phosphotransfer to PleD or to DivK itself (Paul et al, 2008). These experiments were the first demonstration of allosteric regulation of a histidine kinase by its cognate response regulator, and the mechanism of stimulation is still under investigation.

In the newly born stalked cell, activation of DivJ by DivK is thought to result in a positive feedback loop that increases cellular levels of DivK~P and PleD~P, triggering immediate entry into S-phase. In the swarmer progeny, PleC dephosphorylates DivK, which maintains activity of the CckA/ChpT phosphorelay and blocks the proteolysis of CtrA and PdeA (Fig. 2A). After a delay, DivJ accumulates in the swarmer cell, and PleC and DivJ transiently occupy the developing pole together. Perhaps DivK phosphorylation by DivJ helps to switch PleC into kinase mode, which further activates both DivK and PleD, triggering the G1-S transition and differentiation into a stalked cell (Paul et al, 2008) (Fig. 2B).

In this chapter, we have assumed that c-di-GMP functions as a pool of molecules that diffuses freely within a cell or cell compartment. However, a study in *Pseudomonas aeruginosa* showed that two DGCs have similar effects on overall cellular levels of c-di-GMP, but regulate two distinct processes, flagellar motility and production of extracellular polysaccharides (Merritt, et al., 2010). These results suggest that localized production or sensing of c-di-GMP may be involved in cellular outputs. In *Caulobacter*, the DGC PleD becomes localized at the nascent stalked pole during the SW-ST transition and remains at this location for the rest of the cell cycle (Paul, et al., 2004). PleD localization is dependent on phosphorylation of its N-terminal receiver domain by DivJ and PleC, so when PleD is activated to produce c-di-GMP, it is also localized to the stalked pole. PleD may thereby generate a high local concentration of c-di-GMP, which could stimulate downstream processes during the SW-ST transition and contribute to asymmetry in the predivisional cell.

It is unclear what external signals influence c-di-GMP levels in *Caulobacter*, but in other bacteria, c-di-GMP levels have been linked to blue light, antibiotics, and nutrient and oxygen availability (Jenal & Malone, 2006). Therefore, c-di-GMP signaling provides a potential mechanism to integrate the internal drivers of the *Caulobacter* cell cycle with information about the external environment. The phosphodiesterase PdeA is allosterically activated by GTP, providing one potential mechanism linking c-di-GMP to the cell's energy status (Christen et al, 2005). Future work will afford a deeper understanding of how external stimuli might modulate the *Caulobacter* cell cycle through c-di-GMP signaling.

Regulation of flagellum and stalk biogenesis

In addition to being a key cell cycle regulator, CtrA is also at the top of a transcriptional cascade of flagellar gene expression (Smith & Hoover, 2009). In fact, the first *ctrA* mutant was identified in a screen to detect both misregulation of the Class II flagellar gene *fliQ* and temperature-sensitive lethality (Quon et al, 1996). Transcription of Class II flagellar genes is limited to the predivisional phase of the cell cycle by requiring both high levels of CtrA~P (Reisenauer et al, 1999) and the absence of SciP, a swarmer cell-specific protein that represses CtrA-dependent transcription (Gora et al, 2010).

In the canonical view of flagellar biogenesis, a master Class I regulator (in this case CtrA) drives the transcription of Class II genes, which encode proximal components of the flagellum and regulators for the downstream Class III and Class IV genes. Class III and IV genes, encoding distal structural components and flagellins, require the prior transcription of Class II genes. CtrA was first found to recognize an element present in the promoters of Class II

flagellar genes, as well as in the *ccrM* promoter and the origin of replication (Quon et al, 1996; Quon et al, 1998; Stephens et al, 1995). However, chromatin immunoprecipitation microarray (ChIP-chip) experiments revealed that CtrA also binds to the promoters of some Class III and Class IV flagellar genes (Laub et al, 2002), suggesting that the current view of the flagellar transcriptional hierarchy may be oversimplified.

Among the Class II flagellar genes activated by CtrA is *flbD*, which encodes a DNA-binding response regulator with homology to the nitrogen regulatory protein NtrC (Ramakrishnan & Newton, 1990). Response regulators in the NtrC family have three domains, an N-terminal receiver domain, a central AAA+ ATPase domain, and a C-terminal DNA-binding domain. These proteins activate transcription in concert with RNA polymerase containing a specific sigma factor, σ^{54} (Rombel et al, 1998). FlbD works with RNA polymerase containing the *Caulobacter* σ^{54} protein RpoN to transcribe Class III and IV flagellar genes, but FlbD can also behave as a transcriptional repressor (Benson et al, 1994a; Brun & Shapiro, 1992; Ramakrishnan & Newton, 1990; Wingrove et al, 1993). FlbD is phosphorylated *in vivo* and can be phosphorylated *in vitro* using *Caulobacter* extracts (Wingrove et al, 1993), but FlbD is active in *in vitro* transcription assays without phosphorylation (Benson et al, 1994b; Wu et al, 1995). Moreover, a FlbD-specific histidine kinase has not been identified (Muir & Gober, 2001). Instead, FlbD activity is controlled by direct interaction with a trans-acting factor FliX that is thought to sense the assembly of the flagellar basal body (Muir & Gober, 2004).

Caulobacter fliX and *flbD* loss-of-function mutants are nonmotile and fail to transcribe Class III and IV flagellar genes (Mohr et al, 1998; Muir et al, 2001; Wingrove et al, 1993). In addition, mutations that block the assembly of early flagellar structures also prevent transcription of Class III/IV genes (Mangan et al, 1995). Bypass mutations that restore Class III/IV gene expression to cells lacking Class II-encoded structures can be found in both *flbD* and *fliX*, and a bypass mutation in *flbD* can restore motility to a *fliX* null mutant (Mangan et al, 1995) (Muir et al, 2001). These results suggest that FliX is not a structural component of the flagellum, but part of a checkpoint ensuring that early flagellar structures are complete before late flagellar proteins are synthesized. *In vitro*, FliX enhances FlbD-activated transcription by inducing FlbD to form higher-order oligomers (Dutton et al, 2005), similar to activated NtrC (Porter et al, 1993). Interestingly, overexpression of FliX in wild-type cells suppresses FlbD-activated transcription (Muir et al, 2001). Together, these findings suggest that FliX switches from a FlbD-repressing to a FlbD-activating mode upon completion of the Class II flagellar basal body structure. Regulation of a σ^{54}-dependent transcriptional activator by direct binding to another protein is novel (Muir & Gober, 2004), and the precise mechanism of FliX action is still being unraveled.

Caulobacter RpoN functions in concert with a different σ^{54}-dependent transcriptional activator, TacA, to promote stalk biogenesis (Biondi et al, 2006b; Brun & Shapiro, 1992; Skerker et al, 2005). Systematic deletion of each two-component gene in the *Caulobacter* genome revealed that two mutants, *ΔCC0138* and *ΔtacA* were stalkless in rich medium, suggesting that they function in a pathway to regulate stalk biogenesis (Skerker et al, 2005). *CC0138* encodes a hybrid histidine kinase with N-terminal DHp and CA domains, two C-terminal receiver domains, and no obvious sensory domains. A bioinformatics approach identified a histidine phosphotransferase protein encoded by *CC1114*, and phosphotransfer profiling experiments demonstrated that CC1114 (ShpA) preferentially transfers a phosphoryl group between the second receiver domain of CC0138 (ShkA) and the receiver domain of TacA (Biondi et al, 2006b). The constitutively active phosphomimetic protein TacA-D54E rescues the stalk biogenesis phenotype of *shkA* and *shpA* mutants (Biondi et al, 2006b). Together these results

indicate that ShkA and ShpA activate TacA through phosphorylation, leading to stalk biogenesis. Further studies are needed to determine the signals sensed by ShkA and when and where in the cell it is active.

Mutations in other two-component genes, notably *ctrA* and *pleC*, result in cells that are nonmotile and stalkless when grown in rich medium, similar to cells lacking RpoN (Brun & Shapiro, 1992; Quon et al, 1996; Sommer & Newton, 1989). These data can be rationalized by the facts that CtrA directly activates the transcription of *flbD*, *tacA*, and *rpoN* (Laub et al, 2002; Mohr et al, 1998), and PleC promotes CtrA activity by dephosphorylating DivK~P (Biondi et al, 2006a; Tsokos et al, 2011). The stalk biogenesis defects of *ctrA*, *pleC*, and *tacA* pathway mutants can be partially rescued by growth in minimal medium containing low amounts of phosphate, and low-phosphate conditions increase the stalk length of wild-type cells (Gonin et al, 2000). Mutations affecting the Pst phosphate uptake system cause stalk elongation even in the presence of high levels of phosphate, while mutations in the gene encoding the response regulator PhoB prevent stalk elongation in low-phosphate medium (Gonin et al, 2000). Since the *E. coli* Pst proteins deactivate PhoR, the cognate kinase of PhoB, when phosphate is in excess (Wanner, 1993), these results in *Caulobacter* suggest that the lack of phosphate is sensed by the Pst system, leading to phosphorylation of PhoB and a PhoB~P-dependent transcriptional response that promotes stalk elongation.

Despite knowledge of two regulatory pathways, developmental and starvation-induced, that affect the stalk, the relationship between these pathways has yet to be defined, and few proteins directly involved in stalk construction have been identified (Kuhn et al, 2010). Recently, the first mutant to remain stalkless even in low-phosphate medium was discovered (Abel et al, 2011). Deletion of two diguanylate cyclases, *pleD* and *dgcB*, blocks both developmental and phosphate starvation-induced stalk biogenesis, suggesting that these developmental pathways converge on the second messenger c-di-GMP. Additional work is needed to uncover the mechanism linking the c-di-GMP signal to stalk elongation.

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Chapter 2: Mutations that Alter RcdA Surface Residues Decouple Protein Localization and CtrA Proteolysis in *Caulobacter crescentus*

Abstract

Periodic activation and deactivation of the essential transcriptional regulator CtrA is necessary to drive cell cycle progression in *Caulobacter crescentus*. At the onset of DNA replication (the G1-S cell cycle transition), CtrA and the AAA+ protease ClpXP colocalize at one cell pole along with three accessory proteins, RcdA, CpdR, and PopA, and CtrA is rapidly degraded. RcdA is required for polar sequestration and regulated proteolysis of CtrA *in vivo*, but it does not stimulate CtrA degradation by ClpXP *in vitro*, so the function of RcdA is unknown. We determined the 2.9-Å-resolution crystal structure of RcdA and generated structure-guided mutations in *rcdA*. We assayed the ability of each RcdA variant to support CtrA proteolysis and polar protein localization in *Caulobacter*. Deletion of an intrinsically disordered peptide at the C-terminus of RcdA prevents efficient CtrA degradation and blocks the transient localization of RcdA and CtrA at the cell pole. Surprisingly, substitutions in two groups of highly conserved, charged surface residues disrupt polar RcdA or CtrA localization, but do not affect CtrA proteolysis. This is the first report showing that localization of RcdA can be decoupled from its effects on CtrA degradation. In addition, we used epistasis experiments to show that RcdA is still required for regulated CtrA proteolysis when all SsrA-tagged proteins, abundant substrates of ClpXP, are absent from the cell. Our results argue that RcdA stimulates CtrA proteolysis neither by localizing CtrA at the cell pole, nor by preventing competition from SsrA-tagged substrates.

Keywords: *Caulobacter*, ClpXP, proteolysis, cell cycle, protein localization

Abbreviations: aa, amino acids; SW, swarmer cell; ST, stalked cell; PD, predivisional cell

Introduction

Temporally controlled proteolysis of regulatory proteins is a common feature of cell cycle regulation in both eukaryotes and bacteria. A prime example of regulated proteolysis driving cell cycle progression is found in the α-proteobacterium *Caulobacter crescentus*. *Caulobacter* divides asymmetrically to produce morphologically distinct daughter cells. The stalked cell (ST) can immediately reinitiate chromosome replication after the previous cell division, while the swarmer cell (SW) must first differentiate into a stalked cell before starting a new cycle of DNA replication and division.(Collier & Shapiro, 2007) Underlying the differences between the daughter cells is the activity of an essential response regulator, CtrA(Domian et al, 1997; Quon et al, 1996). SW cells contain active CtrA, which directly controls the expression of 95 genes(Laub et al, 2002) and blocks the initiation of DNA replication by binding to five sites in the replication origin.(Quon et al, 1998) Thus, for S-phase to commence, CtrA activity must be eliminated from the developing SW cell. This is achieved redundantly by dephosphorylation and directed proteolysis.(Domian et al, 1997)

CtrA is degraded specifically at the G1-S phase transition by the AAA+ protease ClpXP.(Jenal & Fuchs, 1998) Within this complex, the ClpP subunits have protease activity, while the ClpX subunits mediate substrate recognition and unfolding.(Licht & Lee, 2008) CtrA phosphorylation and proteolysis are coordinately regulated by the same phosphorelay,(Biondi et al, 2006) in which the hybrid histidine kinase CckA phosphorylates the histidine phosphotransferase ChpT. ChpT can then pass the phosphoryl group to CtrA or to the single-domain response regulator CpdR. When the phosphorelay is inactive, CtrA itself is unphosphorylated, and unphosphorylated CpdR promotes CtrA degradation.(Iniesta et al, 2006) RcdA, a protein of unknown function, is required in addition to ClpXP and CpdR for regulated CtrA proteolysis.(McGrath et al, 2006) CpdR is necessary for ClpXP-mediated proteolysis of McpA, a chemoreceptor protein, while RcdA is only known to influence the degradation of CtrA.(Iniesta et al, 2006; McGrath et al, 2006) RcdA is conserved in a subset of the alpha-proteobacteria,(Kainth & Gupta, 2005) but its fold cannot be predicted from its primary sequence.

Despite the fact that ClpXP, CtrA, RcdA, and CpdR are all cytoplasmic proteins, CtrA degradation occurs at a specific location in the *Caulobacter* cell.(Ryan et al, 2002)(Ryan et al, 2004) At the nascent stalked pole in differentiating SW cells, and at the stalked pole of late predivisional (PD) cells, these proteins colocalize, and CtrA is degraded (Fig. 3a). There is a linear dependency for polar localization, such that CtrA requires the presence of RcdA, RcdA requires ClpXP, and ClpXP requires unphosphorylated CpdR.(Iniesta et al, 2006; McGrath et al, 2006)(Iniesta & Shapiro, 2008). Co-immunoprecipitation from *Caulobacter* lysates demonstrates that CpdR associates with ClpXP, and RcdA associates with both ClpXP and CtrA *in vivo*.(Iniesta et al, 2006; McGrath et al, 2006).

Recently a third protein, PopA, was identified that binds to RcdA and links CtrA proteolysis to cellular levels of the second messenger cyclic-di-GMP.(Duerig et al, 2009) PopA contains two receiver domains followed by a domain homologous to diguanylate cyclases.(Jenal & Malone, 2006) However, PopA lacks diguanylate cyclase activity and instead acts as a sensor for cyclic-di-GMP by binding it at an allosteric site that is distinct from the catalytic site.(Duerig et al, 2009) Localization of PopA at the stalked pole of the cell (Fig. 3a), where CtrA is degraded, is dependent on PopA binding to cyclic-di-GMP, but PopA-RcdA binding is independent of cyclic-di-GMP.(Duerig et al, 2009) Mutants lacking PopA or expressing PopA variants that cannot bind cyclic-di-GMP are unable to degrade CtrA at the G1-S transition, and in

these cells, neither RcdA nor CtrA accumulate at the cell pole.(Duerig et al, 2009) Thus, second messenger signaling via PopA along with the CckA-ChpT phosphorelay converge to regulate CtrA proteolysis.

Because RcdA is only known to be required for CtrA proteolysis, and not for the timed degradation of other ClpXP substrates, it was proposed to act as an adaptor for CtrA, receiving cell cycle information and determining when CtrA is a target for degradation.(McGrath et al, 2006) Adaptor proteins bind to the ATPase subunit of a Clp protease and to one or more substrates to enhance their degradation.(Ades, 2004) For example, a quality control system tags incomplete proteins with the SsrA peptide, and these tagged proteins are recognized and degraded by ClpXP.(Gottesman et al, 1998; Keiler et al, 1996) The dimeric adaptor protein SspB binds simultaneously to the SsrA peptide and to ClpX, reducing the Michaelis constant for SsrA-tagged substrates and stimulating their degradation.(Levchenko et al, 2000)(Wah et al, 2002) However, the rate of CtrA proteolysis by purified ClpXP *in vitro* is unaffected by the addition of RcdA, and RcdA does not interact stably with ClpX.(Chien et al, 2007)

In vitro studies suggest that RcdA has a cell-specific function in promoting CtrA degradation, one which is not required in the context of purified proteins. As an example, RcdA could act as a polar localization factor for CtrA, increasing the local concentration of CtrA in the vicinity of ClpXP, but not forming a direct ternary complex with the protease and substrate. Such a factor may exist because CtrA accumulates at the pole in a cell cycle-dependent manner even when it contains a mutation that prevents degradation by ClpXP.(Ryan et al, 2004) Alternatively, RcdA could stimulate CtrA proteolysis by directing ClpXP away from other substrates at the appropriate time in the cell cycle.(Chien et al, 2007) Finally, RcdA could inhibit a negative regulator of CtrA proteolysis (or activate a positive regulator) that is present in the cell, but is not present *in vitro.*

Because RcdA's function has not been revealed by reconstituting CtrA proteolysis *in vitro*, the Ryan lab initiated structure-function studies to determine how RcdA operates in the cell. A collaborator, Dr. Jeremey Wilbur, crystallized RcdA and determined the structure to 2.9 Å. RcdA forms an elongated crescent-shaped dimer with three flexible disordered peptides in each monomer.

Crystallization of RcdA by Dr. Wilbur, yielded rod-shaped tetragonal crystals, and the structure was determined using multiple anomalous dispersion from selenomethionine-substituted crystals (Figure 1a, Table 1, PDB ID: 3CTW). The asymmetric unit contained one RcdA homodimer. RcdA dimers form a crescent shape with approximate dimension of 63Å × 41Å × 21Å. The radius of curvature of the concave side is approximately 36Å. Each monomer of RcdA consists of a three-helix bundle with three intrinsically disordered regions. The first twenty amino acids (aa) of each monomer are disordered (Figure 1b, gray dashed lines), and the first defined residues are a random coil leading into helix α1. Helix α1 has a distortion in helical geometry associated with a Gly-Ala-Gly sequence (aa 46-48) just before the turn leading to helix α2. Helix α2 contains a series of conserved hydrophobic residues starting in the dimerization interface and continuing along its solvent-exposed side. Following helix α2 is an unstructured internal loop of 29 residues (Figure 1b, red dashed lines), initiated by a Pro-Pro motif (aa 93-94) and containing two more prolines. Because of the long internal loop, RcdA has an unusual topology for three-helix bundles with helices α2 and α3 packing parallel to each other. At the end of helix α3 is a short section of random coil and approximately 22 unstructured residues (Figure 1b, green dashed lines). Approximately 50 residues per monomer are disordered within the crystal.

RcdA dimerization buries a large hydrophobic patch (~2000 Å^2), comprised primarily of residues M34, V37, M75, A78, L82, and M92 from each monomer in a two-fold symmetric orientation. The helical interfaces from each monomer pack at an angle near 90º. This is significantly different from the more parallel interaction of BAR domains, the best known three-helix bundle dimers.(Peter et al, 2004) Database searches using the protein structure comparison service SSM(Krissinel & Henrick, 2004) yield many three-helix bundle proteins, but none that dimerize similarly to RcdA. Dimerization in solution was confirmed using calibrated gel filtration (Fig. 4).

We generated structure-guided mutants and tested their ability to restore CtrA proteolysis and polar protein localization to the Δ*rcdA* strain. Deletion of an intrinsically disordered peptide at the C-terminus of RcdA prevents efficient CtrA degradation and blocks the transient localization of RcdA and CtrA at the cell pole. The C-terminal peptide is therefore critical for RcdA's function in the cell. Unexpectedly, we found that some surface residues of RcdA are required for the polar accumulation of RcdA or CtrA but are not required for cell cycle-regulated CtrA proteolysis. These mutants demonstrate that polar accumulation of RcdA or CtrA is not mechanistically coupled to rapid CtrA degradation by ClpXP. Further, we found that RcdA is still required for regulated CtrA degradation even when all SsrA-tagged substrates are eliminated from the cell. Thus, RcdA does not stimulate CtrA proteolysis by directing ClpXP away from this group of alternative substrates.

Table 1. Crystallographic data collection and refinement for RcdA

Statistics						
	Native data	MAD data				
Space group	$P4_3$	$P4_3$				
Cell dimensions (Å)		a=b=75.5 c=81.6		a=b=75.7 c=81.4		
		Peak	Inflection	Remote		
Wavelength (Å)		1.1	0.97957	0.97972		1.01986
Resolution (Å)		44-2.9	44.6-3.2			
R_{merge} (%)		8.5 (29.9)	16.4 (55.0)	15.4 (60.5)		12.5 (48.7)
I/σI	18.1	9.6	8.9	10.8		
Completeness (%)		99.1 (89.6)	99.9	99.9	99.9	
Redundancy		4.7 (3.6)	4.3	4.3	4.2	
Total # Obs. Reflections		44,107	32,822	32,851	32,190	
Refinement						
Resolution (Å)		44-2.9 (2.975-2.90)				
# reflections used during refinement		9,630				
Rwork	27.5 (30.9)					
Rfree	31.2 (33.0)					
R.m.s. deviations						
Bond lengths (Å)		0.011				
Bond angles (º)		1.64				

Fig. 1

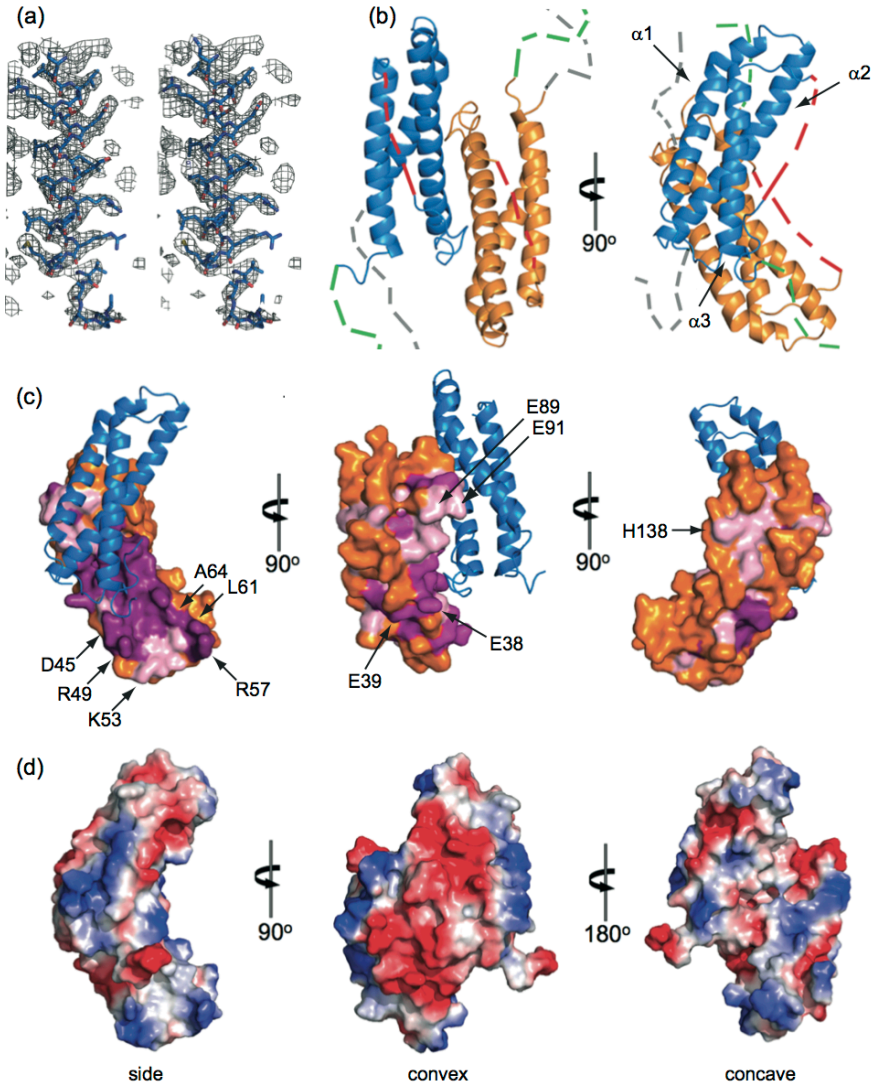


Fig. 1. The crystal structure of RcdA shows novel dimerization and multiple disordered regions. (a) Stereo view of the 2fo-fc electron density (1 sigma) of helix 3 near the C-terminal disordered region. (b) Overview of the RcdA dimer. One monomer is blue and the other is gold. Approximate locations of the N-terminal disordered peptide (gray), the internal disordered loop (red) and the C-terminal disordered peptide (green) are shown. Arrows indicate helices α1, α2 and α3. (c) Conservation of RcdA surface residues, including the dimer interface. Highly conserved (magenta) and moderately conserved (pink) residues are shown on the gold monomer. Positions of substituted residues are indicated with arrows. The paddle region is indicated by the gray circle in the left panel. (d) Qualitative surface electrostatic representation of the RcdA dimer, where red indicates negative charge and blue indicates positive charge.

Results

Deletion of the RcdA C-terminus prevents clearing of CtrA at the G1-S transition

A Postdoc in the Ryan lab, Dr. James Taylor initiated the targeted mutagenesis of RcdA and I carried this work to completion. We sought to identify surface residues of RcdA required for rapid CtrA proteolysis or for polar protein localization, as these amino acids could mediate interactions with other proteins. An alignment of RcdA with its nearest homologs from different genera (Fig. 2) showed that much of the sequence conservation in RcdA lies along the dimer interface and is unlikely to contribute to interactions with other proteins. However, the convex surface of RcdA (Figure 1c, center), extended paddle regions at either end of the RcdA dimer (Figure 1c, circled area), and the unstructured C-terminus also contain highly conserved residues.

We first made site-directed mutations in the gene *rcdA::GFP* to facilitate localization of mutant RcdA-GFP proteins within *Caulobacter* cells. Mutations that disrupted CtrA degradation (Fig. 3) or RcdA-GFP localization (Fig.6) were also created in *rcdA* alone, to confirm results obtained with the GFP fusion proteins, for pulse-chase experiments, and for localization of other fluorescent fusion proteins. Western blots confirmed that the RcdA variants and GFP fusions were expressed at approximately the same level as the wild-type protein (Supplementary Fig. 3)

I expressed each RcdA-GFP fusion protein from the chromosomal *xylX* locus (Meisenzahl et al, 1997) in the Δ*rcdA* strain (KR899), harvested swarmer cells, and allowed them to progress synchronously through the cell cycle. I then probed lysates from the synchronized cultures with antiserum against CtrA to detect CtrA levels as a function of the cell cycle. In these experiments, the wild-type RcdA-GFP fusion protein promoted CtrA proteolysis during the G1-S transition (Fig. 3b) as efficiently as RcdA alone (Supplementary Fig. 4). In contrast, the parent strain lacking RcdA was unable to degrade CtrA at the swarmer-to-stalked cell transition (Fig. 3b).

Because surface-exposed hydrophobic residues are often involved in protein-protein interactions,(Gruber et al, 2007) we replaced the conserved residues L61 and A64 (Figure 1c, left) with the polar amino acid serine to create RcdA-patch-GFP. When expressed from the *xylX* locus in the Δ*rcdA* strain, this protein conferred cell cycle-dependent CtrA proteolysis, similar to wild-type RcdA-GFP (Fig. 3b). Next, we tested the importance of conserved charged residues that protrude from the edges of the paddle regions (D45, R49, K53 and R57, Figure 1c, left). All of these residues were replaced by alanine in the polypeptide already containing the *rcdA-patch* mutations to yield RcdA-paddle-GFP. Δ*rcdA* cells expressing this protein also displayed normal CtrA degradation during swarmer cell differentiation (Fig. 3b). Thus, these conserved residues in the RcdA paddle region are not required for cell cycle-regulated CtrA proteolysis.

Because RcdA is a small, dimeric protein composed almost entirely of alpha-helices, we speculated that it could be phosphorylated on a conserved histidine residue, similar to histidine phosphotransferase proteins in two-component signaling pathways.(Laub & Goulian, 2007) Phosphorylation of RcdA in this manner could then modulate its function or intracellular location. We identified H138 as the only conserved histidine residue protruding from the surface of an alpha-helix (Figure 1c, right) and substituted alanine for this amino acid. The resulting protein, RcdA-H138A-GFP, also conferred normal cell cycle-dependent proteolysis of CtrA (Fig. 3b). Therefore, phosphorylation on H138 is not required for RcdA to promote CtrA degradation.

The most prominent electrostatic feature of the RcdA dimer is large acidic patch on the convex face (CF), containing the conserved residues E38, E39, E89, and E91 of each monomer

(Figure 1c and 1d, center). To determine if these residues are critical for CtrA proteolysis, I replaced all of them with alanines to create RcdA-CF-GFP. Again, these mutations caused no overt defect in CtrA proteolysis during the cell cycle (Fig. 3b). The C-terminus of RcdA beyond residue 150 is unstructured in the crystal, although it contains several amino acids conserved in RcdA homologs (Supplementary Figure 1). We deleted residues 151-169 to create RcdAΔC-GFP and expressed it in Δ*rcdA* cells from the *xylX* locus. During synchronous growth, these cells failed to degrade CtrA during swarmer cell differentiation (Fig. 3b). In the same culture, the chemoreceptor McpA, another ClpXP substrate, was proteolyzed at the expected time, indicating that passage through the cell cycle was unperturbed, and ClpXP function was not globally compromised. We obtained the same results with a Δ*rcdA* strain expressing RcdAΔC alone from the *xylX* locus (Supplementary Fig. 4). Western blots probed with anti-RcdA demonstrated that RcdAΔC was expressed at a level similar to the native RcdA protein in the wild-type strain CB15N (Fig. 4, inset; Supplementary Fig. 3b).

We used size exclusion chromatography to determine if deleting the C-terminus disrupts the gross structure of RcdA (Fig. 4). Purified RcdA and RcdAΔC eluted from a Superdex-200 column at 15.4 ml and 16.1 ml, respectively, corresponding to molecular masses of 42.1 kDa and 34.1 kDa. These values are close to the calculated dimer masses of RcdA (38.1 kDa) and RcdAΔC (34.3 kDa), so it is likely that RcdAΔC folds and dimerizes correctly.

We performed pulse-chase assays on unsynchronized cultures to quantify overall rates of CtrA proteolysis in strains expressing selected RcdA variants. RcdA-CF and RcdA-paddle-GFP each conferred a wild-type rate of CtrA degradation (Table 2), consistent with cell cycle Western blots. In contrast, cells expressing RcdAΔC degraded CtrA ~3-fold more slowly than wild-type (CB15N) or Δ*rcdA xylX::rcdA* cells, but ~1.5 times more quickly than cells lacking RcdA (Table 2). Because CtrA is rapidly proteolyzed during only a small window of the cell cycle, it is likely that profound effects on CtrA abundance at the SW-ST transition correspond to modest effects on the overall CtrA half-life in mixed cultures.

CtrA activity is regulated by redundant mechanisms, proteolysis and phosphorylation. Wild-type cells arrest in the G1 phase of the cell cycle if they overexpress a CtrA variant that both mimics the phosphorylated state and cannot be degraded.(Domian et al, 1997) If an *rcdA* mutation impairs CtrA proteolysis *in trans*, then overexpression of the phosphomimetic protein CtrA-D51E should lead to G1 arrest in that strain. In each strain tested, *ctrA-D51E* was expressed from the P_{xyl} promoter on a high-copy plasmid, while the *rcdA* allele to be tested was expressed from the chromosomal *xylX* locus. Δ*rcdA* cells overexpressing CtrA-D51E became filamentous and predominantly contain only one chromosome (Fig.5). Co-induction of wild-type RcdA, RcdA-H138A-GFP, or RcdA-CF in this strain restored normal morphology and DNA content, whereas induction of RcdAΔC caused filamentation and accumulation of G1-phase cells, similar to the Δ*rcdA* strain (Fig.5). Thus, the disordered C-terminal peptide of RcdA plays a key role in cell cycle-regulated proteolysis of CtrA.

Expression of RcdA-paddle-GFP yielded slightly elongated cells, but did not affect the cellular DNA content (Fig.5). I measured the half-life of the native CtrA protein in the strain Δ*rcdA xylX::rcdA-paddle* and found that it is not significantly different from either the wild-type strain CB15N or from cells expressing wild-type RcdA from the *xylX* promoter (Table 2). We cannot currently account for the change in cell morphology, since RcdA-paddle seems capable of mediating CtrA degradation during the cell cycle (Fig. 3b).

Table 2. CtrA half-lives in *rcdA* mutant strains.

strain	CtrA half-life (min)	standard deviation (min)
CB15N	33.1	4.6
Δ*rcdA*	135.8	23.6
Δ*rcdA xylX::rcdA*	28.1	1.5
Δ*rcdA xylX::rcdAΔC*	90.1	13.5
Δ*rcdA xylX::rcdA-CF*	33.2	2.4
Δ*rcdA xylX::RcdA-paddle-GFP*	31.1	2.6

Each strain was grown in M2G medium containing 0.1% xylose to induce expression of the indicated *rcdA* allele for 12 hours before and during pulse-chase analysis.

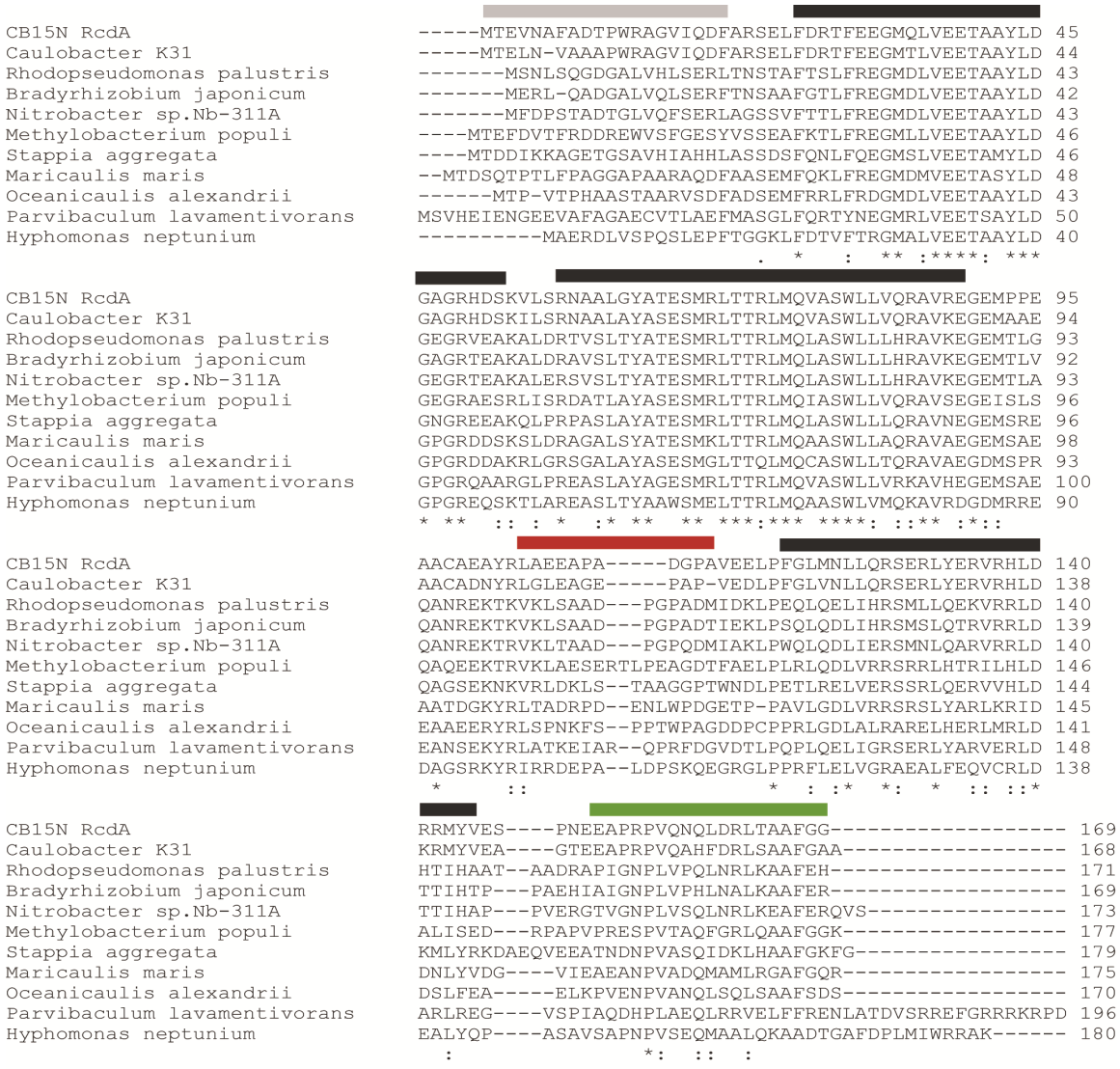


Fig. 2. Sequence alignment generated by ClustalW of RcdA homologs from the indicated species of a-proteobacteria. Black bars above the sequence indicate helices 1-2. The gray, red, and green bars indicate the N-terminal, internal, and C-terminal disordered peptides, respectively. Asterisks below the sequence indicate completely conserved residues, and two dots indicate highly conserved residues.

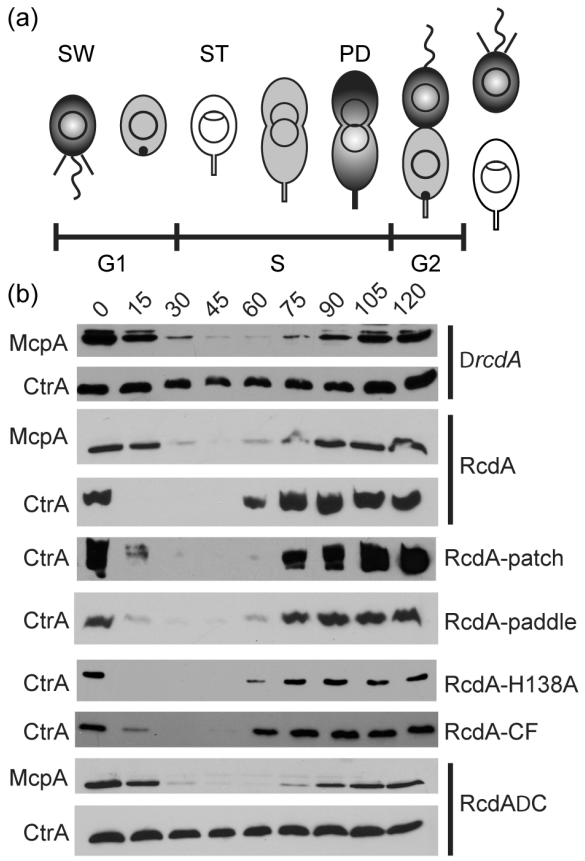


Fig. 3. Cell cycle degradation of CtrA in strains expressing mutant RcdA-GFP fusions. (a) Schematic of the *Caulobacter* cell cycle. The swarmer cell (SW) has a polar flagellum (wavy line) and pili (straight lines) and cannot initiate DNA replication (closed interior circle). As the swarmer cell differentiates into a stalked cell (ST), a proteolytic complex localizes at the cell pole (black dot) and degrades CtrA (dark or light gray shading). The stalked cell initiates DNA replication (theta structure) and elongates into a predivisional cell (PD), which resynthesizes CtrA. In the late predivsional cell, after a barrier is established between the flagellated and stalked compartments, the proteolytic complex again localizes at the stalked pole and degrades CtrA. The swarmer progeny contains CtrA and is replication-silent, while the stalked progeny lacks CtrA and is replication-competent. (b) Synchronized populations of cells expressing the indicated RcdA-GFP fusion proteins from the chromosomal *xylX* locus were sampled at the indicated times (minutes) during passage through the cell cycle. Lysates from equal numbers of cells were analyzed by SDS-PAGE and Western blotting with antisera against CtrA and McpA.

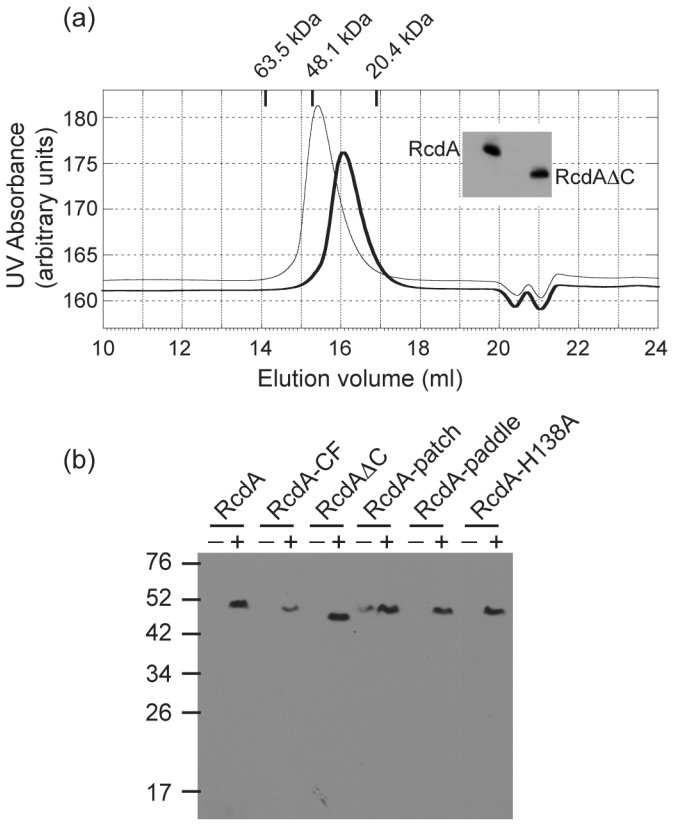


Fig. 4. Expression and dimerization of RcdAΔC. (a) Size-exclusion chromatography of RcdA (thin trace) and RcdAΔC (thick trace) on a Superdex-200 column. Elution peaks of protein standards used to calibrate the column are indicated above. Inset, the wild-type strain CB15N and the mutant Δ*rcdA xylX::rcdA*Δ*C* were grown in PYE/0.03% xylose, and lysates from equal cell numbers were analyzed by SDS-PAGE and Western blotting with antiserum against RcdA. (b) Expression of RcdA mutants. Wild-type strain CB15N and mutant strains expressing the indicated RcdA mutants were grown in PYE/0.03% xylose, and lysates from equal cell numbers were analyzed by SDS-PAGE and Western blotting with antiserum against RcdA.

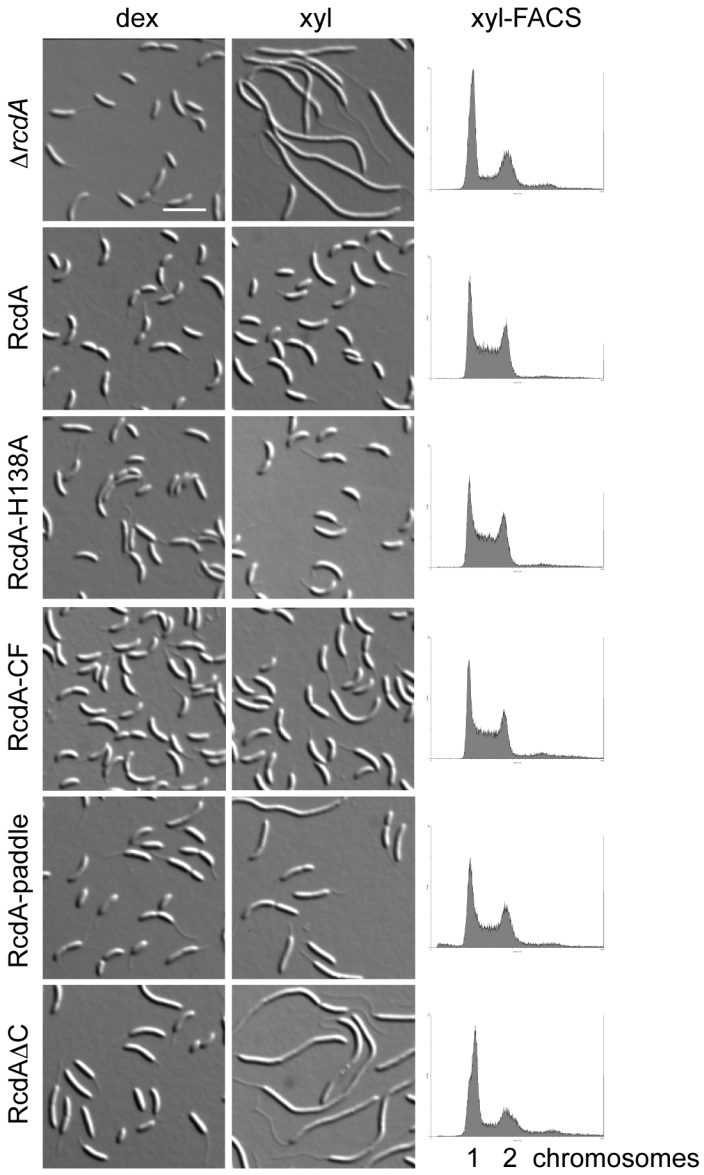


Fig.5. Induction of CtrA-D51E causes G1 arrest in rcdA mutants that are impaired in CtrA degradation. Each strain is Δ*rcdA* + pIDC42, from which CtrA-D51E was expressed under control of the P_{xyl} promoter. The indicated RcdA variants were expressed from the chromosomal *xylX* locus. Left column, DIC images of strains grown in PYE/0.03% dextrose. Middle column, DIC images of strains grown in PYE/0.03% xylose for 8 h. Right column, flow cytometry of xylose-induced cells (50 000 per graph) stained with Sytox green. Scale bar, 5 μm.

Two groups of RcdA surface residues are important for RcdA localization, but not for rapid CtrA proteolysis

The dynamic localization patterns of proteins involved in CtrA degradation have been established by examining fluorescent fusion proteins in living cells using time-lapse microscopy.(Ryan et al, 2002)(McGrath et al, 2006)(Iniesta et al, 2006)(Duerig et al, 2009) During the SW-ST transition, RcdA accumulates at the nascent stalked pole along with CpdR, PopA, and ClpXP, while CtrA is proteolyzed. In early predivisional cells, RcdA and ClpXP only are transiently located at the midcell. Finally, in late predivisional cells, RcdA is sequestered at the stalked pole with ClpXP, CpdR and PopA, and CtrA is cleared from the stalked compartment of the cell (Fig. 3a).

Previous studies have linked these protein localization patterns with CtrA proteolysis. When CpdR cannot be inactivated by phosphorylation, it is constitutively located at the stalked cell pole, and CtrA is degraded more rapidly than in wild-type cells.(Iniesta et al, 2006) A point mutation that prevents PopA from binding to cyclic-di-GMP blocks the localization of PopA and RcdA at the stalked pole and also prevents cell cycle-regulated CtrA proteolysis.(Duerig et al, 2009) These results suggest that localization of accessory proteins at the cell pole, along with ClpXP, is mechanistically important for CtrA proteolysis. We wanted to know if any of our structure-guided mutations affected the polar localization of RcdA.

We expressed each RcdA variant fused to GFP from the chromosomal *xylX* locus and examined mixed cultures using DIC and fluorescence microscopy. GFP fusions to RcdA, RcdA-patch, and RcdA-H138A were properly localized at the cell pole (Fig.6). Midcell localization of RcdA, which is not associated with CtrA proteolysis,(Iniesta et al, 2006) was not explicitly scored. In contrast, RcdAΔC-GFP failed to localize at the cell pole or the midcell of *Caulobacter* (Fig.6). In these mutants, as we expected, polar localization correlated with the ability of the RcdA variant to support CtrA proteolysis.

To determine if the C-terminal peptide of RcdA is sufficient for polar or midcell localization of an unrelated passenger protein, we fused all of the residues following helix α3 (aa 143-169) to the C-terminus of YFP. We observed diffuse signal from this fusion protein in Δ*rcdA* cells (data not shown), so the C-terminal peptide of RcdA is necessary, but not sufficient to direct RcdA to the pole or midcell.

I was surprised to find two RcdA variants that were compromised in polar localization, but which supported CtrA proteolysis. RcdA-CF-GFP completely failed to localize to the pole, while polar localization of RcdA-paddle-GFP was reduced (Fig.6). Wild-type RcdA-GFP was accumulated at the cell pole in 32.3% of swarmer cells, 18.9% of stalked cells, and 27.8% of predivisional cells, while RcdA-paddle-GFP was localized at the pole in only 7.4% of swarmer cells, 1.0 % of stalked cells, and 8.9% of predivisional cells. For each strain, over 500 cells of each type were scored. However, neither RcdA-CF nor RcdA-paddle blocked CtrA degradation during synchronized growth (Fig. 3b), extended the CtrA half-life in mixed cultures (Table 2), or led to G1 arrest in cells expressing CtrA-D51E (Fig.5). These are the first examples of mutations that mislocalize RcdA, but do not block CtrA degradation.

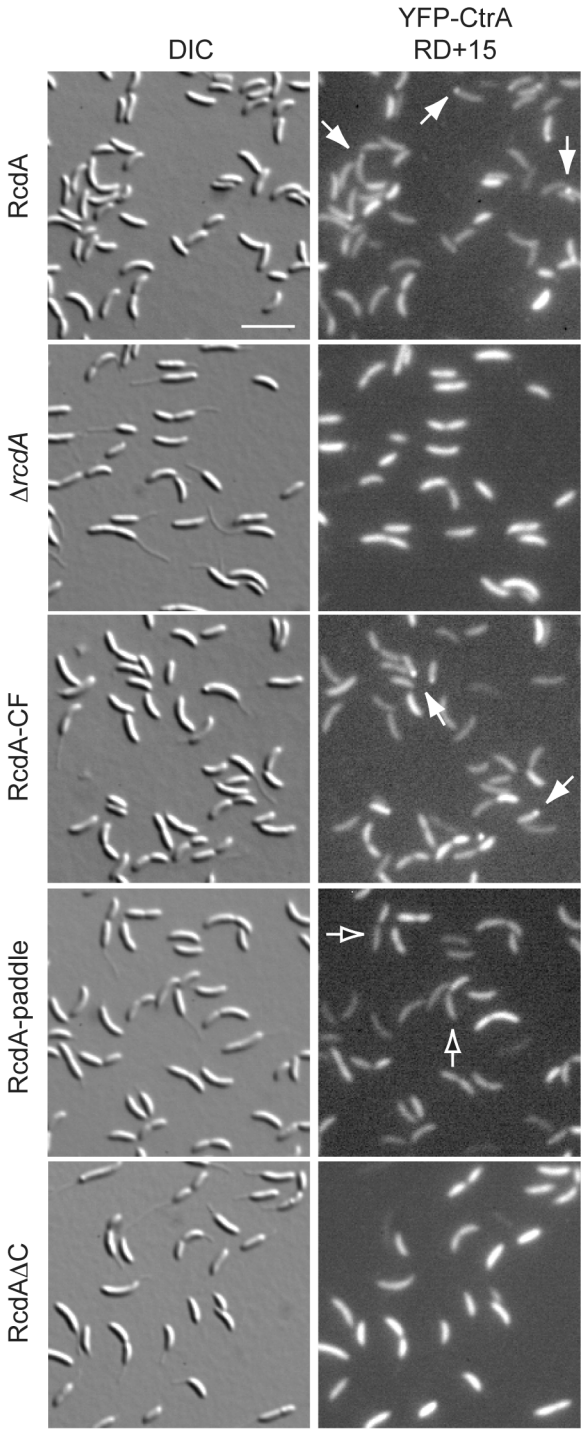


Fig.6. Some RcdA variants that support CtrA proteolysis are impaired in polar localization. Δ*rcdA* cells expressing the indicated RcdA-GFP fusion proteins from the chromosomal *xylX* locus were grown in PYE/0.03% xylose for 2.5 h, and DIC (left column) and fluorescence (right column) images were obtained. Scale bar, 5 μm.

Polar accumulation of CtrA is not required for cell cycle-dependent CtrA proteolysis

CpdR, PopA, and ClpXP can each localize at the cell pole in the absence of RcdA, but polar sequestration of CtrA is dependent upon the presence of RcdA and each of the other upstream factors.(McGrath et al, 2006)(Iniesta et al, 2006)(Duerig et al, 2009) To determine which *rcdA* mutants can support the polar localization of CtrA, we expressed YFP-CtrARD+15, a fusion protein which contains the signals sufficient for CtrA localization and proteolysis,(Ryan et al, 2002) from a low-copy plasmid in Δ*rcdA* cells that also express an *rcdA* allele from the chromosomal *xylX* locus. In cells expressing wild-type RcdA, YFP-CtrARD+15 is located at the pole in a subset of swarmer and predivisional cells, reflecting its transient accumulation during proteolysis (Fig. 7). In contrast, YFP-CtrARD+15 is completely delocalized in cells lacking RcdA (Fig. 7).

I examined YFP-CtrARD+15 in strains where the RcdA variant itself was frequently (RcdA-paddle) or always (RcdA-CF or RcdAΔC) mislocalized. In Δ*rcdA* cells expressing RcdA-paddle or RcdAΔC, I never observed YFP-CtrARD+15 localized at the cell pole (Fig. 7). However, some late predivisional cells expressing RcdA-paddle contained less YFP signal in the stalked compartment than in the swarmer compartment (Fig. 7, black arrows), reinforcing our conclusion that regulated CtrA proteolysis occurs normally in this strain. In cells expressing RcdA-CF, polar localization of YFP-CtrARD+15 was reduced by almost 50%, but not abolished (Fig. 7). Specifically, in cells expressing RcdA-CF, YFP-CtrARD+15 accumulated at the pole in 5.6% of swarmer cells and 2.4% of predivisional cells, as compared to 10.3% of swarmer cells and 4.2% of predivisional cells for the strain expressing wild-type RcdA. (No polar accumulation of YFP-CtrARD+15 was observed in stalked cells, and more than 400 cells of each type were scored for each strain.) Thus, localization of the RcdA variants themselves did not correlate with their ability to promote polar accumulation of CtrA.

Effects of rcdA mutations on PopA interaction and PopA localization

PopA binds to RcdA and is necessary for its polar localization, while PopA localization still occurs in the absence of RcdA.(Duerig et al, 2009) A point mutation in *popA* that blocks its localization to the stalked pole also blocks RcdA localization and CtrA proteolysis, but does not inhibit PopA-RcdA interaction.(Duerig et al, 2009) We therefore asked if our *rcdA* mutants interact with PopA in a bacterial two-hybrid assay. Unexpectedly, RcdA-H138A, RcdA-CF, RcdA-paddle and RcdAΔC all interacted with PopA as well as the wild-type RcdA protein in this system (A. Duerig and U. Jenal, personal communication). Therefore, the degradation and/or localization phenotypes caused by these mutations cannot be attributed to disruption of the RcdA-PopA interaction.

In contrast to a previous report,(Duerig et al, 2009) we found that PopA-GFP localization is perturbed in the Δ*rcdA* strain (Fig. 8 and Supplementary Fig.5). Wild-type predivisional cells typically have a focus of PopA-GFP at each pole; upon cell division, both the swarmer and stalked progeny have a single focus of PopA-GFP at the "old" cell pole. During S-phase, a second focus of PopA-GFP appears at the opposite pole, generating bipolar foci of PopA-GFP in most stalked and predivisional cells. PopA must bind to cyclic diguanylate at its allosteric site to localize at the stalked pole of the cell, where RcdA colocalizes and CtrA proteolysis occurs. Localization of PopA at the pole opposite the stalk during S-phase requires PodJ,(Duerig et al, 2009) which also helps to localize the histidine kinase PleC at the same pole.(Hinz et al, 2003; Viollier et al, 2002)

In our hands, Δ*rcdA* stalked cells most often had only one focus of PopA-GFP at the stalked pole, rather than bipolar foci (Fig. 8b and Supplementary Fig.5). In wild-type

predivisional cells, PopA was predominantly bipolar, but in the Δ*rcdA* strain, predivisional cells with a single focus at the stalked pole outnumbered those with bipolar foci (Fig. 8c). I observed an increased number of swarmer cells with diffuse PopA-GFP in the Δ*rcdA* strain, which may follow from the division of Δ*rcdA* predivisional cells with PopA-GFP localized only at the stalked pole. The strain expressing RcdAΔC, which itself is delocalized and is impaired in promoting CtrA degradation, also had an excess of swarmer cells with delocalized PopA-GFP and stalked cells with PopA-GFP localized only at the stalked pole (Fig. 8).

Two distinct models could account for these results. First, the absence or delocalization of RcdA could have a direct effect on PopA, hindering its ability to accumulate at the pole opposite the stalk. Second, PopA mislocalization could be an indirect result of the failure to degrade CtrA or unknown substrates that depend on RcdA for proteolysis. I examined PopA localization in additional mutants to address this question.

RcdA-CF and RcdA-paddle show no or reduced polar localization, respectively, but they both promote rapid, cell cycle-dependent proteolysis of CtrA. In strains expressing either of these proteins, PopA-GFP displayed the wild-type pattern of localization, with predominantly one focus in swarmer cells and bipolar foci in stalked and predivisional cells (Fig. 8 and Supplementary Fig.5). In contrast, a strain with wild-type RcdA expressing a nondegradable version of CtrA had an excess of stalked cells with a single focus of PopA-GFP at the stalked pole, suggesting that localization of PopA at the pole opposite the stalk is hindered. We infer that PopA mislocalization follows from the failure to degrade CtrA (and perhaps other, unknown targets), not from the mislocalization of RcdA itself.
rcdA

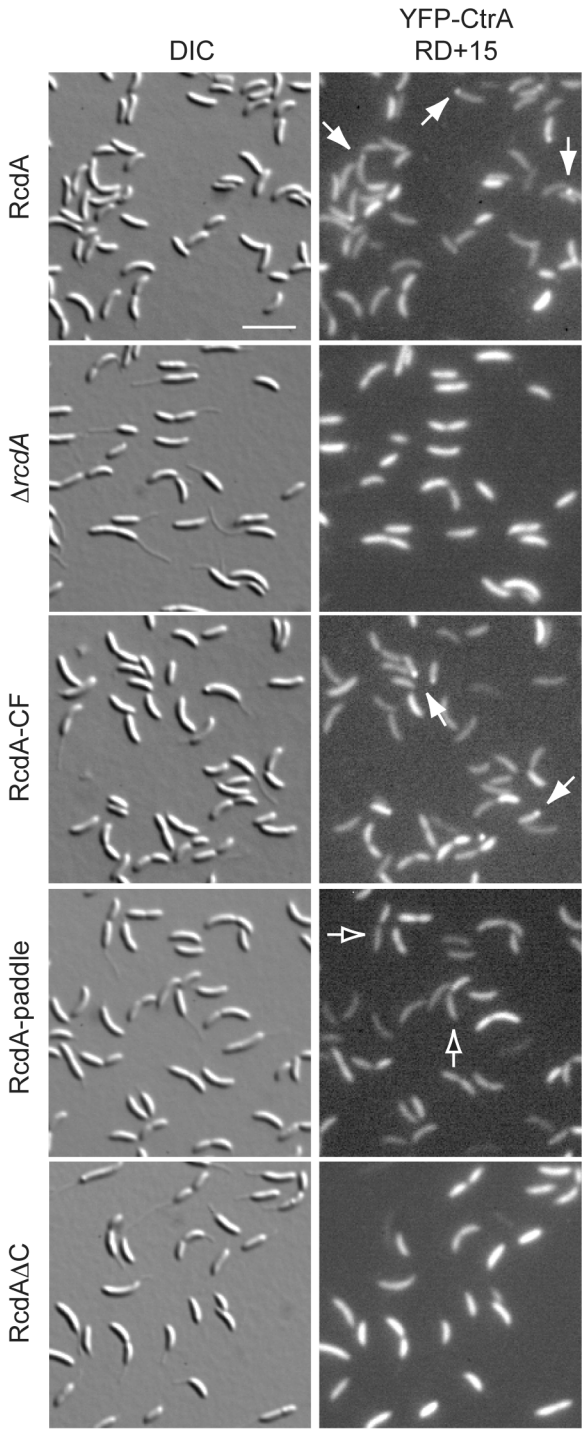


Fig. 7. CtrA localization in rcdA mutants. Δ*rcdA* cells expressing the indicated RcdA variant from the chromosomal *xylX* locus and expressing YFP-CtrARD+15 from the *xylX* promoter on pJT52 were grown in PYE/0.03% xylose for 4 h, and DIC (left column) and fluorescence (right column) images were obtained. Polar accumulation of YFP-CtrARD+15 (white arrows) and predivisional cells in which the stalked compartment is dimmer than the flagellated compartment (black arrows) are indicated. Scale bar, 5 μm.

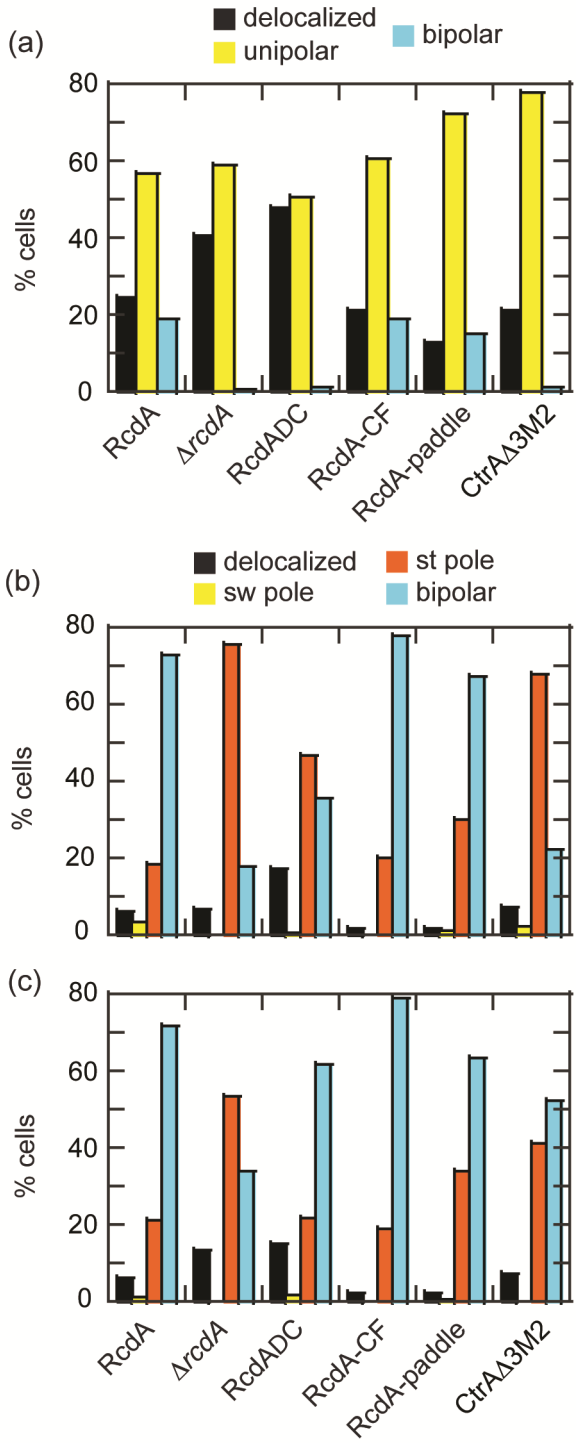


Fig. 8. PopA is mislocalized in strains that fail to degrade CtrA. Δ*rcdA* cells expressing the indicated RcdA variants from the chromosomal *xylX* locus and *popA-EGFP* from a low-copy plasmid were grown in PYE/0.03% xylose for 2.5 h, and DIC and fluorescence images were obtained. PopA localization patterns were scored in swarmer cells (a), stalked cells (b), and predivisional cells (c) of each strain.

rcdA mutations affect CtrA proteolysis downstream of CpdR

When the single-domain response regulator CpdR is unphosphorylated, it stimulates ClpXP localization at the cell pole and proteolysis of CtrA.(Biondi et al, 2006; Iniesta et al, 2006) In cells that contain only the nonphosphorylatable protein $CpdR_{D51A}$, ClpXP is located at the cell pole for a greater fraction of the cell cycle, and the CtrA degradation rate is increased 4-fold.(Iniesta et al, 2006) Mutations in *popA* that block CtrA proteolysis on their own also prevent inappropriate CtrA degradation in the presence of $CpdR_{D51A}$.(Duerig et al, 2009) To determine if mutations in *rcdA* that prevent or slow CtrA degradation also block the effects of $CpdR_{D51A}$, I created strains lacking the native alleles of *rcdA* and *cpdR* that express either *cpdR* or *cpdRD51A* and *rcdA* or *rcdAΔC* from the *xylX* promoter. After inducing expression of both genes, I analyzed CtrA levels by SDS-PAGE and Western blotting.

CtrA levels remained high when $CpdR_{D51A}$ was expressed in the absence of RcdA (Fig. 9, lane 4), or in the presence of RcdAΔC (Fig. 9, lane 12). Only when $CpdR_{D51A}$ was coexpressed with wild-type RcdA were CtrA levels severely reduced (Fig. 9, lane 8). When wild-type CpdR was expressed rather than $CpdR_{D51A}$, normal levels of CtrA were maintained in all *rcdA* backgrounds (Fig. 9, lanes 2, 6, and 10). These results suggest that RcdA acts at a step downstream of CpdR in the pathway controlling CtrA proteolysis.

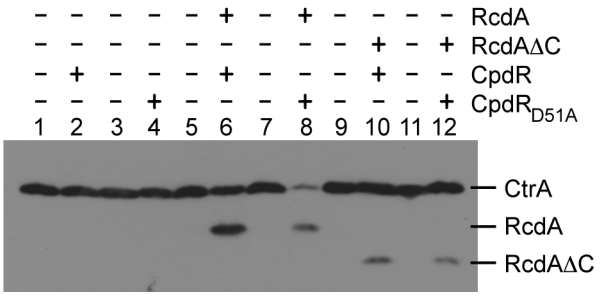


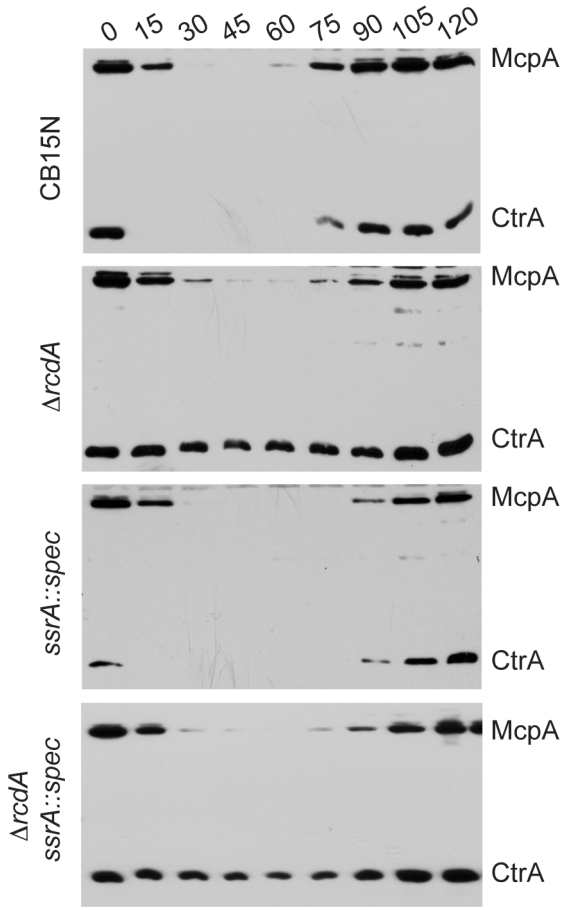
Fig. 9. RcdA acts downstream of CpdR in CtrA proteolysis. Δ*rcdA* Δ*cpdR* strains expressing no RcdA protein (lanes 1-4), wild-type RcdA (lanes 5-8), or RcdAΔC (lanes 9-12) from the *xylX* locus and expressing either CpdR or $CpdR_{D51A}$ from the xylose promoter on a low-copy plasmid were grown for 4.5 hours in PYE/0.03% dextrose (odd lanes) or PYE/0.03% xylose (even lanes). Samples representing equal numbers of cells were analyzed by SDS-PAGE and Western blotting with antisera against CtrA and RcdA.

RcdA does not stimulate CtrA proteolysis by blocking the degradation of SsrA-tagged substrates

Although few native substrates have been identified, *Caulobacter* ClpXP is expected to degrade a wide variety of proteins. RcdA could stimulate CtrA proteolysis indirectly by blocking access to alternative ClpXP substrates.(Chien et al, 2007) There are ~11 000 CtrA molecules and a maximum of ~800 ClpXP complexes in the cell at the time of CtrA proteolysis,(Judd et al, 2003; Osteras et al, 1999) so other substrates could compete for access to ClpXP.

SsrA-tagged proteins comprise a large and diverse group of ClpXP substrates *in vivo*.(Gottesman et al, 1998)(Farrell et al, 2005)(Lies & Maurizi, 2008) In *E. coli*, ~1 in 200 translation events yields an SsrA-tagged protein, producing ~12 000-15 000 of these substrates per generation.(Moore & Sauer, 2005)(Lies & Maurizi, 2008) In *Caulobacter* cells containing an altered *ssrA* RNA which prevented degradation of the tagged substrates, at least 73 different SsrA-tagged proteins were identified by mass spectrometry.(Hong et al, 2007) In addition, the presence of an SsrA-tagged protein and the adaptor SspB can inhibit the degradation of CtrA by ClpXP *in vitro*.(Chien et al, 2007) Thus the ability of ClpXP to degrade SsrA-tagged proteins could influence CtrA degradation *in vivo*.

We used epistasis experiments to test the hypothesis that RcdA stimulates CtrA proteolysis by blocking the degradation of SsrA-tagged proteins. During synchronous growth, CtrA is completely degraded at the swarmer-to-stalked cell transition in wild-type and *ssrA::spec* cells (Fig. 10).(Keiler & Shapiro, 2003) In contrast, CtrA is not completely degraded during swarmer cell differentiation in the single mutant Δ*rcdA* or the double mutant Δ*rcdA ssrA::spec* (Fig. 10). McpA is properly degraded in all of these strains, indicating that they are passing synchronously through the cell cycle and that ClpXP activity is maintained (Fig. 10). Because the double mutant Δ*rcdA ssrA::spec* fails to degrade CtrA rapidly, we conclude that efficient CtrA proteolysis requires RcdA even when competing SsrA-tagged substrates are eliminated.



F**ig. 10. RcdA is required for efficient CtrA proteolysis in the absence of SsrA-tagged substrates.** Swarmer cells of the indicated strains were isolated and allowed to divide synchronously in PYE. Samples were taken at the indicated times (minutes), and lysates from equal numbers of cells were analyzed by SDS-PAGE and Western blotting with antisera recognizing CtrA and McpA.

Discussion

Three proteins, CpdR, PopA, and RcdA, are each required for rapid, cell cycle-dependent proteolysis of CtrA in *Caulobacter*, but to date the mechanisms by which they stimulate CtrA degradation by ClpXP are unknown. In this report we have focused on RcdA, a protein conserved in a subset of the α-proteobacteria, which is required for polar accumulation and rapid degradation of CtrA. We have used structure-guided mutations that alter conserved surface residues of RcdA, coupled with *in vivo* assays of protein localization and degradation, to evaluate proposed models of RcdA function.

Superficially, RcdA resembles the adaptor protein SspB, as both proteins form stable head-to-head dimers, and each protein contains a disordered peptide at the C-terminus that is required to enhance substrate degradation. The interaction between ClpX and SspB is mediated by the disordered C-terminal peptide of SspB,(Dougan et al, 2003; Wah et al, 2003) while the SsrA tag of a substrate protein binds in a conserved cleft within the structured region of SspB.(Levchenko et al, 2003) SspB-mediated tethering of SsrA-tagged proteins increases the local substrate concentration near ClpXP to allow more efficient proteolysis.(Levchenko et al, 2000) However, functional studies show have suggested that SspB and RcdA do not share a common molecular mechanism, as RcdA alone does not enhance CtrA degradation by ClpXP *in vitro* or bind directly to ClpX.(Chien et al, 2007)

We identified three regions of the RcdA protein that are important for its normal localization and/or function in *Caulobacter*. Conserved, charged amino acid residues that protrude from the RcdA paddles, and conserved glutamic acid residues on the convex face, are each important for polar localization of RcdA and CtrA. However, mutations that neutralize the charge in either of these regions do not affect the overall rate or cell cycle timing of CtrA proteolysis. In contrast, the last 19 amino acids of RcdA, which are disordered in the crystal structure, are critical for rapid, timed CtrA degradation, as well as for polar protein localization. The truncated RcdAΔC protein slowed overall CtrA proteolysis by a factor of ~3, prevented CtrA from being cleared during the G1-S transition, and blocked the enhanced CtrA degradation caused by CpdRD51A. We predict that the paddle regions, convex face, and C-terminus of RcdA interact with protein(s) that secure RcdA at the cell pole, but only the C-terminal peptide makes contacts that are essential for CtrA proteolysis. So far, the only protein known to interact directly with RcdA is PopA, (Duerig et al, 2009)yet none of our mutations inhibited binding to PopA. These findings suggest that RcdA contacts one or more unknown proteins to promote CtrA degradation.

What does polar localization of CtrA at the level of light microscopy mean in molecular terms? During degradation, the polar focus of CtrA could represent CtrA molecules bound directly to ClpXP, in the process of being unfolded, or CtrA molecules bound to a different polar protein, awaiting contact with the limited number of ClpXP complexes. In this study, we found that the *rcdA-paddle* mutations abolish polar accumulation of CtrA, but do not significantly affect the rate of CtrA proteolysis. Further, a previous study showed that the non-degradable protein YFP-CtrARD+15-DD, in which the final two amino acids were changed from AA to DD, still localized at the cell pole periodically, at times when CtrA degradation would normally have occurred.(Ryan et al, 2004) Because ClpX is thought to directly recognize the C-terminal amino acids of CtrA, (Chien et al, 2007; Flynn et al, 2003) these results indicate 1) that CtrA accumulation at the cell pole is driven by a process other than direct binding to ClpXP, and 2) that CtrA proteolysis can proceed at a near-wild-type rate without a separate polar recruitment step.

RcdA is necessary for CtrA degradation *in vivo*, but has not *in vitro*,(Chien et al, 2007) and its molecular role is unclear. Could RcdA be the polar localization factor for CtrA? So far, no direct interaction has been detected between RcdA and CtrA.(Duerig et al, 2009) Furthermore, the RcdA-CF protein is itself delocalized, but cells expressing this protein sometimes contain localized CtrA. This result suggests that RcdA may contribute to polar accumulation of CtrA, but it is unlikely to recruit CtrA directly.

Alternatively, it has been suggested that RcdA promotes CtrA degradation by blocking access of other substrates to the protease ClpXP.(Chien et al, 2007) Since CtrA molecules outnumber ClpXP complexes in the cell by ~10:1,(Judd et al, 2003; Osteras et al, 1999) and ClpXP is expected to handle numerous other substrates,(Flynn et al, 2003) CtrA proteolysis may be slower at certain times because ClpXP is occupied with other proteins. An example of an adaptor protein that modulates substrate choice by a AAA+ protease is ClpS, which prevents ClpAP from binding to and degrading SsrA-tagged substrates.(Dougan et al, 2002) Using epistasis experiments, we tested this model with respect to SsrA-tagged proteins, the largest known group of ClpXP substrates.(Hong et al, 2007) When we eliminated all SsrA-tagged substrates from *Caulobacter*, RcdA was still required for rapid cell cycle-dependent degradation of CtrA. Therefore, RcdA does not accelerate CtrA proteolysis *in vivo* by eliminating competition from SsrA-tagged substrates.

In light of these findings, we propose that RcdA either inhibits the activity of a protein that prevents CtrA proteolysis or works in conjunction with a *bona fide* adaptor protein for CtrA. In either case, the disordered C-terminal peptide of RcdA is essential for optimal activity, and RcdA must be able to function without being stably located at the cell pole. This is surprising, because all previous mutations that have disrupted polar protein localization of the substrate, protease, or accessory factors have also inhibited CtrA degradation.

Because ClpXP can degrade CtrA *in vitro* at a rate that would be sufficient to clear CtrA from the cell during the G1-S transition, it was suggested that no positively acting adaptor protein is necessary for CtrA proteolysis *in vivo*.(Chien et al, 2007) However, some results are difficult to explain without postulating the existence of a positive regulatory factor. Specifically, the N-terminal receiver domain of CtrA is necessary for rapid cell cycle-regulated proteolysis *in vivo*,(Ryan et al, 2002) while *in vitro*, the C-terminal DNA-binding domain alone can be degraded by ClpXP.(Chien et al, 2007) Since removal of the receiver domain stabilizes the DNA-binding domain *in vivo*, rather than causing it to be constitutively degraded, we infer that the CtrA receiver domain stimulates cell cycle-dependent proteolysis. So far, however, no proteins have been identified that recognize the CtrA receiver domain and mediate this effect.

We found that cells lacking RcdA or expressing RcdAΔC have reduced PopA localization at the pole opposite the stalk, while RcdA variants that are mislocalized but still permit normal CtrA proteolysis display wild-type PopA localization. A strain expressing the nondegradable protein CtrAΔ3M2 also has reduced PopA localization at the pole opposite the stalk, suggesting the existence of a feedback loop wherein PopA stimulates CtrA proteolysis at the G1-S transition, and this in turn permits PopA to localize at the swarmer pole. PopA accumulation at the swarmer pole requires the localization factor PodJ,(Duerig et al, 2009) a membrane protein that also localizes the histidine kinase PleC at the same site.(Hinz et al, 2003; Viollier et al, 2002) *podJ* transcription is repressed by CtrA,(Crymes et al, 1999; Laub et al, 2000) so stabilizing CtrA may inhibit the accumulation of PodJ protein at the G1-S transition.

Materials and Methods

Bacterial strains, media and plasmids

Strains and plasmids used are listed in Supplementary Table 1. All experiments were performed using derivatives of *Caulobacter crescentus* strain CB15N grown to mid-logarithmic phase. CB15N strains genetically manipulated as described and grown in peptone-yeast extract (PYE) or M2G medium(Ely, 1991) at 28ºC, supplemented with antibiotics as required. Unless otherwise indicated, 0.03% xylose or 0.1% xylose was added to PYE and M2G media, respectively, to induce gene expression from the *xylX* promoter. *E. coli* strains were grown in Luria Broth (LB) at 37°C, supplemented with antibiotics as required.

Mutations were introduced using QuikChange (Stratagene), with pPM53 or pES53 as template (oligonucleotide sequences available on request). GFP fusions to RcdA variants were generated by introducing mutations into pPM53. *rcdA-patch::GFP* (pJT41) carries the mutations L61S and A64S. pJT41 was further mutated, introducing D45A, R49A, K53A and R57A to create *rcdA-paddle::GFP* (pJT50). *rcdA-CF::GFP* (pSS13) contains the mutations E38A, E39A, E89A, and E92A. To generate RcdA expression vectors lacking GFP fusions, stop codons were introduced at the end of the *rcdA* coding sequences in pPM53 and the plasmids derived above. To generate *rcdAΔC*, a BamHI site and a stop codon were introduced after codon 150 of *rcdA*. The BamHI site was used to fuse *rcdA*Δ*C* to *GFP*. pJT52 was generated by moving P_{xyl}-*YFP::ctrARD+15* from pEJ146 into pMR20. The double mutant Δ*rcdA ssrA::spec* was generated by transducing *ssrA::spec*(Keiler & Shapiro, 2003) into Δ*rcdA* cells.

For CpdR epistasis experiments, the D*rcdA* allele was transduced from KR899 into LS2524 (D*cpdR* pP_{xylX}-CpdR) and LS2525 (D*cpdR* pP_{xylX}-$CpdR_{D51A}$).(Iniesta et al, 2006) Subsequently, the wild-type *rcdA* gene was integrated at the *xylX* locus by mating in pSS31, or *xylX::rcdAΔC* was introduced by phage transduction.

For PopA-GFP localization studies, pAD5 (*popA-GFP* under the *popA* promoter)(Duerig et al, 2009) was introduced by mating into D*rcdA*, D*rcdA* strains with mutant *rcdA* alleles integrated at *xylX*, and into KR566, which expresses a stable CtrA allele with a C-terminal M2 tag.

Protein expression and purification

For crystallization, *E. coli* BL21 harboring pES53 were grown in LB to an OD_{600} of 0.6-0.8 and induced to express His6-RcdA with 1 mM isopropyl-thiogalactoside (IPTG) for 4-5 hours. The cell pellet was harvested by centrifugation and frozen at -80ºC. Cell pellets were resuspended in binding buffer (20 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole) and lysed by sonication. Lysates were cleared by centrifugation at 40 000*g* for 45 min, and the supernatant was incubated with Talon affinity resin (Clontech). After 30 min, Talon resin was pelleted, resuspended in fresh binding buffer, and transferred to a column. The resin was washed with 60 ml of wash buffer (20 mM Tris pH 8.0, 500 mM NaCl, 25 mM imidazole), followed by elution of His6-RcdA with 20mM Tris pH 7.0, 350mM imidazole. The His6 tag was removed by incubating the eluted protein with 20 units of thrombin (Sigma) overnight at 4°C. Cleaved RcdA was isolated by gel filtration on a Superdex 200 column in 10 mM HEPES pH 7.0, 100 mM NaCl, then concentrated to 6 mg/ml. Selenomethionine derivatives of RcdA were obtained using the same protocol, except that cell growth and protein expression occurred in M9 minimal medium supplemented with 50 mg/l selenomethionine as previously described.(Van Duyne et al, 1993)

For calibrated gel filtration, RcdA and RcdAΔC were purified as follows. BL21 Tuner cells were grown to an OD_{600} of 0.4 and induced to express His6-RcdA or His6-RcdAΔC with 0.5 mM IPTG for 3 hrs. Cells harvested by centrifugation were frozen at -80ºC. Cell pellets were resuspended in buffer A (50 mM Tris-HCl pH8.2, 100 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 20 mM imidazole) and lysed for 1 hr on ice by the addition of lysozyme (1 mg/ml). Cells were disrupted by sonication and the lysate cleared by centrifugation at 12,000*g* for 20 min at 4ºC. Ni-NTA agarose was added to the lysate, and proteins allowed to bind at 4ºC. The slurry was transferred to a column and washed three times with 30 ml buffer A. Ni-NTA agarose was resuspended in 5 ml buffer A and incubated overnight with 15 U thrombin. Cleaved RcdA or RcdAΔC was eluted from the resin with two additional washes of 10 ml buffer A and added directly to a mono-Q column equilibrated in buffer A. RcdA was eluted using a 40 ml gradient of 0-100% buffer B (buffer A + 1 M KCl).

Crystallization and structure determination

Native or selenomethionine-containing crystals were grown by hanging drop vapor diffusion at room temperature in 1:1 mixtures of protein and 1.5-1.75 M sodium formate, 0.1 M HEPES pH 7.5 or 0.1 M Tris pH 8.5. Tetragonal crystals grew overnight in clusters from a single nucleation point and were separated prior to screening and data collection. Native data were collected at the Advanced Light Source Beamline 5.0.2, (Lawrence Berkeley National Lab) and a 3-wavelength dataset from a single selenomethionine derivative crystal was collected at the Advanced Light Source Beamline 8.3.1. Data was processed with HKL2000,(Otwinowski & Minor, 1997) and selenium sites were located using multiple anomalous dispersion using SHELX.(Sheldrick, 2008) Eight selenium sites were found, and interpretable maps were generated upon density modification in SHELX. Poly-alanine helices were placed into the maps for all six helices in the asymmetric unit. These were not refined but instead were used as a molecular replacement model for the higher-quality native dataset. Molecular replacement solutions were easily found using PHASER,(McCoy, 2007) and the coordinates were built using COOT(Emsley & Cowtan, 2004) and refined in REFMAC.(Murshudov et al, 1997) Initial rounds of refinement were constrained by the selenomethionine experimental phases. Coordinates and structure factors have been submitted (**PDB ID: 3CTW**). Images of RcdA coordinates were generated using PYMOL (Delano Scientific LLC).

Size exclusion chromatography

Gel filtration was performed on a Superdex-200 column (GE Healthcare) that had been calibrated using albumin, ovalbumin, chymotrypsin and blue dextran (GE Healthcare) as standards. The column was run in buffer A + 18% buffer B, and elution peaks measured by absorbance at 280 nm. Molecular masses were calculated according to K_{av} vs. $\ln(M_w)$.

CtrA half-life measurements

Pulse-chase and immunoprecipitation to determine CtrA half-life was performed as described,(Reisinger et al, 2007) except that cultures were grown in M2G supplemented with 0.1% xylose, and cell lysates were precleared using 30µl Pansorbin (Calbiochem).

Microscopy

Fluorescence and DIC microscopy were performed as described.(Reisinger et al, 2007) Cells designated as swarmer cells were half the size of an average predivisional cell of the same strain and had no visible stalk. Stalked cells had a visible stalk, but no midcell pinch marking the division plane. Predivisional cells had both a visible stalk and a midcell pinch. For PopA-GFP localization, >100 cells of each class (swarmer, stalked or predivisional cell) were scored for each strain examined.

Synchronization and immunoblots

Isolation of G1-phase swarmer cells and synchronous growth were performed as described,(Quon et al, 1996) except that cells were grown in PYE, with 0.03% xylose added where necessary for induction of gene expression. Immunoblots were probed with CtrA,(Quon et al, 1996) McpA,(Alley et al, 1993) RcdA,(McGrath et al, 2006) and GFP (Clontech) antibodies at dilutions of 1:10 000, 1:30 000, 1:10 000, and 1:5 000, respectively.

Flow Cytometry

Nucleic acid staining and flow cytometry measurements of DNA content were performed as described.(Sciochetti et al, 2005)

Bacterial two-hybrid assays

Interaction of wild-type RcdA and RcdA variants with PopA was assayed as described (Duerig et al, 2009).

Acknowledgements

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Chapter 3: CtrA proteolysis is accelerated by a multiprotein adapter complex which requires direct binding of the bacterial second messenger cyclic diguanylate.

Absract

In addition to providing a cellular maintenance function by destroying non-functional proteins, proteolysis provides a mechanism for irreversible and complete inactivation of regulatory proteins that specify different outcomes. Proteolysis must be tightly controlled to prevent the destruction of regulatory proteins at inappropriate times or in the wrong cell type. The *Caulobacter* cell cycle transcriptional regulator, CtrA, is degraded by ClpXP prior to initiation of chromosome replication. While the proteins PopA, RcdA and CpdR are known to stimulate CtrA proteolysis *in vivo*, a mechanistic understanding of their functions has proven difficult to attain. In this study, the role of the proteolytic accessory proteins was examined *in vitro*. Further, CtrA mutants were identified that were defective substrates for stimulated proteolysis *in vitro* and in living cells. Proteins that stimulate CtrA proteolysis interacted directly with CtrA, but not with a degradation-defective mutant. These proteins decrease the K_m of the degradation reaction. The bacterial second messenger cyclic diguanylate is required for the direct interaction of CtrA with PopA, and for accelerated proteolysis *in vitro*. The data reported here are consistent with a model in which small molecule binding triggers a multiprotein adaptor complex to increase the affinity of ClpXP for CtrA, and thus stimulate proteolysis at the correct point in the *Caulobacter* cell cycle.

Introduction

Regulated proteolysis of key substrates is a central strategy employed by all cells to adapt to environmental conditions and to respond to developmental cues. In addition to degrading specific target proteins, the AAA+ Clp proteases in bacteria and the 26S proteasome in eukaryotes are also important for general cell maintenance by preventing the accumulation of truncated and misfolded proteins (Gottesman & maurizi, 1992). In both the proteasome and the Clp proteases, a barrel-shaped complex with active sites on the inside is directly responsible for protein degradation. To enter the proteolytic chamber, substrates are recognized, unfolded, and translocated by an ATPase complex that caps the barrel. In Clp proteases, the proteolytic chamber is composed of two stacked heptamers of ClpP, while the ATPase complex is a hexamer of ClpX, ClpA, or ClpC (Baker & Sauer, 2012; Yu & Houry, 2007).

Tight control must be imposed on proteases to prevent the degradation of non-substrate proteins. In fact, the unique class of antibiotics, acyldepsipeptides (ADEPs), function by rendering ClpP promiscuous in substrate selection. ADEPs facilitate oligomerization of ClpP monomers, block associated with cognate ATPases, and widen the entrance to the proteolytic cavity. In this way ADEPs bypass the molecular mechanisms that provide selectivity to the protease and are lethal to many gram positive bacteria (Lee et al, 2010; Li et al, 2010). Many *bona fide* proteolytic substrates are only degraded at specific times or under specific circumstances, as is the case for the *B. subtilis* transcriptional regulator CtsR, which is stable except in response to heat shock when it is degraded by ClpCP (Kruger et al, 2001). Conversely, another transcriptional regulator in B. subtilis, Spx, is stable in conditions that induce disulfide stress, but is degraded by ClpXP under normal laboratory growth conditions (Nakano et al, 2002). These situations present the protease with the greater challenge of not simply recognizing substrates, but also ensuring that transient substrates escape proteolysis except when appropriate.

A common mechanism for exerting control over substrate selection by Clp proteases is exemplified by the *E. coli* protein SspB. This protein acts as adaptor that promotes the

degradation of SsrA-tagged substrates, which arise as a result of failed translation events (Dougan et al, 2003). Traditional adaptors, such as SspB, bind to both the substrate and protease and deliver the substrate to the protease for degradation (Battesti & Gottesman, 2013). SspB recruits substrates to the protease by making physical contacts in with the N-terminal portion of the SsrA peptide, while also interacting with the N-terminal zinc-binding domain of ClpX (Bolon et al, 2004). Additional forms of Clp protease regulation have been observed. For example, in *B. subtilis*, degradation of CtsR by ClpCP is controlled by McsB. McsB is an adaptor, but its activity is dependent both on its own autophosphorylation and on the phosphorylation of ClpP (Elsholz et al, 2011). The subject of this dissertation is an example of proteolytic regulation in the alpha-proteobacterium *Caulobacter crescentus,* in which three proteins and the small molecule cyclic diguanylate (cdG) are required for timed degradation of the cell cycle transcriptional regulator CtrA by ClpXP *in vivo*. Because ClpXP alone can degrade CtrA *in vitro*, the mechanism by which the accessory factors stimulate CtrA proteolysis has remained elusive (see below).

Caulobacter crescentus is an oligotrophic bacterium that divides asymmetrically to produce a motile swarmer (SW) cell and a sessile stalked (ST) cell. The SW cell differentiates into a ST cell prior to replicating its DNA. The ST cell elongates and develops into the predivisional (PD) cell. The predivisional cell retains the stalk at one pole and adopts a SW cell morphology at the opposite pole by assembling a polar flagellum and pili. Upon division of the cytoplasmic membrane, but before cell separation, these morphologically distinct poles give rise to physically separated ST and SW compartments (Collier & Shapiro, 2007; Judd et al, 2003).

When phosphorylated, CtrA regulates the *Caulobacter* life cycle and cell division by directly activating or repressing the transcription of ~100 genes (Laub et al, 2002). In addition, CtrA binds to sites within the chromosomal orgin of replication and prevents the initiation of DNA synthesis in the swarmer cell, as well as in the swarmer compartment of the predivisional cell (Quon et al, 1998). CtrA activity must be transiently eliminated in order for chromosome replication to occur. To this end, CtrA is subject to inactivation by both dephosphorylation and proteolysis just prior to the onset of chromosome replication. These systems of control are redundant. Cells that cannot degrade CtrA are able to progress normally through the cell cycle, as are cells that express a constitutively active phosphomimetic variant of CtrA, CtrAD51E. However, inactivation by one mechanism or another is critical for cell cycle progression. Cells expressing CtrAD51E that are also unable to regulate CtrA through proteolysis become filamentous and are unable to initiate chromosome replication (Domian et al, 1997).

Three accessory proteins, RcdA, CpdR and PopA, are needed to clear CtrA from the cell prior to chromosome replication (Biondi et al, 2006; Duerig et al, 2009; Iniesta et al, 2006; McGrath et al, 2006). The first to be discovered, RcdA, was identified bioinformatically based on its conservation in alpha-protebacterial genomes and the fact that CtrA directly regulates its transcription. A strain lacking RcdA fails to degrade CtrA during SW-ST differentiation (McGrath et al, 2006). RcdA's function could not be predicted from its amino acid sequence. Chapters 2 of this dissertation describe the structural characterization of RcdA and site-directed mutagenesis studies to identify surface features of RcdA important for CtrA degradation. RcdA has no predicted function, but each RcdA monomer forms a three-helix bundle connected by a disordered loop, and the protein exists as a dimer both in solution and in the crystal structure (Taylor et al, 2009). Structure-guided mutagenesis indicates that the disordered C-terminus of RcdA is dispensable for protein dimerization, but is required for efficient CtrA proteolysis *in vivo*.

The second accessory factor to be discovered was CpdR, a single-domain response regulator that is phosphorylated by the same pathway as CtrA (Biondi et al, 2006; Iniesta et al, 2006). The hybrid histidine kinase CckA autophosphorylates during the swarmer and predivisional phases of the cell cycle (Jacobs et al, 2003). The phosphoryl group is passed from a histidine residue in the dimerization and phosphotransfer domain of CckA to a conserved aspartate residue in its C-terminal receiver domain. From here, the phosphoryl group is passed to the histidine phosphotransferase ChpT (Biondi et al, 2006). Finally, ChpT can pass the phosphoryl group to either CtrA or CpdR. While CtrA is active as a transcriptional regulator when it is phosphorylated, CpdR is active in driving CtrA degradation when it is not phosphorylated (Iniesta et al, 2006). This signaling logic dictates that when CckA is active, CtrA will be both stable and active, and when CckA is inactive or functioning as a phosphatase (Chen et al, 2011), CtrA will be both inactivated and degraded. While this work was being performed, it was discovered that unphosphorylated CpdR acts as a canonical adaptor for the ClpXP-mediated proteolysis of PdeA, a cyclic diguanylate phosphodiesterase (Abel et al, 2011; Rood et al, 2012). Degradation of PdeA is one event that contributes to a switch that triggers differentiation of a SW cell into a ST cell, and PdeA proteolysis likely occurs just before or coincident with CtrA proteolysis.

The final accessory protein to be discovered was PopA, a protein comprised of two receiver domains and a C-terminal diguanylate cyclase (DGC) domain (Duerig et al, 2009). The DGC domain of PopA is inactive, but it binds cdG at an allosteric I-site, and cdG binding is required for PopA to stimulate CtrA proteolysis *in vivo*. In contrast, phosphorylation of the PopA receiver domains is not required for CtrA degradation, and it is unknown if PopA is phosphorylated *in vivo*.

The second messenger molecule cyclic diguanylate (cdG) is synthesized by diguanylate cyclase enzymes and broken down by phosphodiesterases. In many bacteria, low cellular cdG levels are associated with a motile lifestyle, while high cdG levels are associated with a sessile or biofilm-attached lifestyle (Jenal & Malone, 2006). In *Caulobacter*, levels of cdG rise from less than 100 nM to approximately 275 nM during differentiation of the motile swarmer cell into a sessile stalked cell (Abel et al, 2013). Further, it has been shown using a cdG biosensor that the concentration of cdG in the SW compartment of the predivisional cell is ~5-fold lower than in the stalked compartment (Christen et al, 2010). When the increase in cdG is abolished by overexpressing a constitutively active phosphodiesterase from *Pseudomonas aeruginosa*, CtrA proteolysis does not occur efficiently, and morphological aspects of the SW-ST transition are delayed or blocked (Duerig et al, 2009). Thus, cdG that is produced and consumed in a regulated manner by specific enzymes orchestrates the G1-S cell cycle transition and the morphological differentiation of a SW cell into a ST cell.

In addition to being the chief organism for studies of bacterial cell cycle control, *Caulobacter* is also prominent in studies of subcellular protein localization. Contrary to expectations, the precise localization of signal transduction pathways and other processes can have profound impacts on the life cycles of a number of bacteria (Nevo-Dinur et al, 2012). Using CtrA or a truncated form of CtrA fused at its amino terminus to the yellow fluorescent protein (YFP), it was discovered that CtrA dynamically localizes to the stalked or incipient stalked pole of the cell just before and during its proteolysis (Ryan et al, 2004; Ryan et al, 2002). Coincident with the discovery of RcdA, it was found that both ClpX and ClpP transiently localize at the same pole during CtrA degradation. Subsequent studies using *Caulobacter* mutants lacking the various accessory proteins or depleted of ClpX revealed a bifurcating hierarchy for localization

of the protease machinery at the cell pole (Biondi et al, 2006; Duerig et al, 2009; Iniesta & Shapiro, 2008; McGrath et al, 2006): PopA accumulates at the stalked pole only when bound to cdG, and PopA localization is one factor needed to recruit RcdA. CpdR localizes at the stalked pole only when it is unphosphorylated, and it recruits ClpX. ClpX and PopA are both needed to localize RcdA, and all of the upstream factors are required for polar accumulation of CtrA.

Due to their striking localization patterns, it has commonly been assumed that the accessory proteins function in CtrA proteolysis merely by ensuring that the protease and substrate are concentrated in the same region of the cell (Battesti & Gottesman, 2013; Jenal, 2009). However, some mutations in RcdA that prevent CtrA accumulation at the pole do not affect the rate of CtrA proteolysis (Taylor et al. 2009). ClpX, CpdR and RcdA can be co-immunoprecipitated along with CtrA from *Caulobacter* cell extracts (Iniesta & Shapiro, 2008; McGrath et al, 2006). However, these experiments do not identify proteins that interact directly with the substrate CtrA, and the immunoprecipitated complexes may contain unknown proteins needed to stimulate CtrA degradation.

Surprisingly, ClpXP is capable of degrading CtrA *in vitro* when no accessory factors are present. The rate of this reaction is theoretically rapid enough to permit clearing of CtrA during SW-ST differentiation, and the addition of RcdA did not affect the *in vitro* degradation rate (Chien et al, 2007). A recent study has shown that the rate of CtrA proteolysis is reduced DNA fragments which contain CtrA binding sites. The inhibition is more dramatic in the presence of SciP, a protein that binds to and modulates the activity of CtrA (Gora et al, 2013) and may also independently regulate transcription (Tan et al, 2010). The effect of accessory proteins that stimulate CtrA proteolysis was not assessed in that study.

In the current work we show that together, CpdR, RcdA, PopA and the small molecule cdG are able to accelerate CtrA proteolysis *in vitro*. This acceleration is observed even in the presence of the inhibitory factors, SciP and DNA. We employed a bioinformatic approach to identify amino acid residues in the CtrA receiver domain that are needed for rapid CtrA proteolysis *in vivo*. We found that these residues are also important for interactions between CtrA and the proteolytic accessory proteins in cell lysates and *in vitro*. In particular, there is a correlation between the ability of a CtrA mutant to undergo accelerated proteolysis and the competence of that mutant to form physical interactions with PopA and RcdA. Finally, we propose a mechanism by which accessory factors act together to stimulate CtrA proteolysis by an adaptor mechanism.

Results

Accessory factors accelerate CtrA proteolysis by ClpXP

The proteins RcdA, PopA and CpdR and the small molecule cdG are each required for rapid CtrA proteolysis in living cells (Biondi et al, 2006; Duerig et al, 2009; Iniesta et al, 2006; McGrath et al, 2006). However, CtrA is readily degraded by ClpXP *in vitro* without other proteins (Chien et al, 2007). The addition of RcdA alone to the ClpXP-CtrA reaction did not increase the rate of CtrA proteolysis (Chien et al, 2007), but these experiments were conducted before the roles of CpdR and PopA in CtrA degradation were recognized. We therefore reconstituted the entire set of proteins and small molecules implicated in CtrA degradation *in vivo* to determine if they change the rate of CtrA proteolysis *in vitro*.

In the absence of any accessory factors, CtrA was degraded by ClpXP *in vitro*. However, addition of PopA, RcdA, CpdR and cdG resulted in an acceleration of CtrA proteolysis (Fig. 1A). When CtrA abundance was plotted over time, the data were well fit by a pseudo-first order

reaction equation. The half-life of CtrA in ClpXP-only reactions was 8.61 +/- 1.8 min, while it was reduced to 3.8 +/- 0.4 min in reactions containing all of the accessory factors.

In vivo studies have shown that the receiver domain and C-terminal 15 amino acids of CtrA (CtrA RD+15) contain the signals necessary for cell cycle-regulated proteolysis (Ryan et al, 2002). In the *in vitro* proteolysis assay, CtrA RD+15 is degraded more slowly than full-length CtrA by ClpXP alone (Fig. 2A), and the accessory factors stimulate its proteolysis to a greater extent, reducing the half-life of CtrA RD+15 from 45 min to 8.6 min (Fig. 2B).

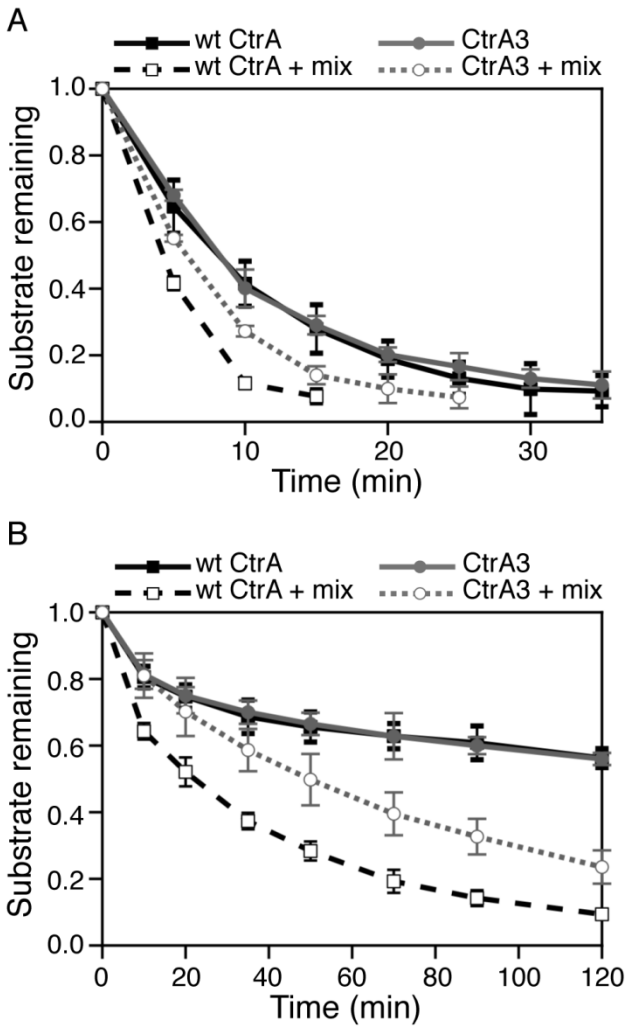

Fig. 1. Regulatory proteins and c-di-GMP stimulate CtrA proteolysis in vitro. (A) *In vitro* degradation of CtrA or CtrA3 visualized by SDS-PAGE. Initial substrate concentration was 1 μm CtrA or CtrA3. Reactions included 0.3 μM $ClpX_6$, 0.6 μM $ClpP_{14}$, an ATP regeneration system (0.9 μg/ml creatine kinase, 4 mM ATP, 16 mM creatine phosphate) and, where indicated, an accessory mix composed of 20 μM cdG and 1 μM each of CpdR, RcdA, and PopA. Quantification of substrate band intensity was plotted over time as the fraction of substrate remaining. (B) Accessory proteins and cdG stimulate CtrA proteolysis in the presence of SciP and DNA with CtrA binding sites**.** Initial substrate concentration was 2.0 μm CtrA or CtrA3. Reactions included 0.25 μM $ClpX_6$, 0.5 μM $ClpP_{14}$, 5 μM P*pilA* DNA, 5 μM SciP, an ATP regeneration system and, where indicated, an accessory mix composed of 20 μM cdG and 1 μM each of CpdR, RcdA, and PopA. Quantification of substrate band intensity over time was plotted as the fraction of substrate remaining.

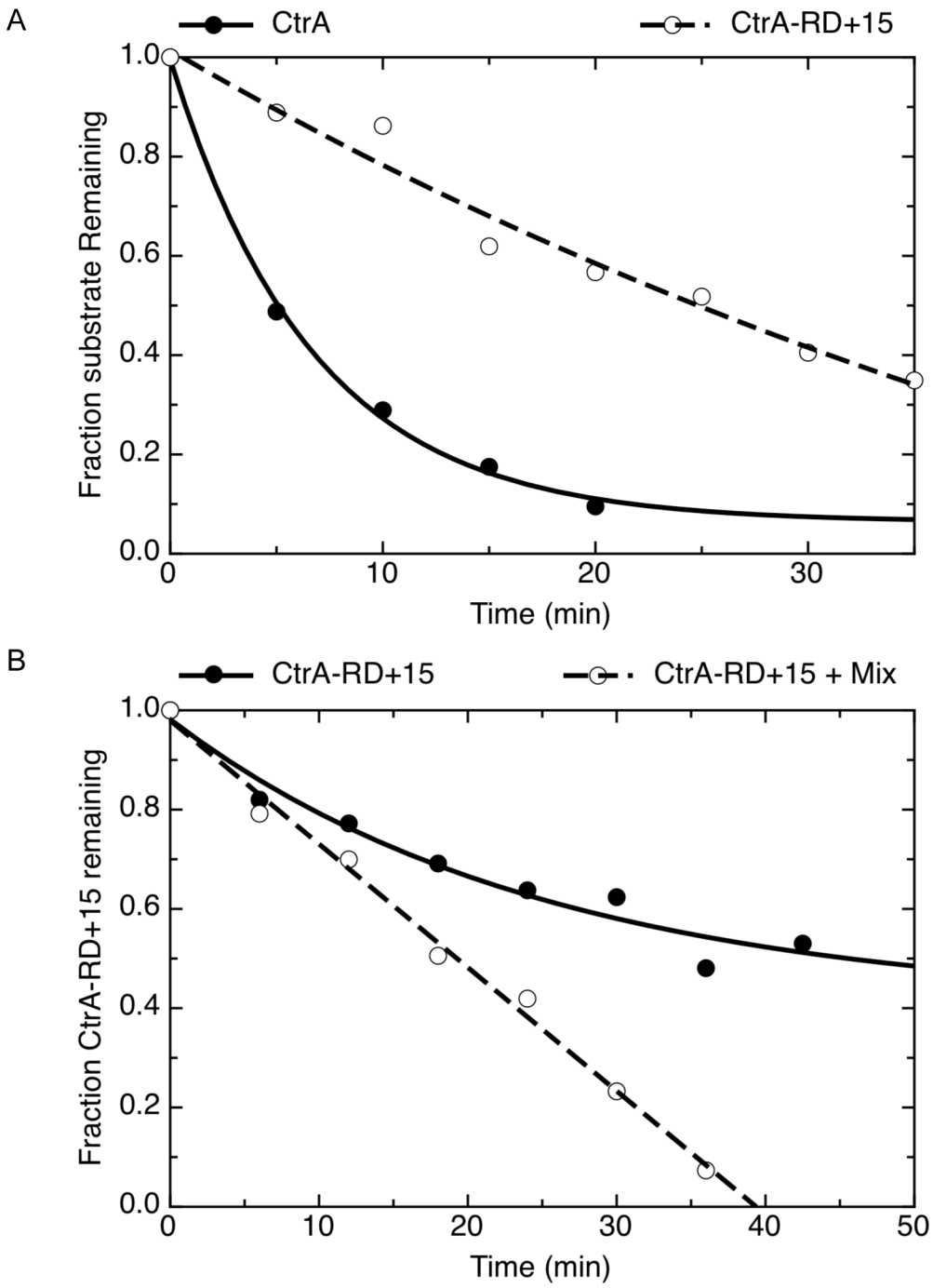

Fig. 2. Characterization of CtrA-RD+15 proteolysis in vitro. (A) CtrA-RD+15 is degraded more slowly than CtrA. Initial concentration of substrate was 5 μm. Reactions included 50 mM KCl, 0.2 μM $ClpX_6$, 0.5 μM $ClpP_{14}$, and an ATP regeneration system. (B) Proteolysis of CtrA-RD+15 is accelerated by the accessory factors. Initial substrate concentration was 5 μm CtrA-RD+15. Reactions included 50 mM KCl 0.25 μM $ClpX_6$, 0.5 μM $ClpP_{14}$, and an ATP regeneration system. Where indicated, reactions were supplemented with an accessory mix composed of 20 μM cdG and 1 μM each of CpdR, RcdA, and PopA.

Accessory factors reduce the K_M of ClpXP for CtrA

To determine how the accessory factors affect the steady-state kinetic parameters of CtrA proteolysis, a fluorescence-based assay was used that measures degradation of GFP fused to proteins of interest. As the substrate, GFP fused to the N-terminus of CtrA-RD+15 was used. In this assay, ClpXP degraded GFP-RD+15 with a V_{max} of 0.68 +/- 0.04 molecules/$ClpX_6$/min and a K_M of 9.9 +/- 2.1 μM. 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/$ClpX_6$/min (K. Joshi and P. Chien, personal communication). If any individual accessory protein or cdG was omitted from the reaction, the observed degradation rate was similar to reactions containing ClpXP and CtrA alone (K. Joshi and P. Chien, personal communication). The reduction of K_M without a significant impact on enzyme turnover is consistent with the accessory proteins functioning as a proteolytic adaptor, which binds both the substrate and protease for efficient substrate delivery. The fact that omission of any single accessory factor caused CtrA to be degraded at the unstimulated rate indicates that each factor is individually required to accelerate CtrA proteolysis, and it suggests that the proteins work together by an all-or-nothing mechanism, rather than each incrementally stimulating the reaction.

Accessory factors overcome the protection from proteolysis provided by DNA and SciP

DNA fragments from CtrA-regulated promoters that contain CtrA binding sites, such as a 50 bp fragment of the *pilA* promoter (P*pilA*), reduce the rate of CtrA proteolysis *in vitro* (Gora et al, 2013). The protective effect of CtrA binding sites is exaggerated in the presence of the protein SciP (Gora et al, 2013), which acts as a transcriptional co-regulator for a subset of genes in the CtrA regulon (Gora et al, 2010; Tan et al, 2010). As expected, CtrA proteolysis by ClpXP alone was significantly slowed by the presence of SciP and a P*pilA* DNA fragment (Fig. 1B). However, the addition of CpdR, RcdA, PopA, and cdG to the reaction substantially accelerated the degradation of CtrA under the same conditions (Fig. 1B). This result suggests either that the accessory factors prevent the protein-protein and protein-DNA interactions that slow CtrA proteolysis, or that the accessory factors along with ClpXP can disassemble the complex between CtrA, DNA, and SciP that inhibits CtrA degradation (Fig. 1B).

Residues in helix α1 of CtrA confer cell cycle-regulated degradation on an otherwise stable CtrA homolog

To identify residues in the CtrA receiver domain that are involved in the regulation of CtrA proteolysis, we constructed a multiple sequence alignment of CtrA homologs from 65 organisms of different genera within the alpha-proteobacteria (Fig. 3). Interestingly, one group of closely related sequences came from bacteria whose genomes encode homologs of RcdA and CpdR, while the other group does not encode these accessory factors (Brilli et al, 2010). We examined the alignment to identify residues in the CtrA receiver domain that are highly conserved in bacterial genomes containing homologs of *rcdA* and *cpdR*, but are divergent in bacteria that lack these genes. We then mapped these residues onto a structural model of the CtrA receiver domain that was based on the known structure of the response regulator Spo0F (PDB: 1NAT, (Madhusudan et al, 1997). 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), while two of the residues are predicted to be partially or completely buried within the protein core (F25 and G40, Fig. 4).

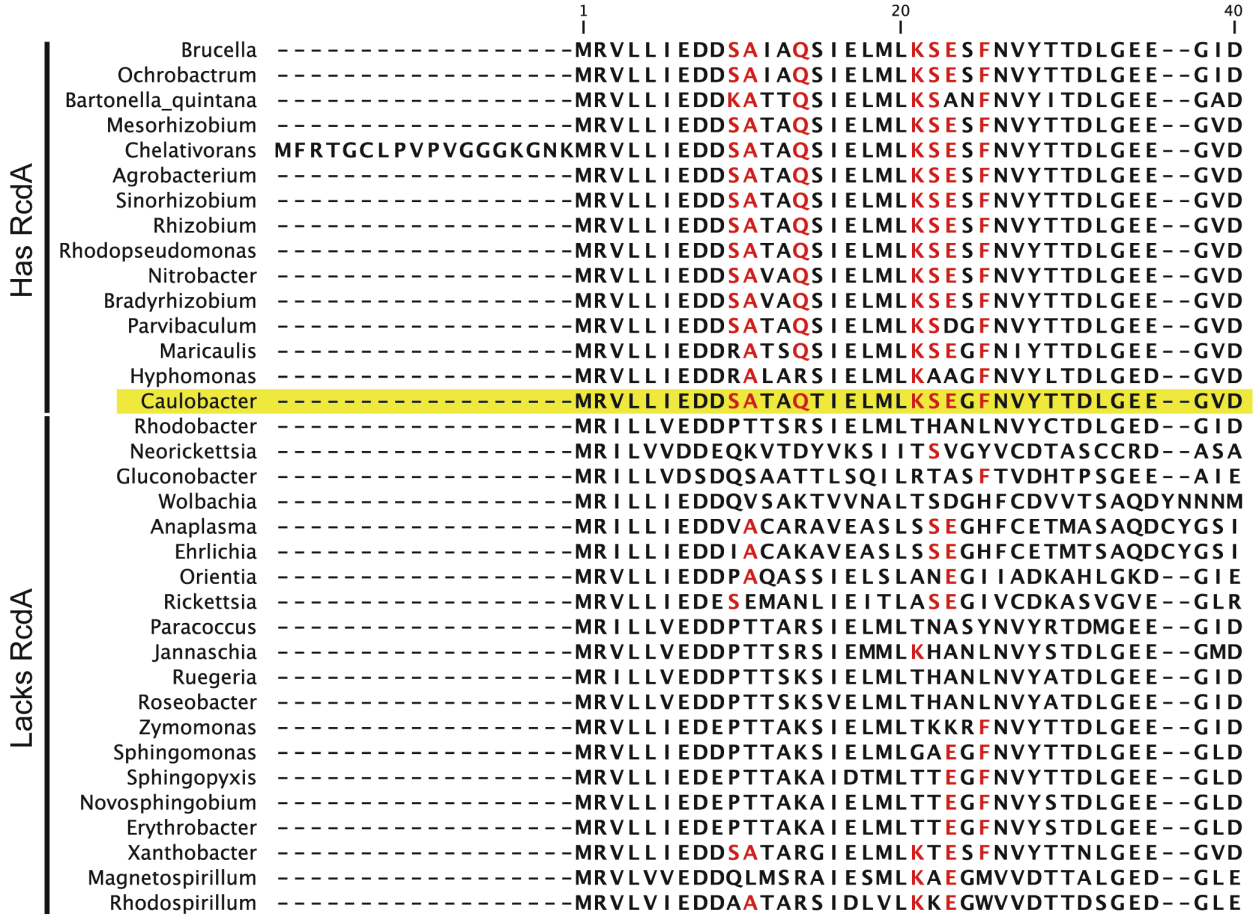

Fig 3. CtrA receiver domain multiple sequence alignment. Shown are the first 40 amino acids of the CtrA receiver domain, aligned with CtrA homologs from 35 genera throughout the alpha-proteobacteria. The *Caulobacter* CtrA sequence is highlighted in yellow. Organisms that encode RcdA homologs are listed above the *Caulobacter* sequence, and those that lack RcdA are listed below (Brilli et. al, 2010). RcdA and CpdR display a strong pattern of co-occurrence. Residues that are highly conserved in organisms that contain homologs of *rcdA and cpdR,* but that are divergent in strains lacking these genes, are shown in red text.

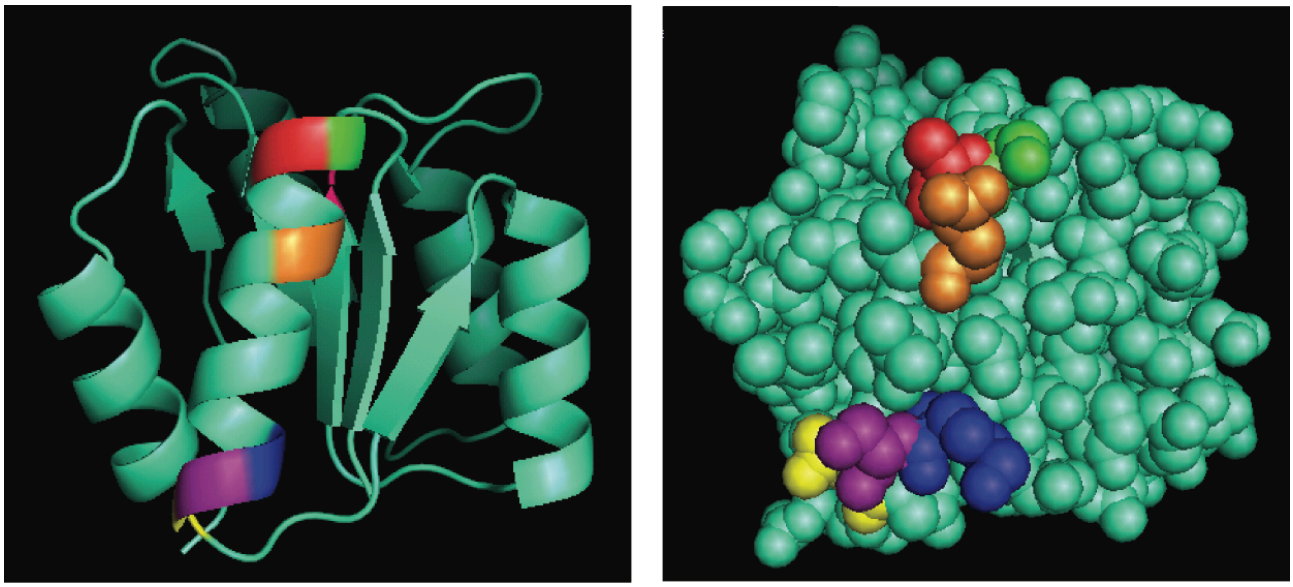

Fig. 4. Surface-exposed residues in the CtrA receiver domain predicted to affect proteolysis. Modeled structure of the CtrA receiver domain based on the solved structure of SpoF (PDB: 1NAT). The ribbon model (left) and space filling model (right) are shown. Residues that are specifically conserved in species that contain *rcdA* and *cpdR* homolgs are colored as follows: S10 (green), A11 (red), Q14 (orange), K21 (violet), S22 (magenta), S23 yellow, F25 (pink).

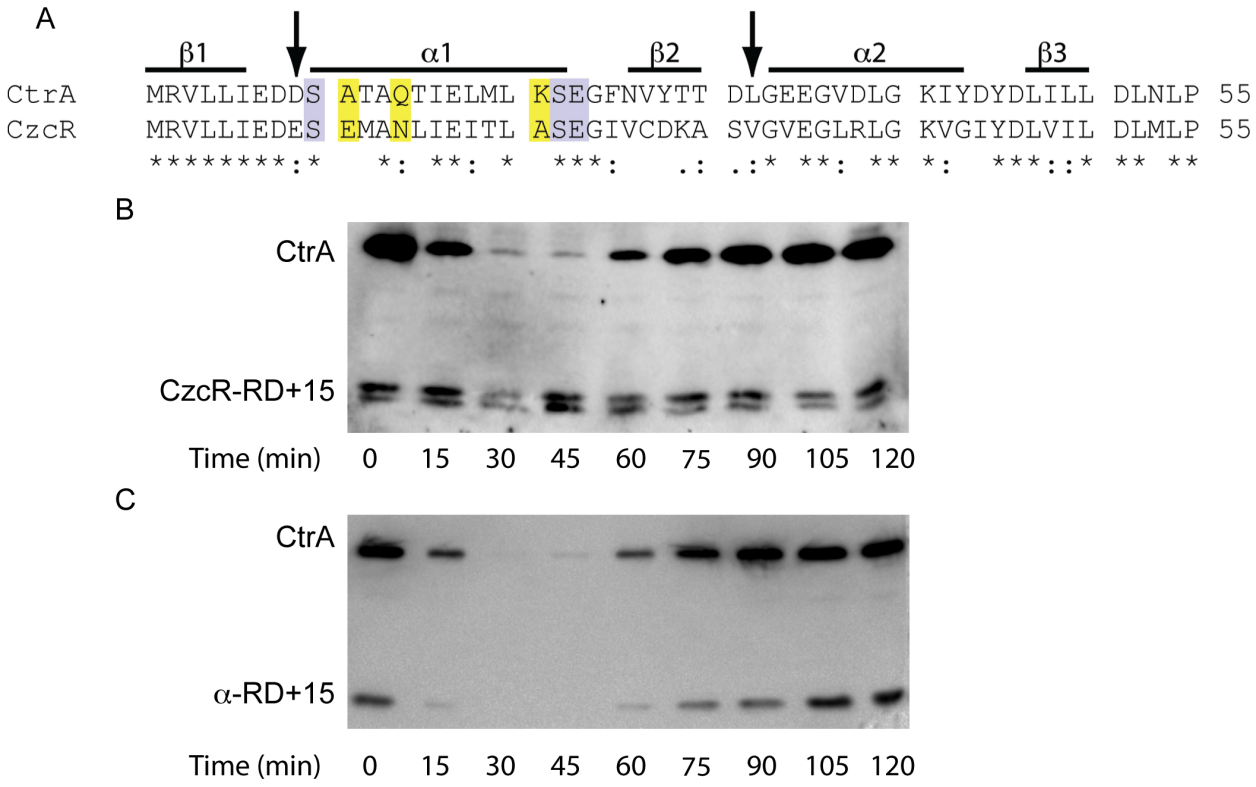

Fig. 5. Amino acid residues 9-31 of CtrA are sufficient to target a stable chimeric protein for proteolysis. (A) Alignment of the CtrA and CzcR N-termini. Residues highlighted in purple or yellow are co-conserved in the alpha-proteobacteria with rcdA and cpdR. Of these, only the residues in yellow differ between the CtrA and CzcR sequences. Arrows indicate residues 9-31 of of CtrA that were substituted into CzcR-RD+15 to create a-RD+15. (B) Anti-CtrA western blot of samples taken as a strain expressing CzcR-RD+15 from the xylX promoter proceeded synchronously through the cell cycle in PYE medium with 0.03% xylose. The last 15 amino acids of CtrA are a strong epitope for the anti-CtrA antibody, which allows detection of CzcR-RD+15. (C) Anti-CtrA western blot of samples taken as a strain expressing a-RD+15 from the xylX promoter proceeded synchronously through the cell cycle in PYE medium with 0.03% xylose.

Based on these results, we examined the *in vivo* degradation pattern of a chimeric protein constructed of CtrA and a distant CtrA homolog CzcR from *Rickettsia prowazekii*. We previously found that while CtrA RD+15 is degraded in *Caulobacter* during SW-ST differentiation, the CzcR receiver domain fused to the CtrA C-terminus (CzcR RD+15) is not specifically degraded at this time (Fig. 5B, (Ryan et al, 2002). In this study, we sought to confer regulated degradation upon CzcR by replacing residues 9-31 with the corresponding sequences from CtrA (Fig. 5A). This region includes both α1 and the subsequent beta-strand β2, which is predicted to pack against helix α1 in the three-dimensional structure of CtrA (Fig. 4).

The resulting chimeric protein α1-RD+15 was expressed from the *xylX* promoter on a medium-copy plasmid (Meisenzahl et al, 1997) in the wild type *Caulobacter* strain CB15N. During synchronous passage through the cell cycle, α1-RD+15 was degraded at the swarmer-to-stalked cell transition, similar to full-length, wild-type CtrA present in the same strain (Fig. 5C). This result indicates that a determinant in residues 9-31 of CtrA, along with the CtrA C-terminus, can confer regulated proteolysis on the otherwise stable CzcR receiver domain.

Residues 26-28 (NVY in CtrA and VCD in CzcR) are different in the two proteins (Fig. 5A), but the NVY sequence does not co-occur specifically with RcdA and CpdR (Fig. 3), and changing these residues within CtrA to the VCD sequence found in CzcR does not cause a defect in CtrA degradation (K. Ryan, personal communication). Interestingly, although CzcR is not degraded, it only differs from CtrA at three of the residues on α1 that are co-conserved 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 directly assess the contribution of the residues identified in the bioinformatic analysis above, we made single and multiple amino acid substitutions within CtrA-RD+15 and expressed the variants from a xylose-inducible promoter in wild-type *Caulobacter*. In most cases, we changed the residue in CtrA to the most common residue occurring at the same position in CtrA homologs from bacteria lacking RcdA and CpdR. The RD+15 variant containing all six amino acid substitutions in α1 was insoluble when overexpressed in *E. coli*, and a GFP fusion to this variant was located in a single randomly-placed focus in *Caulobacter*, similar to aggregated proteins (data not shown, (Lindner et al, 2008). We therefore focused on soluble variants with fewer substitutions.

We performed pulse-chase analyses to measure the rates at which the CtrA-RD+15 variants are degraded during the SW-ST transition. In these experiments, wild-type CtrA-RD+15 was degraded with a half-life of 3.6 +/- 0.3 min. Several CtrA variants with amino acid substitutions showed moderate degradation defects (Fig. 6). The variant containing the substitutions A11T, Q14K, and K21A (CtrA3-RD+15) was degraded the most slowly, with a half-life of 8.5 +/- 2.6 min during SW-ST differentiation. Interestingly, this mutant contains the same subset of amino acid substitutions that differ between the α1 helix of CtrA and the stable homolog CzcR.

Although the CtrA3 mutations slow the degradation of CtrA-RD+15, a full-length CtrA protein with the same substitutions (CtrA3) supports viability in the absence of wild-type CtrA (strain KR3512). In this strain, CtrA3 is expressed from the *xylX* locus on the chromosome and the native *ctrA* gene has been deleted. A fraction of the cells containing only CtrA3 are elongated, indicating a defect in cell division (Fig. 7A,B). This phenotype could indicate an inadequate level of CtrA activity. Indeed, CtrA3 levels in this strain were lower than wild-type

CtrA expressed from its native locus or wild-type CtrA expressed only from the *xylX* locus (strain KR3510, Fig. 7C). In contrast, the levels of CtrA-FLAG (KR3178) and CtrA3-FLAG (KR3179) were similar when each was expressed from the *xylX* locus as the sole copy of CtrA in the cell (Fig. 7D). Fusion of the FLAG peptide to the C-terminus of CtrA obscures hydrophobic amino acids that are expected to bind ClpX and facilitate proteolysis (Domian et al, 1997), and cells expressing CtrA3-FLAG did not exhibit any morphological defects (data not shown). This suggests that CtrA3 may be less stable than wild-type CtrA during parts of the cell cycle when rapid CtrA proteolysis is not occurring. As a result, expression from the *xylX* locus may be inadequate to maintain the levels of CtrA3 required for normal cell division and morphology.

Because ClpXP, the accessory factors, and the substrate CtrA all localize transiently at one pole of the *Caulobacter* cell during proteolysis (Biondi et al, 2006; Duerig et al, 2009; McGrath et al, 2006; Ryan et al, 2004; Ryan et al, 2002) , we examined the intracellular location of a YFP-CtrA3-RD+15 fusion protein, as compared to a comparable fusion protein containing the wild-type CtrA receiver domain. Mixed exponential-phase cultures containing all cell cycle stages were examined by DIC and fluorescence microscopy. In agreement with previous reports (Ryan et al, 2004), YFP-CtrA-RD+15 accumulated at one pole in 11.3% of swarmer cells and accumulated specifically at the stalked pole in 8.9% of predivisional cells (Fig. 8). However, YFP-CtrA3-RD+15 was diffuse in all cells examined, regardless of cell cycle stage (Fig. 8).

Co-immunoprecipitation experiments have shown that CtrA associates with ClpX and RcdA *in vivo* (McGrath et al, 2006), and that CpdR associates with ClpXP (Iniesta et al, 2006). Bacterial two-hybrid experiments further indicated that PopA interacts directly with RcdA (Duerig et al, 2009). Because the amino acid substitutions in CtrA3 cause defects in both proteolysis and protein localization, we examined the ability of CtrA3 to interact with ClpX, RcdA, and PopA *in vivo*. CtrA-FLAG (KR3178) or CtrA3-FLAg (KR3179) was expressed from the xylose locus in a strain lacking the native copy of *ctrA*. In a negative control strain, the unrelated protein Hfq-FLAG was expressed from the *hfq* locus (KR2973) (Iniesta et al, 2010). The FLAG-tagged proteins were immunoprecipitated from cell lysates, and the immunoprecipitates were analyzed by Western blotting with antisera that recognize RcdA, ClpX, or PopA.

Our data confirmed that RcdA and ClpX co-precipitate with wild-type CtrA, and we demonstrated for the first time that PopA also associates directly or indirectly with CtrA *in vivo* (Fig. 9). As expected, Hfq-FLAG failed to interact with any of the tested proteins. When CtrA3-FLAG was used as bait, much less RcdA and PopA were co-precipitated, even though the levels of these proteins in cell lysates were comparable to the strain containing CtrA-FLAG. In contrast, CtrA3 efficiently co-precipitated ClpX, suggesting that the amino acid substitutions in CtrA3 affect specific protein-protein interactions

When purified CtrA3 was used as the substrate in our *in vitro* proteolysis assay, it was degraded at the same rate as wild-type CtrA when only ClpXP was present. The accessory factors RcdA, CpdR, PopA, and cdG accelerated CtrA3 degradation by ClpXP, but not to as great a degree as wild-type CtrA (Fig. 1a). Similarly, in reactions containing the P*pilA* promoter fragment and SciP, the rate of CtrA3 proteolysis was indistinguishable from that of wild-type CtrA when the accessory factors were absent. Although the inhibitory effects of P*pilA* and SciP were reversed by the accessory factors, the proteolysis of wild-type CtrA was stimulated to a greater extent than that of CtrA3 (Fig. 1b). Together, the *in vivo* and *in vitro* data indicate that residues A11, Q14, and K21 are important for regulated CtrA proteolysis and for direct or indirect interactions with the accessory factors RcdA and PopA.

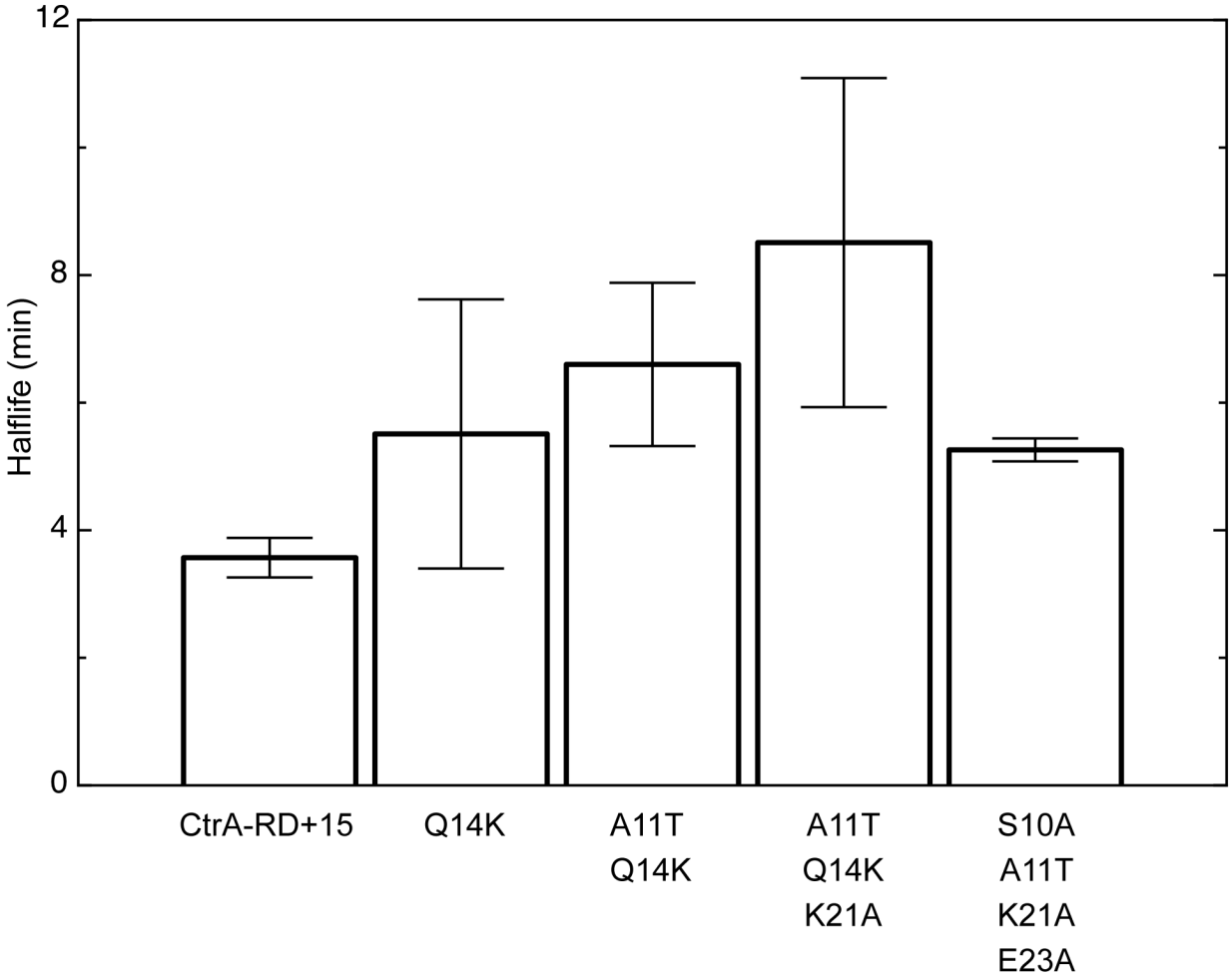

Fig. 6. Degradation rates of CtrA-RD+15 variants during SW-ST differentiation. Swarmer cells were isolated from strains expressing CtrA-RD+15, with the indicated amino acid substitutions, from a xylose- inducible promoter. The swarmer cells were resuspended in medium lacking xylose, and samples of equal volume were collected every 5 minutes during the first half of the cell cycle. Anti-CtrA western blots were used to quantify the levels of the indicated proteins, and half-lives were calculated during SW-ST differentiation. Error bars represent standard deviations from at least 3 independent experiments per mutant.

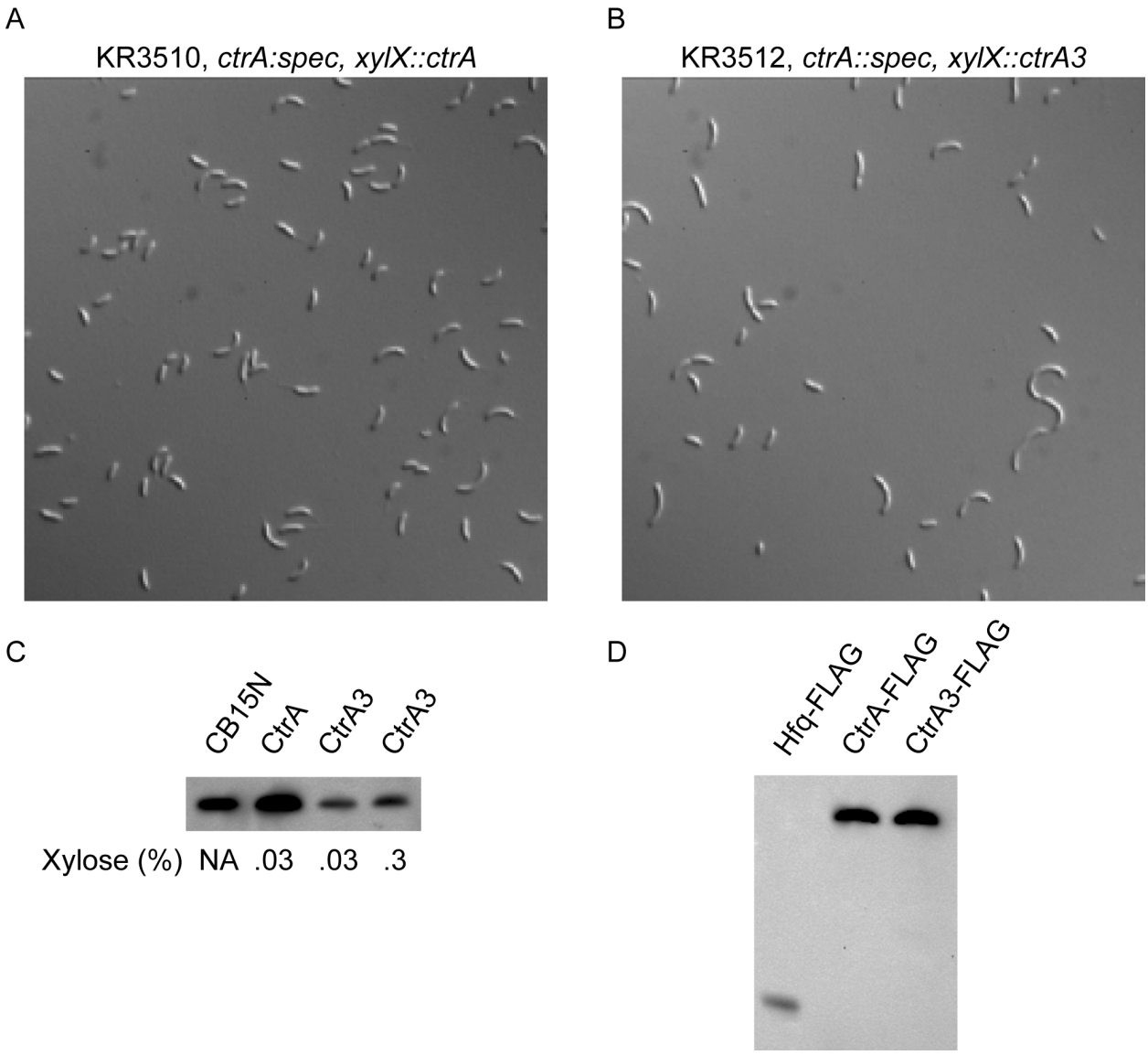

Fig. 7. CtrA3 supports Caulobacter viability. (A) Expression of CtrA was induced from the *xylX* locus in PYE medium supplemented with 0.03% xylose. (B) Expression of CtrA3 was induced from the *xylX* locus in medium supplemented with 0.3% xylose. (C) Anti-CtrA Western blot of equal cell numbers of strains expressing CtrA (KR3510) or CtrA3 (KR3512) from the *xylX* locus in PYE medium supplemented with the indicated concentration of xylose. CtrA expression from its native promoter in the chromosome was included for comparison (CB15N). (D) Anti-FLAG Western blot of equal cell numbers of strains expressing the indicated FLAG fusion proteins.

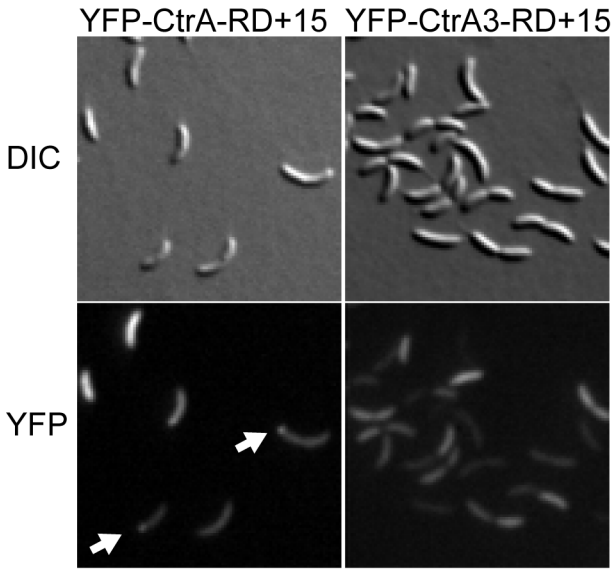

Fig. 8. CtrA3 fails to accumulate at the cell pole. Cells expressing the indicated CtrA-RD+15 variant fused to YFP from a xylose-inducible promoter on a medium-copy plasmid were imaged using DIC and epifluorescence microscopy. Representative cells are shown. Cells with polar foci are indicated with.

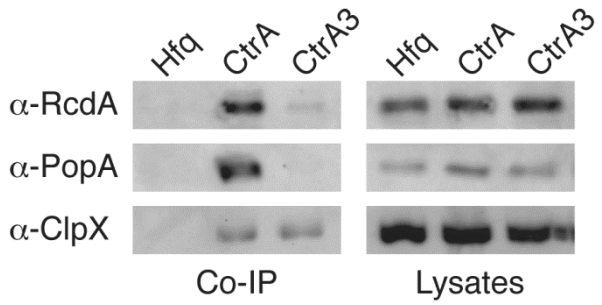

Fig. 9. CtrA3 has an altered network of physical interactions in vivo. The indicated proteins (above image) were fused at their C-termini to the 3xFLAG epitope. These proteins were expressed in *Caulobacter* strains lacking the untagged version of Hfq or CtrA. After formaldehyde crosslinking and immunoprecipitation, precipitated samples were separated by SDS-PAGE and probed with the indicated antibodes (left of image). Lysates from equal numbers of cells that were used in co-immunoprecipitation experiments were analyzed to examine the starting amounts of CtrA-interacting proteins in each strain.

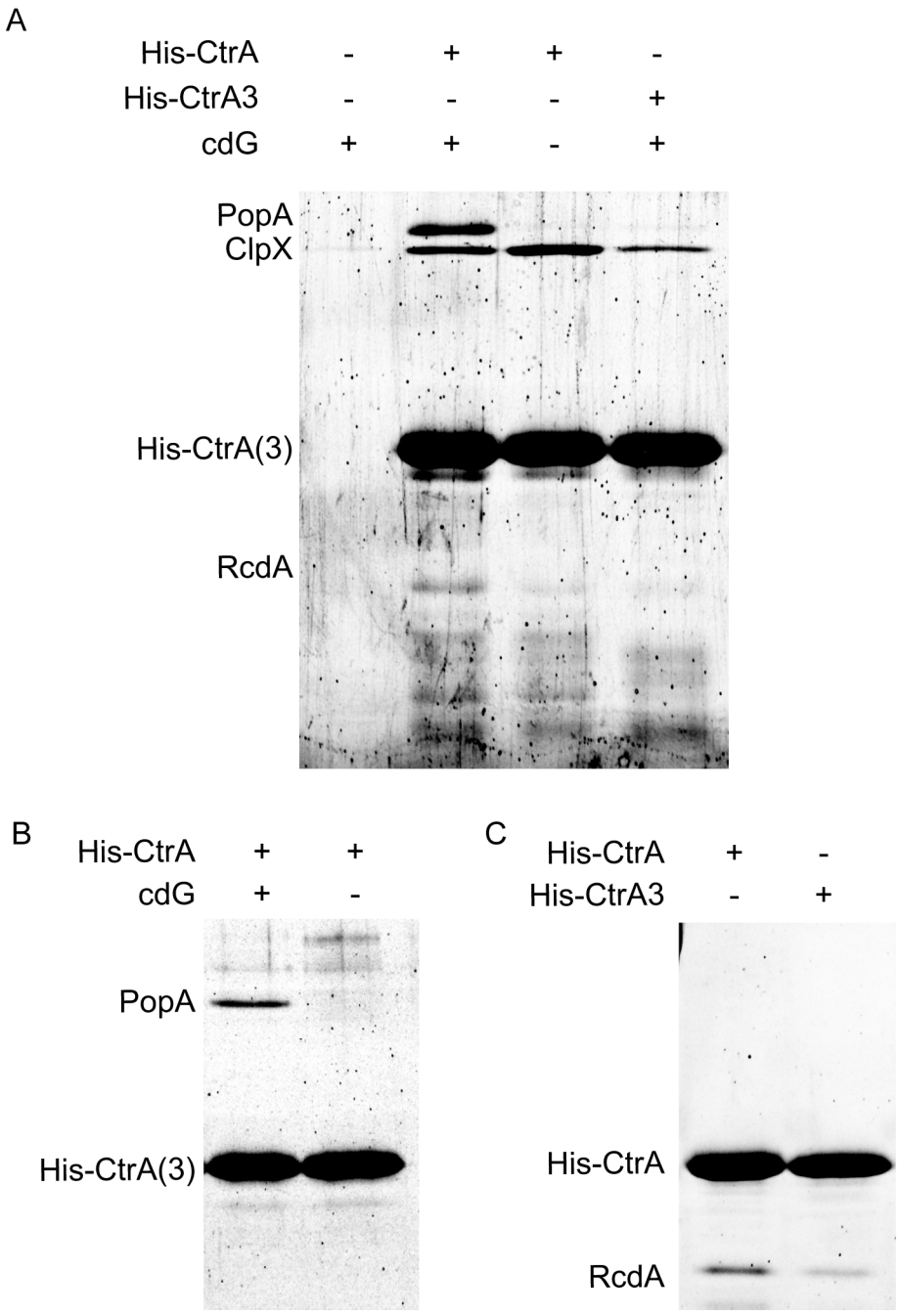

Fig. 10. CtrA interacts directly with accessory proteins. Co-affinity purification experiments were performed by incubating bait proteins (His-CtrA or His-CtrA3) with Ni-NTA agarose beads. After washing to remove unbound bait proteins, the Ni-NTA agarose was resuspended in buffer containing the indicated accessory proteins (prey) with or without 20 μM cdG where indicated. After washing to remove unbound prey proteins, stable complexes were eluted and analyzed by SDS-PAGE and staining with Lumitein fluorescent protein stain (Biotium). (A) His-CtrA or His-CtrA3 complex formation in the presence of PopA, RcdA, CpdR, and ClpX, with or without cdG. (B) Direct binding of His-CtrA to PopA with or without cdG. (c) Direct binding of His-CtrA or His-CtrA3 to RcdA.

Assembly of the proteolytic complex in vitro requires cyclic diguanylate

Interactions among ClpX, CtrA, and the accessory proteins have been investigated using co-immunoprecipitation and bacterial two-hybrid (BACTH) assays, uncovering a network of direct and indirect interactions. Although it is assumed that CtrA must interact with ClpX for it to be unfolded and delivered to ClpP, no studies have demonstrated a direct interaction between CtrA and any single protein in the proteolytic complex. To further define the direct interactions among the protease, substrate, and accessory proteins, we performed co-affinity purification assays using CtrA with an amino-terminal hexahistidine tag (His-CtrA) as bait.

We first assessed the ability of His-CtrA to form a complex with ClpX and the accessory proteins when they were all incubated together in a single binding reaction. When cdG was included in the binding and wash buffers, PopA, RcdA and ClpX were all retained with His-CtrA on the Ni-NTA resin. When cdG was omitted from the experiment, there was a dramatic reduction in the amounts of PopA and RcdA that eluted with His-CtrA. Surprisingly in the presence of the other accessory proteins, but in the absence of cdG, the amount of ClpX that eluted with His-CtrA increased by over 3-fold (Fig. 10A)

Consistent with the results from co-immunoprecipitation experiments shown above, CtrA3 fails to bind PopA and RcdA in the equivalent reaction containing all of the accessory factors, including cdG. Regardless, ClpX is retained with Ni-NTA agarose beads that have been loaded with purified His-CtrA3.

Although CpdR was included in these reactions, we were unable to visualize it consistently in repeated experiments. Further studies are needed to characterize the disposition of CpdR within the entire proteolytic complex.

Individual protein interactions with CtrA

To assess direct interactions with CtrA, His-CtrA was used as bait in reactions with individual regulatory proteins or with specific combinations of regulatory proteins. In these assays, His-CtrA bound directly to ClpX, PopA and RcdA (Fig. 10 B,C, and J. Zik, personal comunication). The interaction between PopA and His-CtrA was completely dependent on cdG. His-CtrA3 bound to ClpX, but failed to bind PopA, even when cdG was included. The amount of RcdA retained was reduced in experiments with His-CtrA3 relative to experiments with His-CtrA. In separate experiments, we found that substituting DD for the terminal AA residues of CtrA abolished the direct interaction with ClpX (J. Zik, personal communication). We conclude from these co-affinity purification experiments that CtrA interacts directly with PopA, RcdA and ClpX, and that the interaction between PopA and CtrA is dependent on cdG. Further, the interactions with PopA and RcdA are each dependent on a conserved set of amino acid residues in the first alpha-helix of CtrA, while the ClpX-CtrA interaction is mediated by the CtrA C-terminus.

Discussion

CpdR, RcdA, PopA and cdG are each necessary, and together are sufficient to accelerate the rate of CtrA proteolysis by ClpXP. The observation that accessory factors dramatically reduce the K_M of the proteolytic reaction (K. Joshi and P. Chien, personal communication) without significantly altering the turnover rate is consistent with a traditional adaptor mechanism. However, published examples of adaptors are generally single proteins (Ades, 2004; Battesti & Gottesman, 2013). This report presents the first evidence of a multiprotein complex functioning as an adaptor to promote proteolysis. The mechanism that regulates the activity of this adaptor is also novel. There are multiple examples of adaptor activity being regulated by

physical interaction with an anti-adaptor protein. For example, in *Bacillus subtilis*, the small protein ComS binds to the adaptor MecA during periods of nutrient deprivation. This disrupts an interaction between MecA and the proteolytic substrate ComK, thus stabilizing ComK and leading to development of competence (Persuh et al, 1999). In this example, the substrate is stabilized when the adaptor protein is bound to its regulator. Additionally, autophosphorylation of the *B. subtilis* adaptor McsB and phosphorylation of the unfoldase ClpC are required for proteolysis of CtsR in response to heat shock. In *E. coli* RssB is an adaptor that promotes the proteolysis of the general stress response sigma factor RpoS. Three anti-adaptors, IraP, IraD and IraM inhibit RssB activity in response to various signaling inputs. One of these signals is the binding of IraP or IraD to the small molecules guanosine pentaphosphate or tetraphosphate (p)ppGpp,(Battesti & Gottesman, 2013). Here we describe a unique mechanism of regulation in which direct binding of the small molecule cdG is required for the function of a multiprotein adaptor complex.

Residues in α1 of CtrA that are co-conserved with CpdR and RcdA play a role in the regulated proteolysis of CtrA. Substituting residues 9-31, comprising α1 and β2, of CtrA for the corresponding structural features in the chimera CzcR-RD+ 15 renders this otherwise stable protein susceptible to cell cycle-regulated proteolysis (Fig. 5B,C). Conversely, mutating three residues in α1 of CtrA to the corresponding amino acids in the CzcR sequence resulted in stabilization of CtrA3-RD+15 at the SW-ST cell transition (Fig. 6). *In vitro* analysis revealed that CtrA3 is defective specifically in ClpXP-mediated proteolysis that is stimulated by the accessory factors (Fig. 1). The rate of CtrA3 degradation was reduced relative to wild-type CtrA in the presence of accessory factors, but there was no significant difference in the basal rates of CtrA and CtrA3 proteolysis when accessories were absent (Fig. 1). The normal cell cycle-dependent polar localization of CtrA was abolished in YFP-CtrA3 RD+15 (Fig. 8). This defect in localization would be predicted for a CtrA3 variant that is unable to form physical interactions with accessory proteins, since cells lacking PopA, RcdA or CpdR fail to localize CtrA.

The physical interactions observed between CtrA and the accessory proteins PopA and RcdA are consistent with an adaptor mechanism. Wild-type CtrA, but not the degradation-defective CtrA3, was competent to interact with with PopA and RcdA *in vivo* (Fig. 9). Likewise, RcdA and PopA interacted directly with CtrA in reactions using purified proteins (Fig. 10). Strikingly, the interaction between PopA and CtrA was strictly dependent on cdG, and RcdA interacted less strongly with CtrA3 than with wild-type CtrA (Fig 10B,C). We also confirmed a previously-reported direct interaction between PopA and RcdA (J. Zik personal communication, (Duerig et al, 2009). Together, these data suggest that the interaction between PopA and RcdA is not dependent on cdG, but when PopA is bound to cdG, a trimeric complex forms between RcdA, PopA and CtrA. We infer, based on the acceleration of proteolysis that occurs in the presence of the accessory proteins, that formation of this complex likely enhances the recruitment of CtrA to the proteolytic pore. However, at this time we cannot rule out a mechanism in which the interaction between PopA, RcdA and CtrA alters the conformation of CtrA, thereby increasing its affinity for the protease. Our model predicts that there is a direct physical interaction between PopA or RcdA and ClpX. Future studies will determine whether such an interaction occurs.

Of the three accessory proteins, only CpdR has been previously shown to function as an adaptor. CpdR binds directly to both ClpX and the phosphodiesterase PdeA, and unlike CtrA, PdeA is absolutely dependent on unphosphorylated CpdR for *in vitro* degradation by ClpXP (Abel et al, 2011). Though not required for basal CtrA proteolysis, CpdR is necessary in its

unphosphorylated state for stimulated degradation of CtrA. At this time it is unclear whether CpdR participates directly in the formation of the adaptor complex that regulates CtrA proteolysis, or if it instead allosterically regulates ClpX, inducing a conformation with enhanced receptivity to PopA/RcdA/CtrA. Work is ongoing to identify the direct physical interactions that mediate CpdR function.

The complete mix of accessory factors increases the rate of CtrA proteolysis in the presence of factors that stabilize CtrA (Fig. 1B). Consistent with previous reports, SciP and a P*pilA* DNA fragment protect CtrA from degradation by ClpXP alone, greatly increasing CtrA stability *in vitro*. Addition of the accessory factors to reactions containing SciP and P*pilA* dramatically accelerated CtrA proteolysis. These results suggest that the accessory factors can either prevent interactions among CtrA, P*pilA*, and SciP that slow CtrA degradation, or they can disassemble these protein-DNA complexes. Future studies will address the mechanism by which the accessory factors overcome this inhibition of CtrA proteolysis.

Since CtrA degradation is accelerated by the accessory factors in reactions lacking SciP and P*pilA*, the role of the accessory factors is not limited to preventing or disrupting the formation of inhibitory complexes. We propose a model in which CtrA interacts directly with the axial pore residues of ClpX via its C-terminal AA residues (J. Zik, personal communication). This interaction is sufficient to account for the basal rate of CtrA proteolysis by ClpX *in vitro*. When PopA is bound to cdG, PopA and RcdA bind to a proteolysis determinant in the α1 helix of CtrA and to either ClpX itself or to CpdR, which is known to interact independently with ClpX (Rood et al, 2012). These interactions create an adaptor which increases the effective concentration of the CtrA C-terminus near the pore residues of ClpX, stimulating degradation.

In the presence of SciP and DNA fragments to which CtrA binds, we propose that the ClpX interaction motif at the C-terminus of CtrA is less accessible to the protease or, by virtue of its conformation, has a lower affinity for the protease. In this situation, binding of PopA-cdG and RcdA to the receiver domain of CtrA may stabilize a conformation of CtrA that is less likely to interact with DNA and SciP. Alternatively, the accessory factors may alter the conformation of CtrA within a DNA-SciP complex that makes the C-terminal motif more accessible to ClpX. Finally, the entire proteolytic complex of ClpXP, CpdR, RcdA, and PopA-cdG may first extract CtrA from the DNA-SciP complex prior to degradation, similar to the extraction of the phage protein MuA from transposition complexes (Levchenko et al, 1995). Understanding precisely the structural changes that mediate this cdG-dependent mechanism of stimulated proteolysis will be a rich field for future research.

Materials and Methods

Bacterial strains, plasmids and culture conditions

All experiments were performed using derivatives of *Caulobacter crescentus* strain CB15N (Evinger & Agabian, 1977) grown to mid-exponential phase. Plasmids were mobilized from *E. coli* to *C. crescentus* by conjugation using *Escherichia coli* strain S17-1 (Ely, 1991), and generalized transduction was performed using ΦCr30 (Ely & Johnson, 1977). CB15N strains were grown in peptone-yeast extract medium (PYE, (Ely, 1991) or minimal glucose medium (M2G, (Ely, 1991) at 30ºC. Where indicated, growth media were supplemented with glucose (0.02%) or xylose (0.03% or 0.3%) to repress or induce, respectively, expression from the *xylX* promoter (Meisenzahl et al, 1997). *E. coli* strains were grown in Luria broth at 37°C, and solid and liquid media were supplemented with antibiotics as described (Reisinger et al, 2007). PCR products were cloned into the pGEMT-easy vector (Promega) before being subcloned into the destination vector. The chimeric protein, α1-RD+15, was constructed using overlap extension

PCR (Higuchi et al, 1988). Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene). Sequences of primers used for amplification or sequence modification are available upon request.

To construct strains expressing either *ctrA* or *ctrA3* at the *xylX* locus, we cloned the indicated open reading frame into pPM53 (P. McGrath, personal communication), which confers resistance to kanamycin, and mobilized it into strain KR1800 (*ctrA::spec* + pCTD14, (Domian et al, 1997) by conjugation. Conjugants were selected on PYE/nal/kan/xylose medium and were passaged for several days in PYE/kan/xylose medium to allow loss of pCTD14, which confers resistance to chloramphenicol. Dilutions were plated on PYE/kan/xylose medium, and individual colonies were screened for resistance to spectinomycin, sensitivity to chloramphenicol, and death on medium containing glucose rather than xylose.

Protein purification

ClpP was expressed from pET21a as a C-terminal fusion to a hexahistidine tag(Chien et al, 2007). RcdA, ClpX, PopA and CtrARD+15 were expressed as N-terminal hexahistidine fusions from the pET28b vector(Chien et al, 2007; Duerig et al, 2009). CtrA and CtrA3 were expressed as N-terminal hexahistidine fusions with a PKA phosphorylation site in pET32a. SciP was expressed from pHIS-DEST, with an N-terminal hexahistidine tag (Gora et al, 2013; Skerker et al, 2005). CpdR was expressed as Hexahistine-Smt3 fusion in pET28b (A.R. Bresnick personal comunication). All proteins were expressed in E. coli Tuner cells. Cells were grown to an OD of A_{600} = 0.6 at 37° C in Terrific broth. IPTG was then added to a final concentration of 0.4 mM and cells were incubated overnight at 18° C. Cells were harvested by centrifugation at 6000 RPM for 5 min, and cell pellets were frozen in Liquid nitrogen and stored at -80° C until use. Proteins were purified by the following protocol with modifications where indicated. Cell pellets were thawed and resuspended in Standard Lysis Buffer (SLB), defined as 50 mM Tris pH 8.2, 100 mM KCl, 1 mM $MgCl_2$ and 10% glycerol. SLB was supplemented with 2 mM BME during lysis and nickel affinity purification. CtrA variants were purified using lysis buffer containing only 50 mM KCl and no $MgCl_2$. ClpP was lysed in P-buffer, defined as 50 mM Na-phosphate pH 8.0, 1 M NaCl, 5 mM Imidazole and 10% glycerol. Cells were lysed by 1-hour treatment with 1 mg/ml lysozyme and 40 units benzonase (Novagen) followed by sonication. Lysates were cleared by three rounds of centrifugation at 20,000 RCF. Imidazole was added to a concentration of 15 mM, except when purifying ClpP, and each histidine tagged protein was incubated with 1 ml Ni-NTA resin (Qiagen) that had been pre-equilibrated with the protein specific lysis buffer. The resin was applied to a gravity column and washed three times with the appropriate lysis buffer, the second of the three washes was supplemented with 300 mM NaCl. Most proteins were eluted with lysis buffer containing 300 mM imidazole. Alternatively, ClpP bound resin was washed with P-buffer with 20 mM imidazole prior to elution of ClpP with 500 mM imidazole. ClpX and RcdA were eluted by overnight cleavage with 20 units of thrombin. After elution with imidazole, CpdR was incubated overnight with Sumo protease (Life Sensors) to cleave the 6His-sumo tag, and a second round of Ni-NTA purification was used to remove the cleaved tag and the histidine tagged Sumo protease. Proteins were further purified by affinity chromatography using HiTrap Q HP columns (GE), except for CtrARD+15, which was further purified using HiTrap DEAE FF columns (GE). Elution was achieved with a gradient of increasing KCl concentration in SLB with 1 mM DTT. SciP was not subjected to further purification after the Ni-NTA step. All proteins were exchanged into PD buffer (25 mM Hepes

pH 7.6, 5 mM $MgCl_2$, 15 mM NaCl, 10% glycerol) with 100 mM KCl and 1 mM DTT prior to freezing in liquid nitrogen and storage at -80° C.

In vitro proteolysis

Proteolysis reactions were initiated by adding the substrate to prewarmed reaction mixtures containing the indicated concentrations of protease and accessory factors. Reaction were conducted at 30°C in PD buffer with 100 mM KCl unless otherwise specified. Samples were removed at the indicated time intervals into tubes containing SDS-PAGE sample buffer and frozen immediately in liquid nitrogen. Samples were analyzed by SDS-PAGE and staining with Lumitein fluorescent protein dye (Biotium) according to manufacturer's instructions. Gels were visualized using a Biorad Gel Doc XL, and band intensities were quantified using Imagelab 4.0 software according to manufactures instructions.

Pulse-chase analyses

CB15N cells harboring a plasmid expressing the wild-type CtrA RD+15 or an amino acid variant were grown in M2G medium. Xylose (0.3%) was added to exponential-phase cultures for two hours before the cells were harvested by centrifugation for 15 min at 9000 rpm in a Sorvall JA-20 rotor, and swarmer cells were isolated by differential centrifugation as described (Reisinger et al, 2007). Swarmer cells were released into M2G medium without xylose, and samples of equal volume were removed at intervals during the first half of the cell cycle. Samples were analyzed by SDS-PAGE and Western blotting with anti-CtrA antiserum (1:10,000, (Quon et al, 1996) and HRP conjugated anti-rabbit antibodies. Western blots were visualized with a Biorad Gel Doc XL and quantified using the Imagelab 4.0 software package according to manufacturer's instructions. The data were fitted to single exponential curves to calculate protein half-lives.

Co-immunoprecipitation of proteins from Caulobacter lysates

KR2973, KR3178, and KR3179 were grown in 60 ml PYE with 0.03% xylose to OD_{660} = 0.3 and harvested by centrifugation (20min, 10500 rpm, Sorvall SS-34 rotor). Cells were resuspended in 1 ml co-IP lysis buffer (20 mM HEPES pH 7.5,100 mM NaCl, 20% glycerol) supplemented with 0.37% formaldehyde to crosslink proteins. After a one-hour incubation at room temperature, the crosslinking reaction was quenched by the addition of glycine to a final concentration of 0.125 M. Cells were washed 3 times and resuspended in co-IP lysis buffer containing 0.5% dodecyl maltoside. Cells were treated with 1 mg/ml lysozyme in the presence of 40 units of benzonase (Novagen) for one hour, followed by sonication to achieve cell lysis. Lysates were centrifuged for 10 minutes at 16000 rpm in a Sorvall SS-34 rotor to remove debris. Lysates were incubated overnight at 4°C with 40 µL anti-FLAG M2 resin (Sigma-Aldrich) pre-equilibrated in co-IP buffer. The resin was washed 2 times with 1 ml Co-IP buffer, and precipitated proteins were released by the addition of 30 µl 2x SDS-PAGE sample buffer and boiling. Equal volumes of co-immunoprecipitates, and cell lysates from equal numbers of cells were analyzed by SDS-PAGE and Western blotting.

Western blots

Each gel lane received the same number of cell equivalents, normalized by OD_{660}, or the same volume of co-immunoprecipitated sample. Samples were boiled in 1x SDS loading buffer, separated by SDS-PAGE, and transferred to Immobilon-P (Millipore). Membranes were dried,

then rewetted using methanol and blocked for 30 min in 5% nonfat milk in TBS (150 mM NaCl, 50 mM Tris-HCl pH 7.4). Membranes were incubated 2 h at room temperature with antiserum against CtrA (1:10,000, (Quon et al, 1996), RcdA (1:10,000, (McGrath et al, 2006), ClpX (1:2,500), SciP (1:10,000, (Tan et al, 2010) or PopA (1:5000, (Duerig et al, 2009). Membranes were washed 4 times for 5 min with TBS/0.05% Tween-20 and incubated with HRP-conjugated anti-rabbit secondary antibodies (Fisher Scientific) at a dilution of 1:5000 in TBS/5% milk for 1 h at room temperature. After washing as above with TBS/0.5% Tween-20, chemiluminescent signals were visualized with Western Lightning (Perkin Elmer) using a Bio-rad Gel Doc XL.

Co-affinity purification

CtrA and CtrA3, each with an amino-terminal hexahistidine tag, were incubated with 20 µl Ni-NTA agarose beads (Qiagen) at 4°C for 1 hour in 1xCAP buffer (25 mM Hepes, pH 7.6, 50 mM KCl) with 10 mM imidazole. After washing two times with 500 ul of 1xCAP/10 mM imidazole, the beads were resuspended using 1xCAP buffer/25 mM imidazole and containing the indicated prey proteins. Assays that included ClpX also included 250 µM ATPγS, and 20 µM cdG was included where indicated. After incubating for one hour, the beads were washed twice with 500 µl CAP buffer with 25 mM imidazole. Bound proteins were eluted with 100 µl of CAP buffer containing 500 mM imidazole. Eluted proteins were separated by SDS-PAGE and stained with Lumitein fluorescent protein stain (Biotium) according to manufacturer's instructions. Gels were visualized using a Bio-rad Gel Doc XL, and band intensities were quantified using Imagelab 4.0 software.

Microscopy

Caulobacter cells were immobilized on agarose pads (1% w/v agarose in M2G medium). Images were acquired using a Nikon Eclipse 80i microscope with a PlanApo 100×/NA 1.40 objective and a Cascade 512B camera (Roper Scientific), driven by Metavue software (Universal Imaging). EYFP fusion proteins were visualized using Chroma filter set 41001.

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Chapter 4: Discussion and synthesis

CpdR, RcdA, PopA and c-di-GMP are each necessary, and together are sufficient to accelerate the rate of CtrA proteolysis by ClpXP. The observation that accessory factors dramatically reduce the K_M of the proteolytic reaction without significantly altering the turnover rate is consistent with a traditional adaptor mechanism. However, published examples of adaptors are generally single proteins (Ades, 2004; Battesti & Gottesman, 2013). This report presents the first evidence of a multiprotein complex functioning as an adaptor to promote proteolysis. The mechanism that regulates the activity of this adaptor is also novel. There are multiple examples of adaptor activity being regulated by physical interaction with an anti-adaptor protein. For example, in *Bacillus subtilis*, the small protein ComS binds to the adaptor MecA during periods of nutrient deprivation. This disrupts an interaction between MecA and the proteolytic substrate ComK, thus stabilizing ComK and leading to development of competence (Persuh et al, 1999). In this example, the substrate is stabilized when the adaptor protein is bound to its regulator. Additionally, autophosphorylation of the *B. subtilis* adaptor McsB and phosphorylation of the unfoldase ClpC are required for proteolysis of CtsR in response to heat shock. In *E. coli* RssB is an adapter that promotes the proteolysis of the general stress response sigma factor RpoS. Three anti-adapters, IraP, IraD and IraM inhibit the RssB activity response to various signaling inputs. One of these signals is binding of IraP or IraD to the small molecules guanosine pentaphosphate or tetraphosphate ((p)ppGpp)(Battesti & Gottesman, 2013). Here we describe a unique mechanism for the regulation of a proteolytic adaptor in which direct binding of the small molecule c-di-GMP is required for the function of a multiprotein adapter complex.

Residues in α1 of CtrA that are co-conserved with CpdR and RcdA play a role in the regulated proteolysis of CtrA. Substituting residues 9-31, comprising α1 and β2, of CtrA for the corresponding structural features in a chimera composed of the CzcR receiver domain fused to the last 15 amino acids of CtrA renders this otherwise stable protein susceptible to cell cycle-regulated proteolysis. Conversely, mutating three residues in α1 of CtrA to the corresponding amino acids in the CzcR sequence resulted in stabilization of CtrA3 RD+15 at the SW-ST cell transition. *In vitro* analysis revealed that CtrA3 is defective specifically in ClpXP-mediated proteolysis that is stimulated by the accessory factors. The rate of CtrA3 degradation was reduced relative to wild-type CtrA in the presence of accessory factors, but there was no significant difference in the basal rates of CtrA and CtrA3 proteolysis when accessories were absent. The normal cell cycle-dependent polar localization of CtrA was abolished in YFP-CtrA3 RD+15. This defect in localization would be predicted for a CtrA3 variant that is unable to form physical interactions with accessory proteins, since cells lacking PopA, RcdA or CpdR fail to localize CtrA.

The physical interactions observed between CtrA and the accessory proteins PopA and RcdA are consistent with an adaptor mechanism. Wild-type CtrA, but not the degradation-defective CtrA3, was competent to interact with with PopA and RcdA *in vivo*. Likewise, RcdA and PopA interacted directly with CtrA in reactions using purified proteins. Strikingly, the interaction between PopA and CtrA was strictly dependent on c-di-GMP, and RcdA interacted less strongly with CtrA3 then with wild-type CtrA. We also confirmed a previously reported direct interaction between PopA and RcdA (Duerig et al, 2009). Together these data suggest that the interaction between PopA and RcdA is not dependent on c-di-GMP, but when PopA is bound to c-di-GMP a trimeric complex forms between RcdA, PopA and CtrA. We infer based on the acceleration of proteolysis that occurs in the presence of the accessory proteins, that formation of

this complex likely leads to the recruitment of CtrA to the proteolytic pore. However, at this time we cannot rule out a mechanism in which the interaction between PopA, RcdA and CtrA alters the conformation of CtrA thereby increasing its affinity for the protease. Our model predicts that there is a direct physical interaction between PopA or RcdA and ClpX. Future studies will determine whether such an interaction occurs.

Of the three accessory proteins, only CpdR has been previously shown to function as an adaptor. CpdR binds directly to both ClpX and the phosphodiesterase PdeA, and unlike CtrA, PdeA is absolutely dependent on unphosphorylated CpdR for *in vitro* degradation by ClpXP. Though not required for basal CtrA proteolysis, CpdR is necessary in its unphosphorylated state for stimulated degradation of CtrA (P. Chien, personal communication). At this time it is unclear whether CpdR participates directly in the formation of the adaptor complex that regulates CtrA proteolysis, or if it instead allosterically regulates ClpX, inducing a conformation that is receptive to the PopA/RcdA adaptor complex. Work is ongoing to identify the direct physical interactions that govern CpdR function.

The complete mix of accessory factors increases the rate of CtrA proteolysis in the presence of inhibitors of CtrA proteolysis. Consistent with previous reports, SciP and a P*pilA* DNA fragment protect CtrA from degradation by ClpXP alone, radically increasing CtrA stability *in vitro*. Addition of the accessory factors to reactions containing SciP and P*pilA* dramatically accelerated CtrA proteolysis. These results suggest that the accessory factors can either prevent interactions among CtrA, P*pilA*, and SciP that slow CtrA degradation, or they can disassemble these protein-DNA complexes. Future studies will address the mechanism by which the accessory factors overcome this inhibition of CtrA proteolysis.

Since CtrA degradation is accelerated by the accessory factors in reactions lacking SciP and P*pilA*, the role of the accessory factors is not limited to preventing or disrupting the formation of inhibitory complexes. We propose a model in which CtrA interacts directly with the axial pore residues of ClpX via its C-terminal AA residues (J. Zik, personal communication). This interaction is sufficient to account for the basal rate of CtrA proteolysis by ClpX *in vitro*. When PopA is bound to cdG, PopA and RcdA bind to the receiver domain of CtrA (specifically α1) and to either ClpX itself or to CpdR which is known to interact independently with ClpX (Rood et al, 2012). These interactions create an adaptor which increases the effective concentration of the CtrA C-terminus near the pore residues of ClpX, stimulating degradation.

In the presence of SciP and DNA fragments to which CtrA binds, we propose that the ClpX interaction motif at the C-terminus of CtrA is less accessible to the protease or, by virtue of its conformation, has a lower affinity for the protease. In this situation, binding of PopA-cdG and RcdA to the receiver domain of CtrA may stabilize a conformation of CtrA that is less likely to interact with DNA and SciP. Alternatively, the accessory factors may alter the conformation of CtrA within its complex with DNA and SciP that makes the C-terminal motif more accessible to ClpX. Finally, the entire proteolytic complex of ClpXP, CpdR, RcdA, and PopA-cdG may first extract CtrA from the DNA-SciP complex prior to degradation, similar to the extraction of the phage protein MuA from transposition complexes (Levchenko et al, 1995). Understanding precisely the structural changes that mediate this cdG-dependent mechanism of stimulated proteolysis will be a rich field for future research.

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