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Regulatory Cohesion of Cell Cycle and Cell Differentiation through Interlinked Phosphorylation and Second Messenger Networks

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SUMMARY

In *Caulobacter crescentus*, phosphorylation of key regulators is coordinated with the second messenger cyclic di-GMP to drive cell-cycle progression and differentiation. The diguanylate cyclase PleD directs pole morphogenesis, while the c-di-GMP effector PopA initiates degradation of the replication inhibitor CtrA by the AAA+ protease ClpXP to license S phase entry. Here, we establish a direct link between PleD and PopA reliant on the phosphodiesterase PdeA and the diguanylate cyclase DgcB. PdeA antagonizes DgcB activity until the G1-S transition, when PdeA is degraded by the ClpXP protease. The unopposed DgcB activity, together with PleD activation, upshifts c-di-GMP to drive PopA-dependent CtrA degradation and S phase entry. PdeA degradation requires CpdR, a response regulator that delivers PdeA to the ClpXP protease in a phosphorylation-dependent manner. Thus, CpdR serves as a crucial link between phosphorylation pathways and c-di-GMP metabolism to mediate protein degradation events that irreversibly and coordinately drive bacterial cell-cycle progression and development.

INTRODUCTION

The development of all living organisms depends on the generation of specialized cells in appropriate numbers. This requires tight regulation of proliferation-differentiation decisions by integrating cell-fate determination processes with replication and cell division. For example, embryonic development and tissue homeostasis requires a strict balance between cells in a multipotent, self-renewing state and differentiated nonreplicative cells (Yadirgi and Marino, 2009). Just how differentiation and prolifer-

ation processes are coordinated remains poorly understood (Bateman and McNeill, 2004; Caro et al., 2007). Many bacteria use complex developmental strategies to optimize their survival. Like their eukaryotic counterparts, bacteria tightly coordinate morphogenetic programs with growth and division, be this to facilitate the transition between a replicative and a terminally differentiated cell form (Errington, 2003; Flårdh and Buttner, 2009; Kaiser, 2008) or to couple cell differentiation to cell proliferation (Curtis and Brun, 2010). In the gram-negative bacterium *Caulobacter crescentus* an asymmetric division produces two daughters with distinct morphologies, behavior, and replicative potential, a motile swarmer cell and a sessile stalked cell. Whereas the newborn stalked cell immediately enters S phase to initiate chromosome replication, the swarmer cell inherits a chromosome that remains quiescent for an extended period termed the G1 phase. Concurrent with the morphological transition of the swarmer cell into a stalked cell, the replication block is suspended and cells proceed first into S phase to double their chromosomes and then into G2 phase to undergo cell division.

C. crescentus uses two-component phosphorylation systems to integrate cellular asymmetry with the processes that determine temporal progression through the division cycle. One of the key components of *C. crescentus* development and cell-cycle control is the essential response regulator CtrA. CtrA functions as a transcription factor for more than 100 cell-cycle genes and acts as repressor of replication initiation by directly binding to the chromosomal origin of replication (Cori) where it occludes replication initiation factors (Laub et al., 2002; Laub et al., 2000; Quon et al., 1998). Upon S phase entry, activated CtrA~P is eliminated by two redundant mechanisms, dephosphorylation, and proteolysis (Domian et al., 1997; Domian et al., 1999), thereby freeing the Cori and enabling replication to start. Cell cycle-dependent degradation of CtrA (Chien et al., 2007; Jenal and Fuchs, 1998) entails a specific spatial arrangement of the proteolytic machinery. During the G1-S transition, CtrA and the ClpXP protease both transiently localize to the incipient stalked cell pole where CtrA is degraded (McGrath et al., 2006; Ryan et al., 2004). Polar localization of ClpXP and CtrA are highly regulated

processes, each of which is governed by dedicated localization factors. ClpXP localization requires CpdR, a single domain response regulator that itself sequesters to the incipient stalked pole subject to its phosphorylation state (Iniesta et al., 2006). CpdR is kept in an inactive, phosphorylated form by the CckA-ChpT phosphorylation cascade that also activates CtrA, thereby coupling CtrA activity and stability (Biondi et al., 2006). Delivery of CtrA to the same pole requires PopA, a protein that sequesters to the incipient stalked pole upon binding of the second messenger cyclic di-GMP (Duerig et al., 2009). PopA activation and localization coincides with an upshift of c-di-GMP during G1-S transition (Christen et al., 2010; Paul et al., 2008), arguing that periodic fluctuations of the second messenger are an integral part of the *C. crescentus* cell-cycle clock. The only diguanylate cyclase (DGC) known to modulate c-di-GMP levels during the cell cycle is the response regulator PleD, which is inactive in swarmer cells but is phosphorylated during differentiation to set off pole morphogenesis (Paul et al., 2008; Paul et al., 2004). Although PleD and PopA are activated simultaneously during the cell cycle, genetic analysis failed to demonstrate PleD interference with PopA-mediated CtrA degradation (Duerig et al., 2009). Hence, although c-di-GMP controls both S phase entry and pole development it is unclear how the second messenger serves to connect these processes and how it integrates with the CckA-ChpT-CpdR phosphorelay, implicated in modulating ClpXP localization.

Here, we isolate critical components of the c-di-GMP regulatory network in *C. crescentus* and uncover the molecular principles that serve to coordinate development and cell-cycle progression. In particular, we identify the phosphodiesterase PdeA as a key regulator of the swarmer cell program. PdeA is limited to the swarmer cell where it antagonizes the activity of the diguanylate cyclase DgcB and blocks differentiation. During the G1-S transition PdeA degradation by ClpXP unleashes DgcB activity coincident with PleD activation. DgcB and PleD together establish the sessile stalked cell program, driving PopA-mediated CtrA degradation to orchestrate pole morphogenesis with the underlying cell cycle. Finally, we demonstrate that CpdR directly controls PdeA degradation by acting as a phosphorylation-dependent adaptor protein for the ClpXP protease. These experiments reveal CpdR as a central component of an interwoven network of phosphorylation, protein degradation, and c-di-GMP-mediated reactions that is designed to coordinately drive cell differentiation and cell-cycle progression.

RESULTS

The Phosphodiesterase PdeA and the Diguanylate Cyclases PleD and DgcB Coordinately Control *C. crescentus* Development

The DGC PleD and the c-di-GMP effector protein PopA have been identified as regulatory components involved in *C. crescentus* pole morphogenesis and cell-cycle progression, respectively (Duerig et al., 2009; Paul et al., 2004). To identify additional proteins of the c-di-GMP network involved in *C. crescentus* cell-cycle control, we individually deleted genes encoding potential diguanylate cyclases and phosphodiesterases and analyzed the resulting mutants with respect to their

morphological features and cell type-specific behavior. Deletion of one of the selected genes, *pdeA* (CC3396), resulted in a severe loss of motility and an increased propensity to attach to surfaces, a phenotype generally associated with increased levels of c-di-GMP (Hengge, 2009) (Figure 1A). Consistent with these observations, the *pdeA* gene codes for an active EAL phosphodiesterase (Christen et al., 2005) and cells lacking PdeA had increased concentrations of c-di-GMP (Figure 1A).

Although *pdeA* mutants were unable to spread on semisolid agar plates, they assembled a flagellum and were motile in liquid media (data not shown). This is reminiscent of c-di-GMP mediated flagellar motility control in *Escherichia coli* (Boehm et al., 2010) and argued that PdeA reduces c-di-GMP to modulate the cell-cycle interval reserved for motility and chemotaxis. The increased surface attachment of mutants lacking PdeA correlates with an increased number of cells exhibiting a polar holdfast (Figure 1A). In agreement with this, *pdeA* mutants assembled holdfast prematurely resulting in newborn swarmer cells that ectopically express an adhesive organelle (Figure 1B). These results suggested that PdeA is a phosphodiesterase specifically required for the motile swarmer cell program and that it acts by delaying the transition to the sessile cell type.

To identify components interacting with PdeA, we isolated motile suppressors of the *pdeA* mutant. One suppressor allele mapped to gene *CC1850*, which codes for a protein with an N-terminal coiled coil and a C-terminal GGDEF domain. Biochemical studies demonstrated that CC1850 exhibits Mg^{2+} -dependent and Ca^{2+} -sensitive diguanylate cyclase activity and that the protein is a constitutive dimer in vitro (Figure S1 available online). We thus renamed this protein DgcB. A *dgcB* deletion strain showed increased motility and reduced attachment levels (Figure 1A). Deletion of *dgcB* in a *pdeA* background restored motility, attachment and holdfast biogenesis indicating that the two proteins functionally interact to coordinate pole development (Figure 1A). Importantly, *dgcB pdeA* double mutants restored the proper cell-cycle timing of holdfast formation (Figure 1B) indicating that DgcB is active in G1 swarmer cells and responsible for premature holdfast synthesis observed in a *pdeA* mutant. In contrast, PdeA does not seem to functionally interact with the DGC PleD as *pleD pdeA* double and *pdeA* single mutants showed the same motility and holdfast timing defect and attachment of cells lacking both the PdeA and PleD remained as low as observed for *pleD* single mutants (Figure 1). Since PleD is only active in sessile stalked cells (Paul et al., 2008), this raised the possibility that PdeA activity is limited to swarmer cells.

PleD and DgcB are both required for optimal attachment and holdfast biogenesis. Whereas single mutants showed delayed holdfast formation, an intermediate level of surface attachment and intermediate stalk length, strains lacking both DGCs showed markedly reduced c-di-GMP levels and a complete failure to elongate stalks, synthesize holdfast, and attach to surfaces (Figure 1 and Figure S2). Importantly, deleting *pdeA* in mutants lacking both cyclases did not restore attachment, holdfast formation, and c-di-GMP levels (Figure 1A). Together this indicated that *C. crescentus* cell fate determination is governed by the coordinated action of the phosphodiesterase PdeA and the diguanylate cyclases PleD and DgcB.

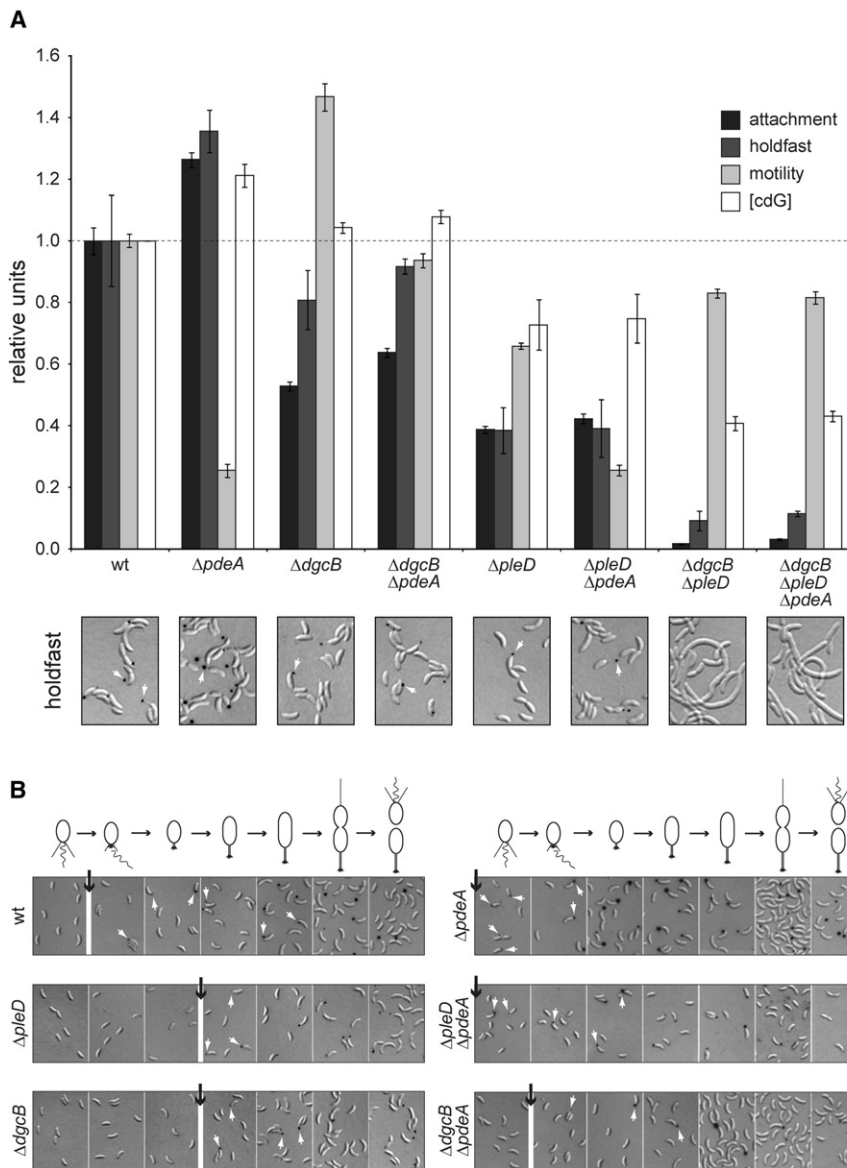


Figure 1. DgcB, PleD, and PdeA Antagonistically Regulate *C. crescentus* Polar Development

(A) Behavior of *C. crescentus* wild-type and c-di-GMP signaling mutants (see also Figures S1 and S2). Surface attachment (black bars), fraction of cells producing a holdfast (dark gray), colony size on motility plates (light gray), and cellular concentration of c-di-GMP (white) are indicated. Representative examples of pictures of DIC images with overlaid fluorescent holdfast staining (black spots) are shown underneath the graph for all strains. Error bars represent the standard error of the mean. Note that *pleD* mutants form smaller colonies on semisolid agar plates, despite of their reported hypermotility phenotype in liquid media (Aldridge et al., 2003; Burton et al., 1997). All deletions were complemented by providing a copy of the respective gene in *trans* (Figure S4 and data not shown).

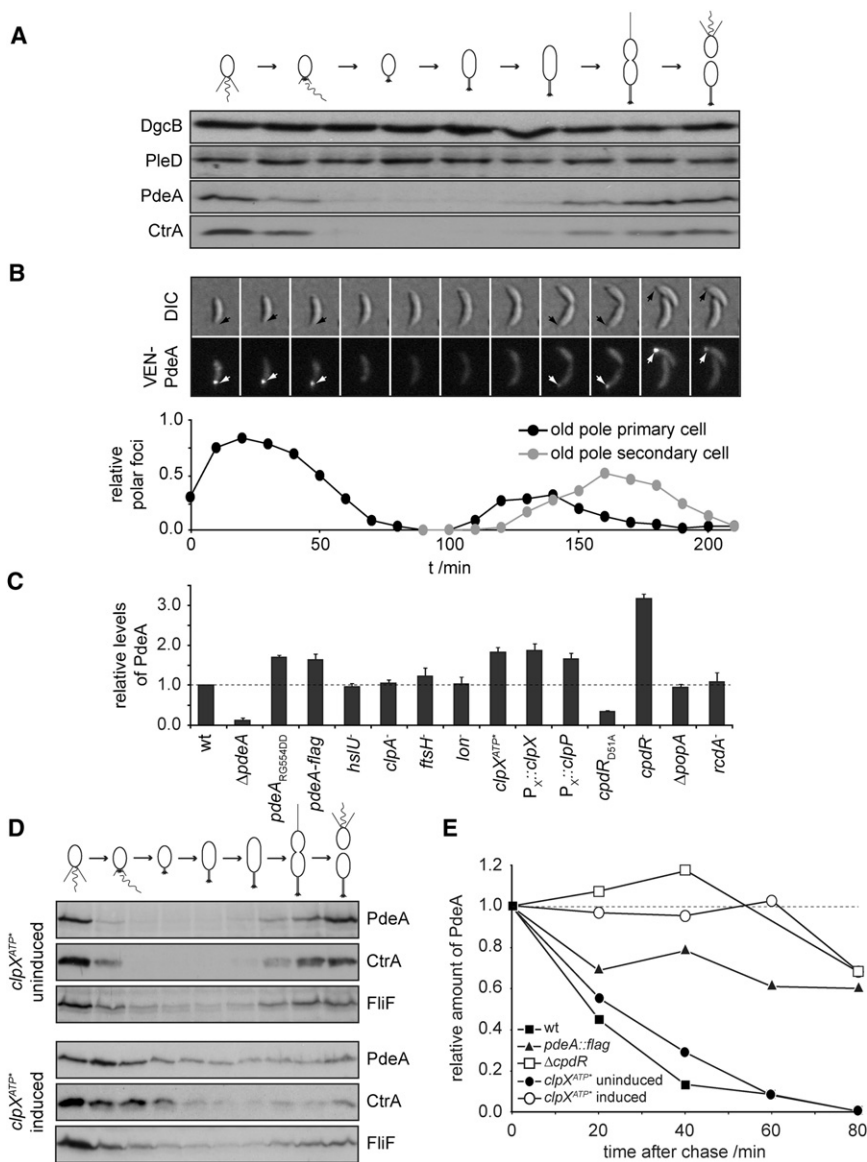
(B) Timing of holdfast synthesis during the *C. crescentus* cell cycle. Fluorescently labeled holdfast structures are shown as in (A). Cell-cycle progression is shown schematically above the micrographs. Small white arrows highlight holdfasts; black arrows indicate the time point of holdfast appearance. See also Figures S1, S2, and S4.

Regulated Proteolysis Limits PdeA to the Motile Swarmer Cell Type

The functional analysis discussed above suggested that DgcB is active both in swarmer and in stalked cells, while PdeA seems to act exclusively in swarmer cells. With the help of polyclonal antibodies raised against purified PdeA and DgcB, we analyzed the distribution of both proteins. Whereas DgcB is present throughout the cell cycle, PdeA was only observed at the very beginning and end of the cell cycle (Figure 2A). This indicated that PdeA is present in swarmer cells but is specifically removed prior to S phase entry, reminiscent of the cell cycle-dependent distribution of CtrA (Domian et al., 1997) (Figure 2A). This was confirmed by the analysis of the cellular distribution of fluorescently tagged PdeA, which transiently localized to the ClpXP occupied stalked cell pole before being cleared (Figure 2B). To identify the protease responsible for PdeA degradation, several

mutants lacking known ATP-dependent proteases were analyzed. PdeA levels were normal in *hslU*, *clpA*, *ftsH*, and *lon* mutants, but levels were increased in cells depleted for ClpP or ClpX and in cells expressing a dominant negative copy of *clpX*, *clpX^{ATP*}* (Potocka et al., 2002) (Figure 2C). A similar increase was observed when the highly charged FLAG tag was fused to the C terminus of PdeA or when the two amino acids at the PdeA C terminus, Arg-Gly, were altered to Asp-Asp. This indicated that, similar to other ClpXP substrates, the

C terminus of PdeA serves as a protease recognition motif (Figure 2C). To substantiate a specific involvement of ClpXP in cell cycle-dependent degradation of PdeA, PdeA levels were analyzed in synchronized cells containing an inducible dominant negative allele of *clpX* (*clpX^{ATP*}*). Induction of *clpX^{ATP*}* in synchronized swarmer cells resulted in a marked stabilization of both PdeA and CtrA during the G1-to-S transition (Figure 2D). In contrast, stability of FliF, a protein specifically degraded by the ClpAP protease during G1-S (Grünenfelder et al., 2004), was not affected. Finally, we determined the overall stability of PdeA by pulse-chase experiments in nonsynchronous populations (Figure 2E). PdeA was degraded rapidly in wild-type cells with a half-life of about 20 min, but, as expected, induction of *clpX^{ATP*}* resulted in complete stabilization of PdeA. Likewise, PdeA-FLAG was considerably more stable than nontagged PdeA (Figure 2E). Together, these data indicated that PdeA is present in



swarmer cells but is specifically degraded during the G1-to-S transition by the ClpXP protease complex.

CpdR, but Not PopA and RcdA, Is Required for Cell Cycle-Dependent Proteolysis of PdeA

Temporal control of CtrA degradation involves several factors controlling the coordinated spatial organization of the ClpXP protease and its substrate. This includes CpdR, which is involved in recruiting ClpXP to the old cell pole upon S phase entry (Iniesta et al., 2006) as well as PopA and RcdA, which are required for the localization of CtrA to the same subcellular site (Duerig et al., 2009; McGrath et al., 2006). To test whether ClpXP degrades PdeA via the same pathway, we analyzed the dynamic distribution of PdeA throughout the cell cycle. An N-terminal fusion of PdeA to the fluorescent protein Venus was found at the flagellated pole of the newborn swarmer cell (Figure 2B, Figure S3A,

Figure 2. Cell Cycle-Dependent Degradation of PdeA by the ClpXP Protease

(A) Immunoblots of synchronized cultures of *C. crescentus* were stained with anti-DgcB, anti-PleD, anti-PdeA, or anti-CtrA antibodies as indicated.

(B) Subcellular localization of PdeA during the *C. crescentus* cell cycle. DIC and fluorescence images of synchronized *C. crescentus* ΔpdeA cells expressing an N-terminal Venus-PdeA fusion protein. Black arrows mark the old cell pole; white arrows indicate polar PdeA (see also Figure S3 and Movie S1). The relative numbers of fluorescent foci at the old pole of the primary cell (black) and the old pole of the newborn secondary cell (gray) are depicted over time (n = 58).

(C) Quantification of cellular PdeA levels as determined by immunoblots from *C. crescentus* wild-type and mutant strains. The dominant negative *clpX* allele (*clpX*^{ΔTP*}) was induced for 3 hr, and ClpX and ClpP depletion strains (P_{xy1}::*clpX*, P_{xy1}::*clpP*) were grown in the absence of xylose for 10 hr prior to sample harvest. Data were normalized to wild-type PdeA levels. Experiments were performed as independent triplicates. Error bars represent the standard error of the mean.

(D) Analysis of ClpX-dependent degradation of PdeA during the cell cycle. Strains containing the dominant negative *clpX* allele (*clpX*^{ΔTP*}) under the control of the xylose inducible promoter P_{xy1} were analyzed during the cell cycle in the presence (induced) and absence (uninduced) of xylose. Specific antibodies were used to determine levels of ClpXP (PdeA and CtrA) and ClpAP substrates (FliF).

(E) PdeA stability as determined by pulse/chase. The dominant negative *clpX* allele (*clpX*^{ΔTP*}) was induced with xylose for 3 hr prior to the radioactive labeling. All samples were normalized to radio-labeled PdeA present immediately after adding the chase solution.

See also Figures S3 and S4 and Movie S1.

and Movie S1). During the swarmer-to-stalked cell transition, VEN-PdeA disappeared from the pole coincident with its proteolytic removal (Figures 2A and 2B, Figure S3A, and Movie S1). VEN-PdeA reappeared in the predivisive cell but upon cell constriction quickly localized to the pole in the stalked compartment, while retaining a dispersed distribution in the swarmer compartment (Figure 2B, Figure S3A, and Movie S1). This suggested that VEN-PdeA redistributes to the ClpXP occupied old cell pole to be degraded upon entry of cells into S phase. Consistent with this, fusion of YFP to the C terminus of PdeA resulted in a protein that mimicked the spatial behavior of VEN-PdeA but persisted at the old cell pole due to shielding of the C-terminal ClpXP degradation motif (Figure S3B).

To test whether any of the regulators involved in CtrA degradation are required for PdeA turnover, we analyzed PdeA levels during the cell cycle in the respective mutant strains. PdeA

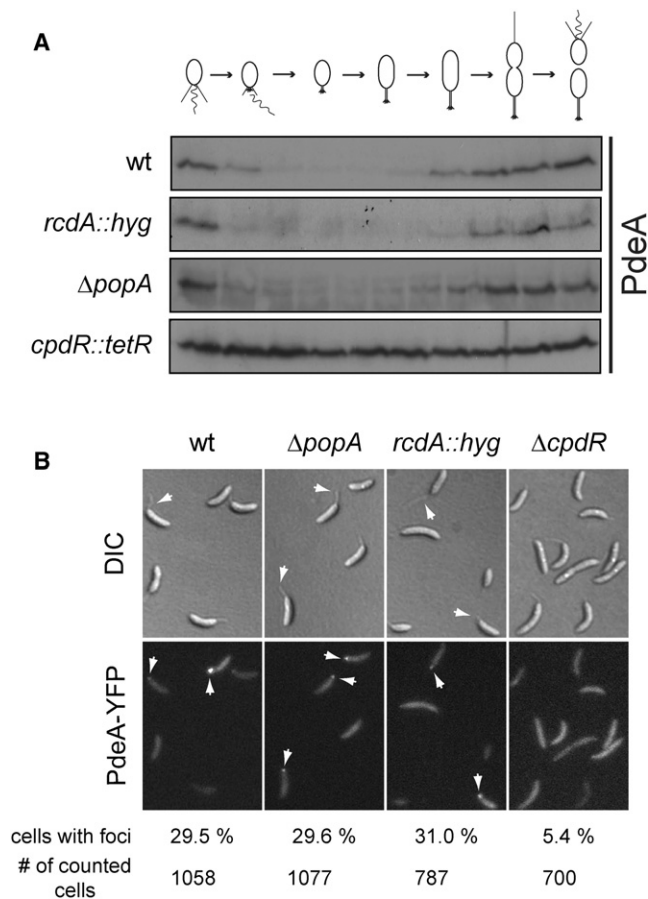


Figure 3. Polar Localization and Degradation of PdeA Requires CpdR

(A) Cell cycle-dependent degradation of PdeA requires CpdR but not PopA or RcdA. Synchronized cultures of *C. crescentus* wild-type and mutant strains were analyzed by immunoblots with anti-PdeA antibodies.

(B) Localization of PdeA to the cell pole requires CpdR but not PopA or RcdA. PdeA-YFP localization was analyzed in *C. crescentus* wild-type and mutant strains (see also Figures S4 and S5). White arrows in the DIC images mark stalked cell poles, and arrows in the YFP channel highlight polar PdeA foci. The relative number of cells with polar foci is shown below the corresponding micrographs.

See also Figures S4 and S5.

was degraded in *rcdA* and *popA* mutants, but was completely stabilized in a mutant lacking CpdR (Figures 2C, 2E, and 3A). Moreover, in the presence of CpdR_{D51A}, a nonphosphorylatable, constitutively active CpdR variant shown to destabilize other ClpXP substrates (Iniesta et al., 2006), PdeA levels were severely reduced (Figure 2C). Because specific factors involved in CtrA localization are not required for PdeA degradation, we tested whether PdeA localization is dispensable for its turnover during G1-S or whether an alternative degradation pathway exists for PdeA degradation. PdeA-YFP was readily detected at the stalked pole in wild-type cells and in mutants lacking PopA or RcdA, but was delocalized upon deletion of *cpdR* (Figure 3B). This change was independent of the ability of CpdR to localize ClpXP as cells depleted of ClpX or ClpP showed normal PdeA localization (Figure S5). Together, these data indicated that polar

localization of PdeA is independent of the protease and of factors necessary for CtrA localization and suggested that PdeA exploits CpdR directly to regulate its localization and degradation.

CpdR Acts as Phosphorylation-Dependent Adaptor for ClpXP-Mediated Degradation of PdeA

During the G1-to-S transition the unphosphorylated form of CpdR localizes to the old cell pole to recruit the ClpXP protease complex (Iniesta et al., 2006). As CpdR is important for PdeA degradation this suggested that this response regulator might initiate PdeA degradation through localization to the ClpXP containing pole. Alternatively, CpdR could act as an adaptor for PdeA delivery to the ClpXP protease. To distinguish between these possibilities, we performed in vitro degradation experiments with purified ClpXP, PdeA, and CpdR. As shown in Figure 4A, ClpXP alone was not capable of degrading PdeA; however, addition of CpdR dramatically increased degradation of PdeA in an ATP-dependent manner. This is in line with the in vivo requirement of CpdR for PdeA degradation by ClpXP and provides a mechanistic framework for CpdR playing a direct and specific role in PdeA recognition by the protease. Interestingly, CpdR mediated degradation of PdeA is enhanced by GTP (Figure S6A), indicating that GTP binding to the allosteric GGDEF domain of the phosphodiesterase might stimulate both PdeA activity (Christen et al., 2005) and its subsequent degradation by ClpXP.

CpdR is regulated during the cell cycle by phosphorylation via the CckA-ChpT phosphorelay (Biondi et al., 2006) (Figure 7A). The finding that CpdR is necessary for PdeA degradation by ClpXP in vitro led us to examine whether this activity depends on the phosphorylation status of CpdR. As shown in Figure 4B, PdeA was readily degraded by ClpXP in the presence of non-phosphorylated CpdR. When CpdR was preincubated with the CckA sensor histidine kinase, the ChpT phosphotransfer protein and ATP, PdeA degradation was strongly reduced. No significant reduction of PdeA degradation was observed when CpdR was preincubated with the phosphorelay mix lacking ChpT (Figure 4B). Importantly, CpdR_{D51A}, a mutant lacking the phosphoryl acceptor site facilitated PdeA degradation even in the presence of the upstream phosphorelay components (Figure 4C). In vivo, the activation of CpdR hinges on its dephosphorylation during the swarmer to stalk transition when PdeA is rapidly turned over. As a more faithful representation of these events, we investigated if specific dephosphorylation of CpdR~P could lead to activation of PdeA degradation in vitro. Phosphorylated CpdR was incapable of delivering GFP-PdeA for degradation; however, addition of a kinase-dead, phosphatase-active mutant of CckA (CckAH322A) (Chen et al., 2009) siphoned phosphates from CpdR~P and rapidly restored PdeA degradation (Figure 4D). Together, these experiments demonstrated that CpdR facilitates ClpXP-mediated PdeA degradation in vitro and that this activity is controlled by its phosphorylation state in a manner that precisely mirrors its regulation in vivo.

Protein Interaction Map of PdeA and Its Antagonist DgcB

The in vitro degradation experiments with PdeA indicated that CpdR acts as facilitator or adaptor protein for ClpXP-mediated

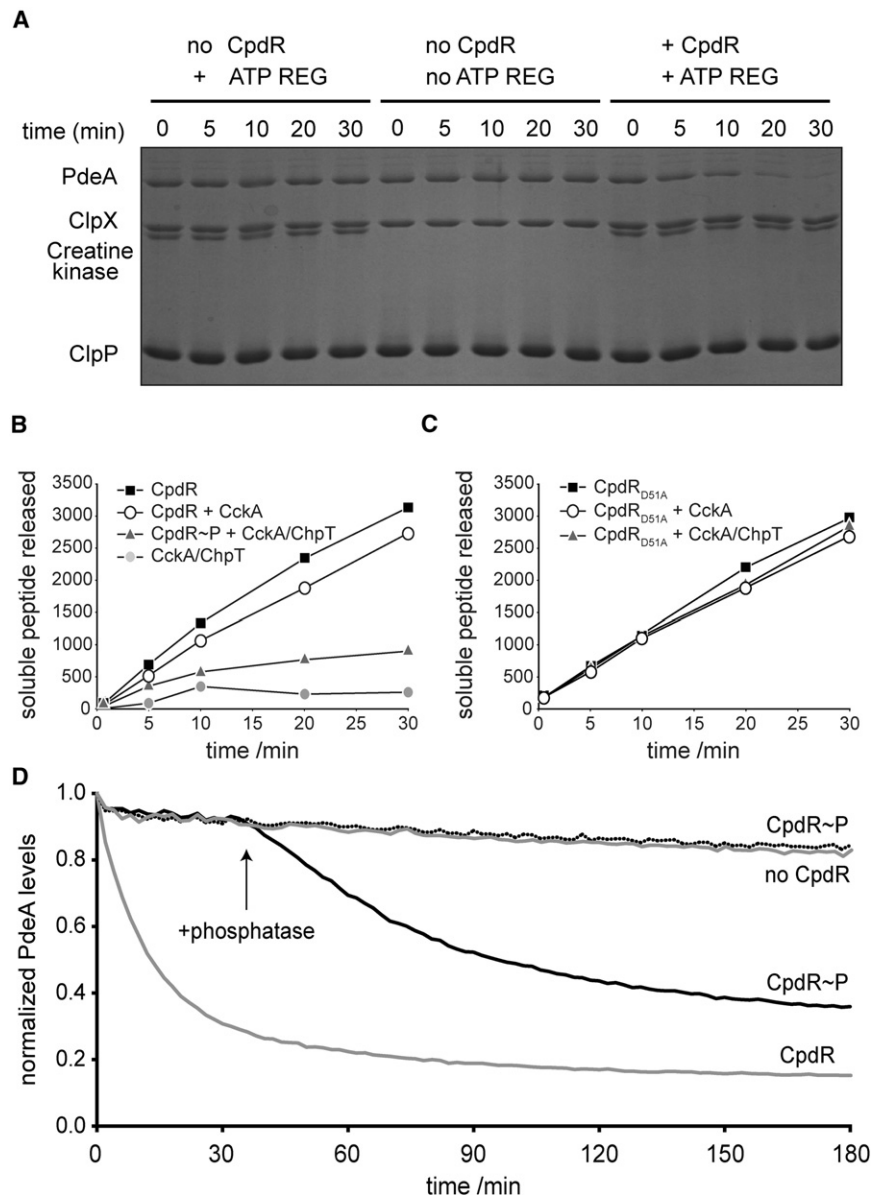


Figure 4. CpdR Is a Phosphorylation-Dependent Adaptor for PdeA Degradation

(A) CpdR is required for ClpXP mediated PdeA degradation in vitro. Purified PdeA (1 μ M) was incubated with 0.4 μ M ClpX and 0.8 μ M ClpP, either in the absence of CpdR, in the presence of 1 μ M CpdR without addition of an ATP regeneration system, or in the presence of both 1 μ M CpdR and an ATP regeneration system.

(B and C) Quantification of the soluble peptide release upon degradation of 35 S labeled PdeA as a function of time. In addition to 2 μ M PdeA, all reactions contain 0.2 μ M ClpX, 0.4 μ M ClpP, 1 mM GTP, and an ATP regeneration system (see also Figure S6).

(B) Only unphosphorylated CpdR stimulates PdeA degradation by ClpXP. CpdR, contains unphosphorylated CpdR without the phosphorelay; CpdR + CckA, contains unphosphorylated CpdR and the histidine kinase CckA without the phospho-transfer protein ChpT; CpdR~P + CckA/ChpT, CpdR was preincubated with CckA/ChpT for 10 min prior to PdeA addition; CckA/ChpT, contains the phosphorelay but no CpdR.

(C) The nonphosphorylatable CpdR_{D51A} stimulates PdeA degradation even in the presence of a phosphodonor. D51A, contains the non-phosphorylatable CpdR_{D51A} variant without phosphorelay; D51A + CckA, contains CpdR_{D51A} and the histidine kinase without the phospho-transfer protein; D51A + CckA/ChpT, contains CpdR_{D51A} and the CckA/ChpT phosphorelay.

(D) Dephosphorylation of CpdR drives PdeA degradation. Phosphorylation of CpdR (CpdR~P) with the CckA/ChpT phosphorelay deactivates delivery of PdeA. Addition of the CckA_{H322A} phosphatase (arrow) reactivates PdeA degradation. Degradation was monitored by following loss of fluorescence of a GFP-PdeA fusion protein substrate.

See also Figure S6.

degradation of PdeA. Such a role implies that PdeA directly interacts with both CpdR and the chaperone subunit of the protease. To examine the direct interactions between CpdR, PdeA, and ClpX we made use of the bacterial two-hybrid system (BacTH) that exploits complementary fragments (T18 and T25) of *Bordetella pertussis* adenylate cyclase (Karimova et al., 1998). As shown in Figure 5A, at least one combination of fusions scored positive for PdeA interaction with ClpX and CpdR, respectively. PdeA also strongly interacted with itself, indicating an oligomerization-dependent activation mechanism. Interestingly, the BacTH assay revealed a strong interaction between PdeA and its functional counterpart DgcB (Figure 5A). Since DgcB failed to interact with any of the other components tested, this interaction appears to be specific. PdeA-DgcB interaction was validated in vitro with purified

DgcB and PdeA proteins (data not shown). Finally, a weak but reproducible interaction was observed between PdeA and PleD (Figure 5A).

These data define a protein-protein interaction network that includes PdeA, its diguanylate cyclase antagonists, and the components mediating cell cycle-dependent PdeA degradation (Figure 5B). The observation that most of the interaction partners of PdeA localize to the cell pole during the G1-S transition led us to analyze the spatial distribution of DgcB during the cell cycle. A functional DgcB-GFP fusion protein (Figure S4) specifically localized to the flagellated pole of newborn swarmer cells (Figure S3C and Movie S2). During cell differentiation, DgcB is rapidly released from the incipient stalked pole only to transiently relocate to the same pole later in the cell cycle. At the same time DgcB also localizes to the pole opposite the stalk, where it persists until the newly born swarmer progeny initiates differentiation into a sessile stalked cell (Figure S3C and Movie S2). Since PdeA is only present in swarmer cells, the two proteins

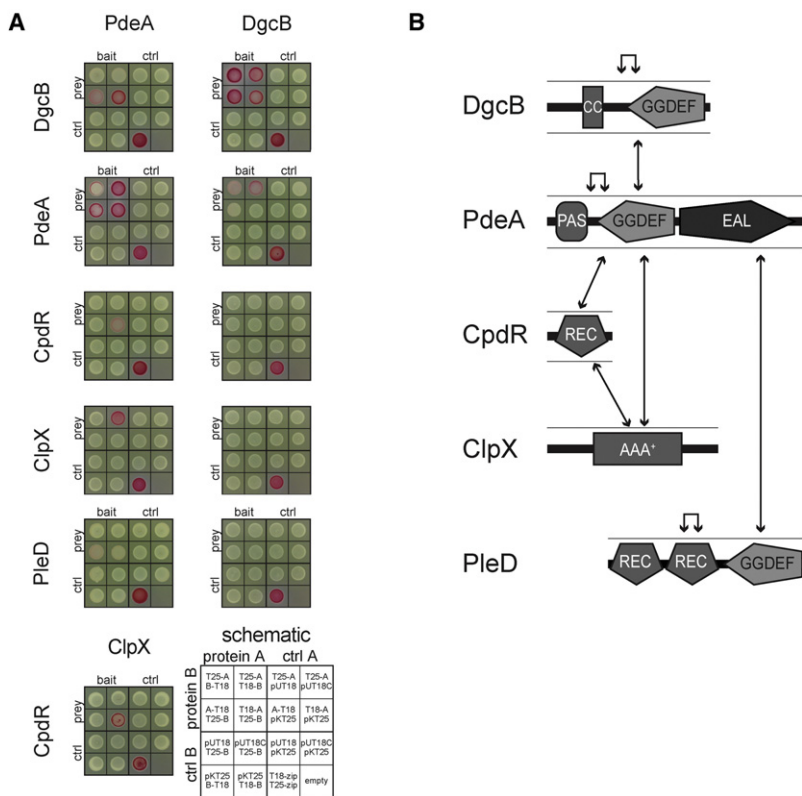


Figure 5. PdeA Interaction Network

(A) Bacterial two-hybrid assays depicting PdeA and DgcB interaction partners (Karimova et al., 1998). The loading scheme is indicated in the lower right corner.

(B) Schematic summary of the interactions shown in (A). Individual protein domains are indicated. Arrows connect interaction partners as defined in (A).

likely interact at the cell pole at this cell-cycle stage. However, polar localization of PdeA and DgcB did not show interdependence (data not shown), arguing that the two proteins use distinct localization mechanisms to reach the pole. Future studies will have to address the role of PdeA and DgcB interaction and co-localization for the activities of these key regulators of *C. crescentus* development.

Simultaneous Activation of a Diguanylate Cyclase and Degradation of a Phosphodiesterase Coordinate Pole Development and S Phase Entry

The epistasis data shown in Figure 1 strongly indicated that DgcB and PleD together are responsible for the increase in c-di-GMP concentration necessary for the motile to sessile transition. The data also suggested that the phosphodiesterase PdeA specifically antagonizes the diguanylate cyclase DgcB, but does not interfere with PleD, because their activities are temporally separated during the cell cycle. This led us to propose that the upshift of c-di-GMP observed during G1-S results from at least two simultaneous events, PleD phosphorylation, and PdeA degradation. To test the contribution of each of these events, we analyzed strains expressing a stable PdeA mutant (*pdeA-flag*) and found a reduction in surface attachment propensity and an increase in cell motility both in *C. crescentus* wild-type and mutants lacking either DgcB or PleD (Figure 6A). As expected from the epistasis experiments (Figure 1), a mutant lacking both diguanylate cyclases was not affected by the *pdeA-flag* allele. Because reduced surface attachment correlates with reduced levels of holdfast formation we examined

the timing of holdfast appearance and found that holdfast formation was substantially delayed in wild-type expressing PdeA-FLAG (Figure 6B), as was loss of motility (data not shown). This is similar to the behavior of a *dgcB* mutant (Figures 1), arguing that loss of DgcB and stabilization of its phosphodiesterase antagonist produces the same developmental delay. Expression of PdeA-FLAG allele had an even more dramatic effect in a mutant lacking PleD, where holdfast formation was barely detectable during the first cell cycle of newly differentiated swarmer cells (Figure 6B). This strongly argues for a model where the simultaneous activation of the diguanylate cyclase PleD and degradation of the phosphodiesterase PdeA are responsible for the correct timing of pole morphogenesis during the *C. crescentus* cell cycle.

We next asked whether interference with PdeA degradation has an effect on CtrA degradation during the G1-S transition. As observed earlier, cell cycle-dependent degradation of CtrA was unaltered in a *pleD* mutant (Figure 6C). Likewise, CtrA degradation was not affected significantly in cells expressing PdeA-FLAG (Figure 6C). However, CtrA degradation during G1-S was severely affected in a *dgcB pleD* double mutant or in *pleD* single mutants expressing PdeA-FLAG (Figure 6C). Inefficient degradation of CtrA during the G1-S transition correlated with an increased overall stability of CtrA as determined in non-synchronized cultures of the respective mutant strains (Figure S7). Together these data suggested that an increase in c-di-GMP concentration promotes both S phase entry and pole morphogenesis in a coordinated fashion. The necessary c-di-GMP upshift results from two simultaneous processes, PleD activation and PdeA degradation, both of which are orchestrated by interlinked phosphorylation pathways that together determine progression of *C. crescentus* cells through the asymmetric division cycle (Figure 7).

The finding that CpdR is required for the degradation of both CtrA and PdeA raised the possibility that during G1-S transition, CpdR could act on CtrA stability solely through modulation of c-di-GMP levels. However, a *cpdR pdeA* double mutant failed to degrade CtrA excluding the possibility that persisting PdeA primarily accounts for stabilized CtrA in the $\Delta cpdR$ background (data not shown). This is consistent with the observation that PopA localization to the incipient stalked pole is not affected in mutants lacking CpdR (Duerig et al., 2009). Finally, expression of the strong heterologous diguanylate cyclase YdeH from

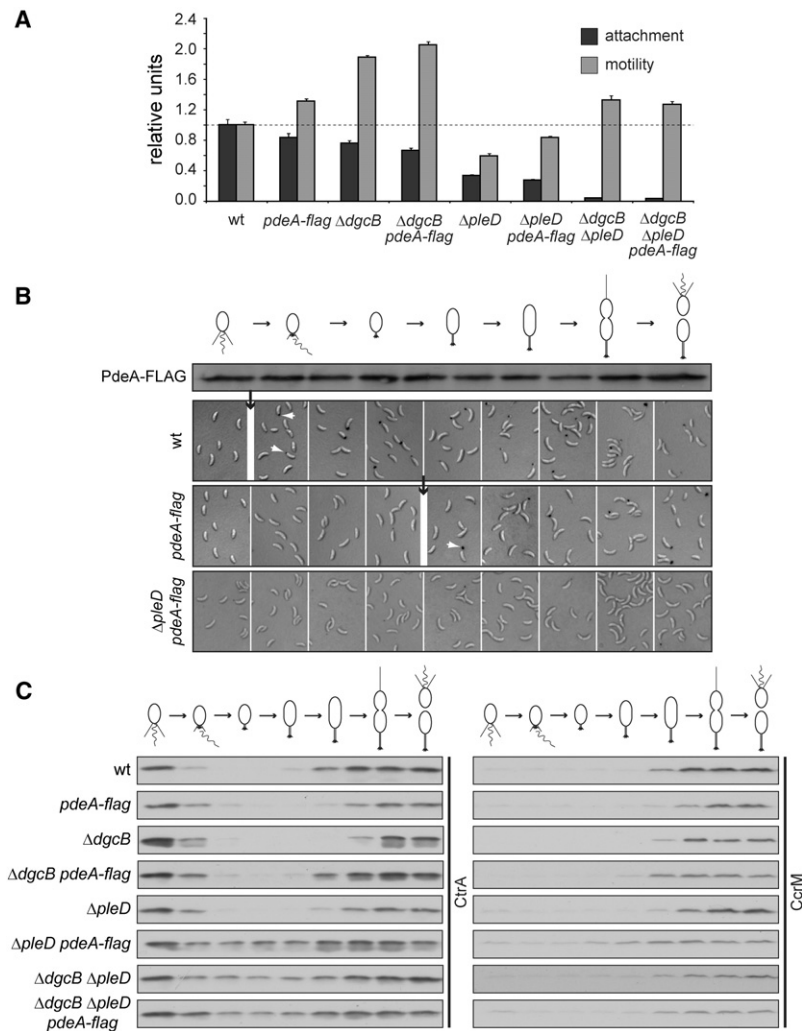


Figure 6. Pole Development and Cell-Cycle Progression Requires PdeA Degradation

(A) Attachment and motility of *C. crescentus* wild-type and mutant strains expressing a stabilized form of PdeA. The mean of eight (attachment) and four (motility) independent colonies is depicted. Data are presented as relative values of the wild-type. Error bars represent the standard error of the mean.

(B) Cell cycle-dependent holdfast formation in strains expressing a stabilized form of PdeA. Small white arrows highlight labeled holdfasts; black arrows indicate the time point of holdfast appearance. Distribution of stabilized PdeA-FLAG during the cell cycle is indicated in the immunoblot stained with anti-PdeA antibodies.

(C) Cell cycle-dependent degradation of CtrA in strains with altered c-di-GMP metabolism. Synchronized swarmer cells of wild-type and mutants were followed throughout the cell cycle. CtrA protein levels were analyzed in immunoblots. Immunoblots with an anti-CcrM antibody are shown as control for cell-cycle progression. See also Figure S7.

E. coli (Boehm et al., 2009) in the *cpdR* mutant failed to restore CtrA degradation (data not shown). From this we conclude that the single domain response regulator CpdR is a bifunctional protein that operates as protease localization factor and at the same time acts as specific adaptor protein for certain ClpXP substrates like PdeA.

DISCUSSION

The coupling of cell morphogenesis and proliferation allows *C. crescentus* to generate specialized cell types to optimize survival. Our results uncover how differentiation and cell-cycle progression are coordinated in this organism by tight cohesion of phosphorylation and c-di-GMP signaling networks. Signaling through this network culminates in successive protein degradation events that robustly and irreversibly commit cells to S phase and to a sessile lifestyle. At the top of this regulatory cascade are sensor histidine kinases, which drive and maintain the oscillatory cell-cycle program. In particular, the CckA/ChpT phosphorelay is responsible for the activation and stabilization of CtrA in swarmer and predivisional cells (Biondi et al., 2006) (Figure 7).

CpdR-mediated delivery of PdeA to the polar ClpXP complex contributes to the upshift in c-di-GMP, the activation of PopA, and thus the recruitment of CtrA to the ClpXP-occupied cell pole (Figure 7).

The phosphate flux through the CckA-ChpT pathway reverses prior to S phase entry, contributing to CtrA and CpdR dephosphorylation and, ultimately, replication initiation. This activity is coordinated with a second phosphorylation pathway involved in G1-S transition that triggers the synthesis of c-di-GMP through phosphorylation of the PleD diguanylate cyclase (Aldridge et al., 2003; Paul et al., 2004) (Figure 7). The cell type-specific activity of this pathway relies on the spatial dynamic behavior of two sensor histidine kinases, PleC and DivJ, which position to opposite poles of the *Caulobacter* predivisional cell and differentially segregate into the daughter progeny (McAdams and Shapiro, 2003). PleC is a phosphatase in swarmer cells but during cell differentiation adopts strong kinase activity and, together with the newly synthesized DivJ kinase, promotes a rapid upshift of c-di-GMP through the activation of PleD (Aldridge et al., 2003; Paul et al., 2004). Reversal of PleC activity is implemented by the essential single domain

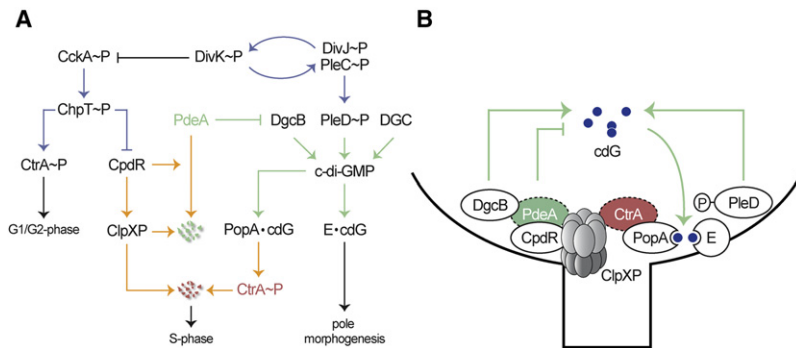


Figure 7. Model for the Integration of Protein Phosphorylation, Degradation, and c-di-GMP Pathways to Coordinate *C. crescentus* Pole Morphogenesis with Cell-Cycle Progression

(A) Regulatory network controlling *C. crescentus* pole morphogenesis and cell-cycle progression. Blue lines indicate phosphorylation reactions, yellow lines indicate processes involved in the regulation of proteolysis, and green lines indicate signaling via c-di-GMP. Postulated diguanylate cyclases (DGC) and c-di-GMP effector proteins (E) are indicated. Red and green protein names specify ClpXP substrates.

(B) Spatial arrangement at the incipient stalked pole of proteins involved in cell-cycle control and development. PdeA and CtrA are recruited to the cell pole by CpdR and PopA. CpdR-mediated degradation of PdeA together with PleD activation increases the concentration of c-di-GMP to activate PopA as well as yet unknown effector-proteins (E) required pole morphogenesis.

response regulator DivK that acts as an allosteric activator of both PleC and DivJ autokinase activity in sessile stalked cells (Paul et al., 2008). At the same time, activated DivK downregulates the CckA-ChpT pathway to contribute to the removal of active CtrA during G1-S transition (Biondi et al., 2006; Tsokos et al., 2011) and through this mechanism helps to adjust the activity of the two cell type-specific phosphorylation pathways during the *C. crescentus* cell cycle (Figure 7).

While DivK links the two phosphorylation networks at the level of the kinase activities, our work reveals CpdR as an additional key component coordinating these two pathways. Although CpdR was originally identified as a polar recruitment factor for the ClpXP protease, we show here that CpdR also serves as a specific adaptor to deliver the PdeA phosphodiesterase to ClpXP prior to S phase entry. Adaptor proteins for AAA+ proteases increase the stringency of substrate selection and alter priorities of target degradation (Kirstein et al., 2009). Before this work, the only characterized adaptor in *C. crescentus* was SspB that delivers *ssrA*-tagged substrates to the ClpXP protease (Chien et al., 2007). However, in contrast to the SspB adaptor case, delivery of PdeA by CpdR is dependent on the phosphorylation status of the adaptor. Although cell cycle-regulated activation is unprecedented for ClpXP adaptors, phosphorylation dependent changes in adaptor function have been described before (Kirstein et al., 2006; Mika and Hengge, 2005). Thus, it appears that coupling of adaptor phosphorylation with adaptor activity is a conserved mechanism to control specific substrate delivery. The observation that CpdR itself is degraded by the ClpXP protease (Iniesta and Shapiro, 2008) suggests that the ClpXP pathway can be rapidly inactivated in S phase by the simultaneous removal of adaptor and substrate protein.

Several lines of evidence argue that DgcB and PdeA function as antagonists and that PdeA, due to its dominance over DgcB, establishes the motile program in swarmer cells. How does PdeA “neutralize” DgcB in swarmer cells? A simple explanation would be that PdeA is catalytically more active than its antagonist. The direct physical coupling of PdeA and DgcB could enhance this effect. Similar to the concept of “metabolic channeling” (Conrado et al., 2008), such an arrangement could increase the efficiency of PdeA control over DgcB by preventing diffusion of

c-di-GMP into the surrounding cytoplasm. This “futile cycle” mechanism, although seemingly wasteful, may provide for a rapid response to environmental signals that can override the internal cell-cycle control. Alternatively, PdeA could directly control DgcB activity through allosteric or inhibitory effects resulting from the simple physical interaction between the two proteins. The observation that a PdeA active site mutation shows the same phenotype as a *pdeA* deletion mutation argues against such a scenario (data not shown). We have shown that PdeA activity is allosterically stimulated by GTP binding to its regulatory GGDEF domain (Christen et al., 2005). While the kinetic parameters suggest that PdeA is fully induced under physiological conditions, certain conditions could lead to a (local) drop in GTP that would be readily transduced into an increase in c-di-GMP by downregulating PdeA. Clearly, we are just beginning to understand how CpdR, PdeA, PleD, PopA and ClpXP collaborate to drive development and cell-cycle progression. Understanding the dynamic nature of these complexes at the cell pole (Figure 7) will be the aim of future work.

EXPERIMENTAL PROCEDURES

More-detailed descriptions of experimental procedures and a list of all plasmids and strains (Table S1) are provided in the Supplemental Experimental Procedures.

Microscopy

Fluorescence and differential interference contrast (DIC) microscopy were performed on a DeltaVision Core (Applied Precision, USA)/Olympus IX71 microscope equipped with an UPlanSapo 100×/1.40 Oil objective (Olympus, Japan) and a coolSNAP HQ-2 (Photometrics, USA) CCD camera. Cells were placed on a PYE pad solidified with 1% agarose (Sigma, USA). Images were processed and analyzed with softWoRx version 5.0.0 (Applied Precision, USA) and Photoshop CS3 (Adobe, USA) software.

Bacterial Two-Hybrid Experiments

Bacterial two hybrid screens were performed according to Karimova et al. (1998). Full open reading frames or gene fragments were fused to the 3' end of the T25 (pKT25), the 3' end of the T18 (pUT18C) or the 5' end of the T18 (pUT18) fragment of the gene coding for *Bordetella pertussis* adenylate cyclase. Two microliter of a MG1655 *cyaA::ftr* culture containing the relevant plasmids were spotted on a MacConkey Agar Base plate supplemented with kanamycin, ampicillin and maltose, incubated at 30°C.

Protein Expression and Purification

E. coli BL21 (DE3) pLys (Stratagene, USA) carrying the *dgcB* expression plasmid were grown in LB medium to an OD₆₀₀ of 0.5 before expression was induced by adding isopropyl 1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.5 mM. Cells were harvested by centrifugation, resuspended in 20 mM HEPES (pH 7.5), 100 mM KCl, 20 mM imidazole running buffer and lysed by passage through a French pressure cell. Clarified crude extract was loaded on a HP HisTrap column (GE Healthcare, UK) attached to an ÄKTApurifier (GE Healthcare, UK). Protein was eluted by raising the imidazole concentration to 500 mM in running buffer. Further purification and buffer exchange were performed by size-exclusion chromatography on a Superdex200 HR26/60 column (GE Healthcare, UK) with 20 mM HEPES (pH 7.5), 100 mM KCl as running buffer. PdeA and CpdR were expressed as C-terminal fusions to a his-tagged SUMO domain in BL21DE3 pLysS cells. Purification of the fusions, cleavage of the SUMO domain, and separation of the cleaved protein were performed as before (Wang et al., 2007). GFP-PdeA was purified via standard Ni-NTA protocols (QIAGEN). Subsequent purification was performed via size-exclusion chromatography (Sephacryl S-300 for PdeA and GFP-PdeA; Superdex-75 for CpdR) with 20 mM HEPES, 100 mM KCl, 5 mM MgCl₂, 5 mM Beta-Mercaptoethanol, 10% Glycerol (pH 7.4) used as the buffer (H-buffer). Monomeric fractions were concentrated and stored at -80°C. Radiolabeled PdeA was produced by labeling with [³⁵S] labeled L-methionine (Wang et al., 2007) and purification as above, with the exception of the gel-filtration step. ClpX and ClpP were purified as before (Chien et al., 2007).

Protease Assay

PdeA degradation was assayed at 30°C in H-buffer (20 mM HEPES, 100 mM KCl, 5 mM MgCl₂, 5 mM Beta-Mercaptoethanol, 10% Glycerol [pH 7.4]). For typical qualitative reactions, 1 μM PdeA was incubated with 1 mM GTP, 0.4 μM ClpX₆, 0.8 μM ClpP₁₄, 1 μM CpdR, and an ATP regeneration system (Chien et al., 2007). Aliquots were removed at indicated times and quenched by addition of SDS loading dye and immediately frozen. Samples were separated by SDS-PAGE and stained with Coomassie for visualization. For quantitative assays, degradation of radiolabeled PdeA was monitored by measurement of TCA soluble peptides (Wang et al., 2007). Degradation of GFP-PdeA was performed in the same conditions as above with the exception that GFP fluorescence was continuously monitored in 384-well plates with a Spectramax M5 (Molecular Devices). Phosphorylation of CpdR was performed as before (Chen et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, one table, and two movies and can be found with this article online at doi:10.1016/j.molcel.2011.07.018.

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