Cytokinesis signals truncation of the PodJ polarity factor by a cell cycle-regulated protease

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We demonstrate that successive cleavage events involving regulated intramembrane proteolysis (Rip) occur as a function of time during the *Caulobacter* cell cycle. The proteolytic substrate PodJ_L is a polar factor that recruits proteins required for polar organelle biogenesis to the correct cell pole at a defined time in the cell cycle. We have identified a periplasmic protease (PerP) that initiates the proteolytic sequence by truncating PodJ_L to a form with altered activity (PodJ_S). Expression of *perP* is regulated by a signal transduction system that activates cell type-specific transcription programs and conversion of PodJ_L to PodJ_S in response to the completion of cytokinesis. PodJ_S, sequestered to the progeny swarmer cell, is subsequently released from the polar membrane by the membrane metalloprotease MmpA for degradation during the swarmer-to-stalked cell transition. This sequence of proteolytic events contributes to the asymmetric localization of PodJ isoforms to the appropriate cell pole. Thus, temporal activation of the PerP protease and spatial restriction of the polar PodJ_L substrate cooperatively control the cell cycle-dependent onset of Rip.

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Introduction

Proteolysis is integral to the regulatory networks of diverse biological systems (Gottesman, 2003; Jenal and Hengge-Aronis, 2003; Ehrmann and Clausen, 2004). In response to specific cues, proteases can stimulate, modulate, or eliminate the activities of their respective substrates. In the α -proteobacterium *Caulobacter crescentus*, proteolysis contributes to the defined fluctuations of many regulatory and structural proteins over the course of the cell cycle (Figure 1A and B) (Grunenfelder *et al.*, 2001; Ryan *et al.*, 2004; Skerker and Laub,

2004). The swarmer cell cycle begins with a chemotactic swarmer cell, which has pili and a single flagellum at one pole of the cell. After a period of motility, the swarmer cell differentiates into a sessile stalked cell, replacing these polar organelles with a stalk containing holdfast at its tip for surface adherence. The stalked cell prepares for cell division by replicating its chromosome and assembling a flagellum and the pili secretion apparatus at the pole opposite to the stalk. Asymmetric division then produces a stalked cell and a swarmer cell; the swarmer cell synthesizes pili and starts the swarmer cell cycle again, whereas the stalked cell initiates its cell cycle by immediately beginning another round of chromosome replication and division. Timely proteolysis is critical to the normal progression of these orchestrated processes. For example, degradation of the essential CtrA response regulator during the swarmer-to-stalked transition and in the stalked compartment of the dividing cell helps ensure that chromosome replication initiates at those points in the spatial-temporal matrix (Domian *et al.*, 1997; Ryan *et al.*, 2004). By varying the levels of target proteins, proteases can serve as regulatory switches for different physiological states.

The interplay of proteolysis and other regulatory mechanisms drives the developmental program integral to the *Caulobacter* cell cycle (Quardokus and Brun, 2003; Holtzendorff *et al.*, 2004). The polarity determinant PodJ contributes a spatial component to this program (Figure 1A and B) (Wang *et al.*, 1993; Viollier *et al.*, 2002a; Hinz *et al.*, 2003; Lawler *et al.*, 2006). The *podJ* gene encodes a 974-aa protein with a cytoplasmic N-terminal domain, a single transmembrane segment, and a periplasmic C-terminal region. Transcription of *podJ* is controlled by the master regulators CtrA, GcrA, and DnaA, such that full-length PodJ (PodJ_L) is synthesized only in the early predivisional cell (Crymes *et al.*, 1999; Holtzendorff *et al.*, 2004; Hottes *et al.*, 2005). PodJ_L localizes to the incipient swarmer pole (Figure 1A) and recruits factors required for pili biogenesis, including the PleC histidine kinase/phosphatase and components of the pili assembly machinery (Viollier *et al.*, 2002a; Hinz *et al.*, 2003; Lawler *et al.*, 2006). As the cell divides, the periplasmic domain of PodJ_L is degraded, giving rise to a truncated form (PodJ_S) that stays attached to the membrane at the flagellated pole of the progeny swarmer cell (Viollier *et al.*, 2002a; Hinz *et al.*, 2003). PodJ_S is needed for chemotaxis and holdfast formation, presumably because, by analogy to PodJ_L, it recruits polar factors required for those processes (Viollier *et al.*, 2002a; Lawler *et al.*, 2006). PodJ_S is cleared from the cell pole during the swarmer-to-stalked transition, before PodJ_L is synthesized again and localized to the opposite pole (Viollier *et al.*, 2002a; Hinz *et al.*, 2003; Chen *et al.*, 2005). This proteolytic clearance of PodJ_S depends on the functionally conserved membrane metalloprotease MmpA (Chen *et al.*, 2005). MmpA and its homologs, in both prokaryotes and eukaryotes, participate in regulated intramembrane proteolysis (Rip), which releases membrane-bound

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Figure 1 PodJ levels and localization vary over the cell cycle between wild-type (left) and $\Delta perP$ (right) strains. **(A)** Schematic diagrams depict PodJ localization during the *Caulobacter* cell cycle. Red circle indicates PodJ_L, whereas green circle indicates PodJ_S. SW, swarmer cell with polar pili (straight lines) and flagellum (wavy line); ST, stalked cell; PD, predivisional cell. **(B)** Cell extracts from a synchronous population of cells were analyzed for the presence of PodJ_L, PodJ_S, McpA, and CtrA by immunoblots. Samples were taken every 20 min, as indicated above the blots. Timing of the cell cycle corresponds to that depicted in panel A. Molecular mass standards, in kDa, are indicated to the left. **(C)** Cells with *yfp-podJ* replacing the endogenous *podJ* allele were examined by differential interference contrast (DIC) and fluorescence microscopy. Localization of YFP-PodJ in individual cells is represented in the schematic panels with orange circles.

substrates from the membrane and has been adapted as a control mechanism in diverse biological systems (Brown *et al*, 2000; Urban and Freeman, 2002; Weihofen and Martoglio, 2003). In *Caulobacter*, Rip by MmpA contributes to the asymmetric distribution of PodJ: loss of MmpA inhibits PodJ_S degradation and leads to bipolar localization of PodJ isoforms (Chen *et al*, 2005). Thus, the combination of transcriptional regulation, protein localization, and proteolysis restricts the presence of PodJ isoforms to a specific spatial and temporal pattern during the *Caulobacter* cell cycle.

Changes in PodJ's protein levels and localization during the cell cycle correlate with its functions in polar organelle development. PodJ is required to localize the PleC histidine kinase/phosphatase (Viollier *et al*, 2002a; Hinz *et al*, 2003), which, together with the DivJ histidine kinase and the DivK response regulator, constitute the basic components of a signal transduction system that monitors cytokinesis (Figure 2A, left) (Sommer and Newton, 1991; Matroule *et al*, 2004; McGrath *et al*, 2004). In the predivisional cell, the DivJ kinase is located at the stalked pole, where it phosphorylates the DivK single-domain response regulator; diffusion of phosphorylated DivK to the swarmer pole results in its dephosphorylation by PleC at that pole (Hecht *et al*, 1995; Wu *et al*, 1998; Wheeler and Shapiro, 1999; Jacobs

et al, 2001; Lam *et al*, 2003; Matroule *et al*, 2004). Hence, DivJ and PleC act in opposition, spatially and catalytically, on DivK's phosphorylation state between the two cell poles (Matroule *et al*, 2004; McGrath *et al*, 2004). Cytokinesis, resulting in the formation of a diffusion barrier (Judd *et al*, 2003), traps dephosphorylated DivK in the swarmer compartment, leading to induction of a swarmer-specific developmental program in that compartment (Matroule *et al*, 2004). If cell compartmentalization is blocked, dephosphorylated DivK fails to accumulate, and the swarmer-specific program does not initiate (Matroule *et al*, 2004). PodJ's recruitment of PleC to the correct pole is central to the functionality of this DivJ–PleC–DivK signaling system. Null alleles of *podJ* and *pleC* cause similar developmental defects: for example, pili synthesis, a component of swarmer progeny development that occurs after cell division, is abolished in both *pleC* and *podJ* null mutants (Wang *et al*, 1993; Viollier *et al*, 2002a, b; Hinz *et al*, 2003).

We report here that proteolytic processing of PodJ_L to PodJ_S is an integral component of the swarmer development program, initiated by the DivK response regulator after completion of cytokinesis. We also show that the DivJ–PleC–DivK monitoring system controls the expression of a newly identified gene, *perP*, which encodes a periplasmic

Figure 2 Conversion of PodJ_L to PodJ_S depends on compartmentalization, as signaled by DivJ, PleC, and DivK. **(A)** Schematic diagrams depict the subcellular locations of DivK, PleC, DivJ, and PodJ molecules in wild-type (left) and *divK_{D90G}* (right) strains. Cytokinesis occurs in the presence of the essential cell division protein FtsZ, but is inhibited in its absence. Wild-type DivK requires compartmentalization for release from the swarmer pole, whereas DivK_{D90G} bypasses that requirement, allowing the swarmer pole to develop a stalk even when division is blocked (Matroule *et al*, 2004). **(B)** Immunoblots show contrasting PodJ levels in wild-type (left) and *divK_{D90G}* (right) cells in the presence (+) or absence (-) of FtsZ. Asynchronous cultures of cells with *ftsZ* under the control of a chromosomal xylose-regulated promoter were grown in the presence of xylose at 30°C. The cells were then washed and resuspended in media containing glucose or xylose to inhibit or induce FtsZ production, respectively. Samples were taken every hour, starting immediately after the wash.

protease required for efficient truncation of PodJ_L. Cell cycle-dependent transcription of *perP* plays a critical role in regulating its function, as untimely expression of *perP* prevents PodJ_L accumulation and, consequently, inhibits pili formation. In addition, *perP* expression is activated by the CtrA response regulator, reinforcing the hypothesis that CtrA integrates signals from DivJ, PleC, and DivK (Wu *et al*, 1998; Sciochetti *et al*, 2002; Ausmees and Jacobs-Wagner, 2003). Because PodJ is required for PleC localization, polar PleC activity affects *perP* expression, and PerP promotes PodJ_L processing, we propose a novel regulatory mechanism in which a sequential proteolytic pathway involving Rip is under temporal and spatial control.

Results

Proteolytic conversion of PodJ_L to PodJ_S depends on DivK signaling the completion of cytokinesis

Given that proteolytic conversion of PodJ_L to PodJ_S occurs at the time of cell division, and that PodJ_S is the only isoform present in swarmer cells (Viollier *et al*, 2002a; Hinz *et al*, 2003), we asked if PodJ_L processing is a component of the swarmer cell-specific developmental program. First, as determined by Quardokus and Brun (2004), we confirmed that PodJ_L processing depends on cytoplasmic compartmentalization (Figure 2B, left). Steady-state levels of PodJ isoforms were monitored after inhibition of cytokinesis using a strain in which the essential cell division gene *ftsZ* is under the control of a xylose-inducible promoter (Wang *et al*, 2001). In the presence of xylose, cells divided normally, and PodJ levels stayed constant. When xylose was removed, depleting FtsZ and blocking cell division, PodJ_S levels dropped dramatically, suggesting that PodJ_L was no longer being converted to PodJ_S, while any remaining PodJ_S disappeared because it continued to be degraded.

Knowing that the D90G mutation (*divK341* allele) in the DivK response regulator can relieve the cytokinesis dependence of swarmer cell development (Figure 2A, right) (Sommer and Newton, 1991; Hecht *et al*, 1995; Hung and Shapiro, 2002; Matroule *et al*, 2004), we next examined PodJ levels in the *divK_{D90G}* mutant when FtsZ was depleted. In the mutant, levels of PodJ_L and PodJ_S in non-dividing cells remained comparable to the levels in dividing cells (Figure 2B, right), indicating that PodJ_L processing bypassed the requirement for compartmentalization. Thus, proteolytic processing of PodJ_L appears to be a developmental event that occurs in response to DivK signaling the completion of cytokinesis and the creation of separate swarmer and stalked progeny compartments.

The distinct phosphorylation states of DivK in the two progeny compartments, generated by the antagonistic effects of the DivJ kinase at the stalked pole and the PleC phosphatase at the swarmer pole, form the basis of the cytokinesis signal (Matroule *et al*, 2004; McGrath *et al*, 2004). To confirm that the DivJ–PleC–DivK monitoring system regulates PodJ_L processing, we compared steady-state levels of PodJ_L in cells with mutations in *divK*, *divJ*, or *pleC* to that in wild-type cells (Figure 3A, left). In the *divK_{D90G}* and Δ *divJ* mutants, PodJ_L levels were reduced relative to the wild-type standard, as expected: both mutations tilt the equilibrium toward premature initiation of the swarmer cell-specific program, including PodJ_L processing. DivK_{D90G} itself does not localize to the flagellar pole, while dephosphorylated DivK predominates in the absence of DivJ and also fails to localize (Wheeler and Shapiro, 1999; Jacobs *et al*, 2001; Lam *et al*, 2003; Matroule *et al*, 2004). These conditions mimic the situation when dephosphorylated DivK is released from the flagellar pole in the swarmer compartment following cell division (Jacobs *et al*, 2001; Matroule *et al*, 2004).

Figure 3 Proteolytic processing of PodJ is affected in multiple mutants. (A) Immunoblots show that PodJ_L levels in $\Delta divK$, $\Delta divJ$, and $\Delta pleC$ mutants differ from that in wild-type cells, regardless of whether *podJ* is expressed from its native promoter (P_{podJ} -*podJ*) or from a xylose-inducible promoter (P_{xyl} -*podJ*) on the chromosome. Cells were grown at 30°C. (B) Efficient conversion of PodJ_L to PodJ_S requires *perP*. Immunoblot on the left compares steady-state levels of PodJ_L and PodJ_S in wild-type, $\Delta perP$, $\Delta mmpA$, and $\Delta mmpA \Delta perP$ strains. Immunoblot on the right shows PodJ levels in wild-type and $\Delta perP$ strains when they carry a complementing plasmid (+) or the vector alone (–). The complementing plasmid contains *perP* under the control of its own promoter (P_{perP} -*perP*). (C) Constitutive *perP* expression prevents PodJ_L accumulation. *perP* was placed under the control of a xylose-inducible promoter on the chromosome (P_{xyl} -*perP*). Wild-type, $\Delta perP$, $\Delta mmpA$, and $\Delta mmpA \Delta perP$ strains carrying the construct were grown in the presence (+) or absence (–) of xylose and harvested for immunoblot analysis. (D) Schematic diagram depicts sequential proteolytic processing of PodJ, first by PerP, then by MmpA, and finally by an unknown cytoplasmic protease.

On the other hand, the $\Delta pleC$ mutation leads to accumulation of phosphorylated DivK, preventing its release from the flagellated pole and initiation of swarmer cell development (Wheeler and Shapiro, 1999; Jacobs *et al*, 2001; Matroule *et al*, 2004). As a consequence, PodJ_L processing should be inhibited. Indeed, we observed accumulation of PodJ_L in $\Delta pleC$ cells (Figure 3A). This elevated level of PodJ_L was reduced when the $\Delta divJ$ mutation was combined with the $\Delta pleC$ mutation (Figure 3A), because DivJ and PleC act in opposition on DivK. We also compared PodJ_S levels in the various mutants to that in wild-type and $\Delta mmpA$ cells (Figure 3A). As previously reported (Chen *et al*, 2005), PodJ_S degradation was inhibited in the $\Delta mmpA$ mutant,

leading to higher steady-state levels, but it did not differ significantly from wild type in strains with mutations in *divK*, *divJ*, or *pleC*.

To eliminate the possibility that variation in PodJ_L levels resulted from changes in *podJ* transcription, we placed *podJ* under the control of a xylose promoter on the chromosome and examined protein levels when the gene was constitutively induced (Figure 3A, right). Similar results were obtained as when *podJ* was under the control of its own promoter. Therefore, the DivJ–PleC–DivK signaling pathway does not modulate PodJ_L levels by affecting *podJ* expression; rather, it may regulate post-transcriptional events such as proteolytic processing of PodJ_L.

A periplasmic protease participates in PodJ_L processing

We postulated that the DivJ–PleC–DivK system controls the expression of factors that regulate PodJ_L processing. To find such factors, we performed microarray analysis on *pleC*, *podJ*, and *divJ* mutants, comparing their steady-state mRNA levels to that in wild-type cells (Figure 4). We identified a set of 26 genes whose transcript levels appeared reduced in *pleC* and *podJ* and elevated in *divJ* mutants. In addition, the expression patterns of these genes during the *Caulobacter* cell cycle were consistent with participation in the swarmer progeny program (Figure 4) (Laub *et al*, 2000): their mRNA levels were elevated in swarmer cells, dropped during the swarmer-to-stalked transition, and stayed low until the time of cell division. The expression patterns of these swarmer-specific genes were also similar over the course of a modified cell cycle in the $\Delta divK_{D90G}$ mutant (Figure 4). The $\Delta divK_{D90G}$ mutant was isolated as a cold-sensitive extragenic suppressor of a *pleC* mutation (Sommer and Newton, 1991) and, at the restrictive temperature, fails to degrade the master response regulator CtrA and to initiate chromosome replication (Hung and Shapiro, 2002). When $\Delta divK_{D90G}$ swarmer cells were allowed to start the cell cycle synchronously at the restrictive temperature, transcript levels of these swarmer-specific genes stayed elevated, as the cell cycle was stalled due to the continual presence of active CtrA. Only when the cells were shifted to the permissive temperature, allowing CtrA to be degraded and the cell cycle to progress, did the transcript levels drop. Thus, the DivJ–PleC–DivK pathway appears to trigger expression of this set of genes in the swarmer compartment.

One member in particular among the set of DivK-regulated genes was a candidate for controlling PodJ_L processing. The *CC1307* gene encodes a protein with a putative signal sequence or membrane anchor at its N-terminus (Bendtsen *et al*, 2004) and a conserved aspartic protease motif in its periplasmic domain (Marchler-Bauer and Bryant, 2004). As PodJ_L processing involves truncation of its periplasmic domain (see Figure 3D), we suspected that the *CC1307* gene product is the perpetrating protease and named it *perP*, for periplasmic protease of PodJ. To confirm this, we constructed a strain with an in-frame deletion of *perP* and examined its PodJ levels by immunoblot analysis (Figure 3B). Compared to that in wild-type cells, PodJ_L levels were elevated in the $\Delta perP$ mutant, whereas PodJ_S levels were reduced to being almost undetectable, suggesting that conversion of PodJ_L to PodJ_S was inhibited in the $\Delta perP$ mutant. We were able to complement this PodJ_L processing defect in the $\Delta perP$ mutant by introducing a plasmid carrying *perP* under the control of its

Figure 4 Microarray analysis of total RNA in *pleC*, *podJ*, *divJ*, and *divK_{D90G}* mutants shows variations in expression profiles. For the *pleC*, *podJ*, and *divJ* strains, RNA was extracted from asynchronous populations of mutant cells and compared to that of wild-type CB15. Blue indicates a decrease and yellow indicates an increase relative to the CB15 reference. For the *divK_{D90G}* mutant, swarmer cells were isolated and grown at the restrictive temperature (18°C) for 300 min, then shifted to the permissive temperature (33°C) for another 80 min. Samples were taken at the indicated times and compared to reference RNA from a mixed population of wild-type cells grown at 30°C. Expression profiles from the wild-type cell cycle are included for comparison (Laub *et al*, 2000; Hottes *et al*, 2005). For the wild-type and *divK_{D90G}* cell cycles, yellow and blue indicate increase and decrease, respectively, relative to the average expression value for that gene during the cycle. Schematics depict stages of the cell cycle; circle or theta structure in the cell represents quiescent or replicating chromosome, respectively. HK, histidine kinase; RR, response regulator.

own promoter (Figure 3B, right). However, the complementing plasmid reduced steady-state levels of PodJ_L in both wild-type and Δ *perP* strains, raising the possibility that improperly regulated expression of *perP* from the plasmid affects PodJ_L accumulation (see below).

We also examined PodJ levels in a Δ *mmpA* Δ *perP* double mutant and found increased levels of PodJ_L, similar to that in the Δ *perP* mutant (Figure 3B). Notably, PodJ_S levels in the double mutant were similar to that in wild type and intermediate between those in the Δ *mmpA* and Δ *perP* single mutants. We interpret these results as follows: PodJ_L is normally truncated by PerP to produce PodJ_S, which is then cleaved by MmpA and released from the cell membrane for subsequent degradation in the cytoplasm (Figure 3D) (Chen *et al*, 2005). Sequential degradation of PodJ by the PerP and

Figure 5 Pulse-chase analysis shows inhibition of PodJ_L processing in the Δ *perP* mutant. Mixed populations of wild-type or Δ *perP* cells were pulse-labeled with [³⁵S]methionine for 7.5 min and then chased with excess unlabeled methionine and casamino acids. Samples were taken at 15-min intervals after the chase and immunoprecipitated with antibodies to the N-terminal domain of PodJ. Immunoprecipitates were resolved by SDS-PAGE, as shown, to determine the relative levels of PodJ_L. Fraction of PodJ_L remaining over time was plotted on a logarithmic scale (base 2) and fitted according to exponential decay. A representative analysis is shown.

MmpA proteases forms the first two steps of the Rip pathway in *Caulobacter*. In the absence of PerP, conversion of PodJ_L to PodJ_S is inhibited, preventing PodJ_S accumulation. Nevertheless, PodJ_L may still be processed by proteases other than PerP, albeit significantly less efficiently, and this leaky processing may lead to some PodJ_S accumulation when PodJ_S degradation fails to occur in the absence of the MmpA protease.

To demonstrate that PerP participates in the proteolytic processing of PodJ_L, we performed pulse-chase analysis and compared PodJ_L stability in wild-type and Δ *perP* strains (Figure 5). In wild-type cells, newly synthesized PodJ_L stayed relatively stable for about 45 min and then rapidly disappeared, with a half-life of approximately 15 min. As previously reported (Hinz *et al*, 2003; Chen *et al*, 2005), the disappearance of PodJ_L corresponded to the concomitant appearance of PodJ_S, implicating proteolytic conversion. This stability profile is consistent with PodJ_L being synthesized in the early predivisional cell and remaining intact until cytoplasmic compartmentalization (Judd *et al*, 2003), when it is truncated. In the Δ *perP* mutant, PodJ_L was relatively stable for at least 120 min after the chase, with negligible appearance of PodJ_S. These results indicate that PerP is required for the efficient proteolytic conversion of PodJ_L to PodJ_S.

We determined that PerP directly truncates PodJ_L by performing *in vitro* cleavage assays with purified domains of PerP and PodJ_L (Figure 6). The periplasmic domain of PodJ_L (PodJ_{PERI}) was tagged with His₆ at the N-terminus and purified as described previously (Viollier *et al*, 2002a). PerP, without its first 28 amino acids, including a hydrophobic leader sequence, was tagged with His₆ at both termini to generate PerP_{ALS}. PerP_{ALS} was purified and cleavage reactions

Figure 6 PerP cleaves the periplasmic domain of PodJ (PodJ_{PERI}) *in vitro*. **(A)** Schematics depict full-length PerP and PodJ_L and the purified fragments PerP_{ALS} and PodJ_{PERI}. PerP_{ALS} was tagged with His₆ and T7 epitope at the N-terminus and with His₆ at the C-terminus. PodJ_{PERI} was tagged with His₆ at the N-terminus. Numbered arrows point to relevant amino-acid residues. LS, hydrophobic leader sequence; TM, transmembrane segment. **(B)** Reaction mixtures containing purified PerP_{ALS} (lane 1), PodJ_{PERI} (lane 2), or both (lanes 3–5) were incubated at 37°C for 4 (lane 3), 8 (lane 4), or 20 h (lanes 1, 2, 5). Samples were resolved by SDS-PAGE and visualized by Coomassie blue staining. The MW lane contains molecular weight markers, indicated on the left in kDa. Diagram below the gel shows cleavage of PodJ_{PERI} by PerP_{ALS} into PodJ_{PERI}-N and PodJ_{PERI}-C. The PodJ_{PERI}-N band is not visible here but is detectable by silver staining (data not shown).

were carried out as described in Materials and methods. In the presence of PerP_{ALS}, the 33-kDa PodJ_{PERI} was cut into two fragments, PodJ_{PERI}-C (~27 kDa) and PodJ_{PERI}-N (~6 kDa); PodJ_{PERI}-N was not detectable by Coomassie blue staining but was visible by silver staining (data not shown). Cleavage of PodJ_{PERI} appeared to progress linearly with time, and while the assays shown in Figure 6 required long incubation times, there is precedent: *in vitro* cleavage of anti-sigma factor RseA by the DegS periplasmic protease required 4 h (Walsh *et al*, 2003). Furthermore, PerP_{ALS} was purified from inclusion bodies and may have a low fraction of active proteases. We assigned the 27-kDa fragment as the one containing the C-terminus of PodJ_{PERI} for two reasons. First, previous studies suggested that removal of 20–30 kDa from PodJ_L's C-terminus yields PodJ_S (Viollier *et al*, 2002a; Chen *et al*, 2005), and the size of the removed fragment is consistent with that of PodJ_{PERI}-C. Second, we examined *Escherichia coli* cells expressing a PodJ_L construct containing GFP at its N-terminus by immunoblotting (Supplementary Figure S6). The construct was cut into two fragments when the cells coexpressed full-length PerP: one fragment was only detectable with antibodies to GFP, and the other, exactly the same size as PodJ_{PERI}-C, was only detectable with antibodies to the C-terminal domain of PodJ_L. The 6-kDa fragment (PodJ_{PERI}-N) released in the *in vitro* assay, we believe, remains attached to

the transmembrane domain *in vivo*. Our experiments thus suggest that PerP cleaves PodJ_L near the N-terminus of its periplasmic domain to produce membrane-bound PodJ_S and PodJ_{PERI}-C (Figure 3D). PodJ_{PERI}-C is then presumably cleared from the periplasm by other proteases *in vivo*.

PerP expression affects PodJ_L distribution and function

To ascertain that PerP is responsible for PodJ_L processing at a specific time during the *Caulobacter* cell cycle, we monitored PodJ levels over the course of the cycle in the Δ perP mutant (Figure 1B, right). As expected, PodJ_L was the only isoform detected in the mutant, and its level remained relatively constant throughout the cell cycle. This is in contrast to the situation in wild-type cells, where PodJ_L and PodJ_S levels varied in a defined pattern over the cycle (Figure 1B, left). We also examined other proteins whose levels fluctuated in a specific manner over the course of the cell cycle, including the CtrA response regulator (Domian *et al*, 1997) and the McpA chemoreceptor (Tsai and Alley, 2001) (Figure 1B), as well as the PleC kinase/phosphatase and pili components Pila and CpaE (Viollier *et al*, 2002b) (data not shown). Their patterns of cell cycle-dependent variation were the same in both wild-type and Δ perP cells. Thus, PerP appears to participate specifically in the proteolytic processing of PodJ_L.

The *podJ* gene has been shown to be required for pili biogenesis, holdfast formation, and efficient chemotaxis (Wang *et al*, 1993; Viollier *et al*, 2002a; Hinz *et al*, 2003), with PodJ_L specific for pili formation and PodJ_S for holdfast and chemotaxis functions (Viollier *et al*, 2002a). Because *perP* expression and PerP activity appeared to be stringently restricted to a specific time in the cell cycle, coincident with the completion of cytokinesis, we asked if altering the timing of *perP* expression would alter polar organelle phenotypes. Accordingly, we placed *perP* under the control of a xylose-inducible promoter on the chromosome, allowing *perP* to be expressed at all times in the cell cycle. In the presence of xylose, PodJ_L failed to accumulate (Figure 3C) and, as a consequence, pili synthesis failed to occur (data not shown). Thus, the timing of *perP* expression is critical for pili formation. Notably, constitutive conversion of PodJ_L to PodJ_S by PerP did not lead to immediate degradation by MmpA, and PodJ_S degradation was still blocked when MmpA was absent (Figure 3C). These results suggested that PodJ_S degradation is regulated by a mechanism independent of PerP, whereas PodJ_L processing is dependent on regulation of *perP* expression.

Previous analysis of the Δ mmpA protease mutant showed that PodJ degradation contributes to its asymmetric localization during the *Caulobacter* cell cycle (Chen *et al*, 2005). Thus, we predicted that inhibition of PodJ_L processing in the Δ perP mutant would lead to bipolar localization of PodJ_L because PodJ_L at the stalked pole would not be degraded before new PodJ_L is synthesized and localized to the incipient swarmer pole (Figure 1A). Assessment of YFP-tagged PodJ localization in Δ perP cells affirmed this prediction (Figure 1C). Cells expressing *yfp-podJ* as the only form of *podJ* from its native chromosomal locus were examined by fluorescence microscopy. YFP-PodJ localized to both poles in a subset of the Δ perP cells, whereas it was found at only one pole in wild-type cells. However, we did not observe a discernable alteration in polar organelle formation in Δ perP strains (data not shown), suggesting that, as is the case for

the CtrA global regulator (Domian *et al*, 1997), multiple levels of control are operative on PodJ function.

Regulation of *perP* transcription

Because timely expression of *perP* is critical to PodJ_L accumulation and its associated functions, we examined the regulation of *perP* expression more closely. We created a reporter construct using a *lacZ* gene without its own start codon on a low-copy-number plasmid. The *lacZ* gene was transcriptionally fused to an approximately 350-bp sequence upstream of the *perP* gene and translationally fused to the first eight codons of *perP* (Figure 7A). The reporter plasmid was introduced into different strains to assess expression levels by β -galactosidase assays. We first measured β -galactosidase activities in strains with mutations in *divJ*, *pleC*, or *divK* (Figure 7B). In agreement with the microarray analysis, expression of the *perP* reporter construct was significantly lower in $\Delta pleC$ cells compared to wild type; lower *perP* expression correlated with reduced PodJ_L processing in the $\Delta pleC$ strain (Figure 3A). In contrast, expression was higher in the $\Delta divK_{D90G}$ strain at the permissive temperature, consistent with increased PerP activity and reduced PodJ_L accumulation in that strain. Although PodJ_L processing appeared enhanced in the $\Delta divJ$ strain, *perP* expression did not differ significantly from the wild-type level. We do not have a definitive explanation for this, but one possibility is that the $\Delta divJ$ mutation has different post-translational effects on PerP and LacZ. Regardless, PleC and DivJ did appear to act in opposition: expression of the *lacZ* reporter in the $\Delta pleC \Delta divJ$ double mutant was intermediate between those in the $\Delta pleC$ and $\Delta divJ$ single mutants, consistent with the steady-state levels of PodJ_L in those mutants.

The opposing localization of DivJ and PleC is required for correct functioning of the DivJ–PleC–DivK signaling system (Matroule *et al*, 2004; McGrath *et al*, 2004). Because PodJ is necessary for PleC localization to the swarmer pole, deletion of the *podJ* gene disrupts many PleC-controlled functions (Viollier *et al*, 2002a; Hinz *et al*, 2003). Indeed, according to our microarray analysis, the $\Delta podJ$ mutation affected *Caulobacter* transcriptional profile in much the same way the $\Delta pleC$ mutation did, albeit to a smaller magnitude (Figure 4). Examination of *perP* expression using the *lacZ* reporter construct confirmed this requirement for PodJ (Figure 7C). The $\Delta podJ$ mutation reduced *perP* expression to about 43% of wild-type level, whereas the $\Delta pleC$ mutation reduced expression to about 10%. These effects are consistent with the idea that PleC can still dephosphorylate DivK in the $\Delta podJ$ mutant, but the phosphatase activity is no longer concentrated at the swarmer pole. Although normal *perP* expression requires PleC localization by PodJ, it does not require PodJ_L processing by PerP: the *lacZ* reporter construct generated similar activity in the $\Delta perP$ mutant as in wild type (Figure 7C).

Previous studies suggested that DivJ, PleC, and DivK act through the CtrA master response regulator (Wu *et al*, 1998; Sciochetti *et al*, 2002; Ausmees and Jacobs-Wagner, 2003). In addition, data from previous microarray analyses indicated that the cell cycle-dependent expression of *perP* is regulated by CtrA (Laub *et al*, 2000, 2002). To confirm the transcriptional effect of CtrA on *perP*, we measured expression of the *lacZ* reporter in strains with temperature-sensitive mutation in *ctrA* or *cckA*, encoding a kinase that is required for the

Figure 7 *perP* transcription is regulated by signaling proteins that affect cell cycle progression. (A) A *lacZ* gene without its own start codon was translationally fused to the first eight codons of *perP* (as annotated in GenBank) and transcriptionally fused to a 350-bp upstream sequence. The fusion construct was placed on a low-copy-number vector to generate plasmid pJC327. Partial nucleotide sequence of the *perP* promoter is shown. Underlining indicates possible CtrA binding site (Ouimet and Marczyński, 2000), whereas box indicates a candidate methylation site (Chen and Shapiro, 2003). Bent arrow indicates the putative transcriptional start site, based on Affymetrix data (PT McGrath *et al*, unpublished). (B) β -Galactosidase assays indicate variations in *perP* expression in $\Delta pleC$, $\Delta divK_{D90G}$, $\Delta divJ$, and $\Delta pleC \Delta divJ$ backgrounds. Mixed populations of cells carrying pJC327 were grown at 30°C and harvested for analysis. β -Galactosidase activities were normalized according to that in wild-type cells. (C) Expression of the *lacZ* reporter construct was reduced in the $\Delta podJ$ but not the $\Delta perP$ strain. (D) *perP* expression requires active CtrA. Wild-type, *ctrA401* (Ts), and *cckA1* (Ts) cells carrying pJC327 were grown at the permissive temperature (28°C) and then shifted to the restrictive temperature (37°C). Samples were harvested at 0, 2, and 4 h after the shift to measure β -galactosidase activities, which was normalized according to that in wild-type cells at 0 h. (E) CtrA is a positive regulator of *perP* transcription. Expression of the *lacZ* reporter construct was determined in strains carrying an empty vector or a plasmid with *ctrA* under the control of a xylose-inducible promoter (*P_{xyI}-ctrA*). Cells were grown in the presence of glucose and then shifted to media containing xylose. Samples were harvested 0, 2, and 4 h after the shift. Activities were normalized according to that at 0 h in cells carrying the vector alone. Error bars indicate standard deviations.

phosphorylation of CtrA (Jacobs *et al*, 1999, 2003) (Figure 7D). Even before shifting to the restrictive temperature (0 h), *P_{perP}-lacZ* expression in either mutant was substantially lower, less than 10%, compared to wild type.

Shifting to the restrictive temperature only further reduced the level of expression. On the other hand, when *ctrA* was constitutively expressed from a plasmid, expression of the *lacZ* reporter increased (Figure 7E). Therefore, CtrA appears to be a positive regulator of *perP* expression.

Discussion

Results presented here demonstrate that successive cleavage events involving Rip occur as a function of time during a bacterial cell cycle and are dependent on cytokinesis. The first proteolytic event in the series occurs following the establishment of two compartments (Judd *et al*, 2003) in the predivisional cell, when the newly identified PerP protease truncates PodJ_L to PodJ_S at the flagellated pole (Figure 3D). We have shown that this first cleavage step is temporally coordinated with cytokinesis via transcriptional regulation of *PerP*. Thus, integration of the temporal activation of the PerP protease and the spatial restriction of the polar PodJ_L substrate results in the controlled onset of the proteolytic sequence. The membrane metalloprotease MmpA then performs Rip on PodJ_S in the progeny swarmer cell as it differentiates into a stalked cell, releasing PodJ_S into the cytoplasm for further degradation (Figure 3D). The sequential actions of PerP and MmpA on PodJ illustrate how the Rip pathway has been adapted to regulate the asymmetric distribution of a polarity factor over the course of the *Caulobacter* cell cycle.

The proteolytic processing of the polarity determinant PodJ_L to PodJ_S occurs in the swarmer cell compartment of the predivisional cell when the DivJ–PleC–DivK phosphotransfer system signals the completion of cytokinesis. Correct accumulation and efficient truncation of PodJ_L both depend on the timely expression of *perP*, which encodes a periplasmic protease. We have shown both *in vivo* and *in vitro* that PerP clips the periplasmic domain of PodJ_L to produce PodJ_S. The DivJ–PleC–DivK system controls the timing of PodJ_L processing by ensuring that *perP* is transcribed at a specific time in the cell cycle. These results suggest a model of PodJ processing in which PodJ, PleC, and PerP form the components of a regulatory circuit (Figure 8). First, localized PodJ_L recruits the PleC histidine kinase/phosphatase to the incipient swarmer pole (Viollier *et al*, 2002a; Hinz *et al*, 2003; Lawler *et al*, 2006). Second, upon establishment of two compartments prior to cell separation, correct polar PleC localization controls the accumulation of the pili subunit PilA (Viollier *et al*, 2002a) and appears to be responsible for the monitoring system in which cytokinesis activates *perP* expression in the swarmer compartment. We propose that

once a diffusion barrier forms between the two nascent daughter compartments, the phosphorylation level of DivK declines in the swarmer section, where PleC resides, activating *perP* transcription through the CtrA response regulator. Third, PerP converts PodJ_L to PodJ_S in the swarmer cell by proteolytic truncation. Thus, PodJ_L localizes PleC, PleC mediates the onset of PerP expression, and PerP cleaves PodJ_L (Figure 8).

After PodJ_L is converted to PodJ_S, a distinct mechanism appears to regulate the timing of subsequent PodJ_S proteolysis, as suggested by the continued presence of PodJ_S when *perP* was constitutively expressed (Figure 3C). This mechanism probably signals the MmpA membrane metalloprotease to initiate PodJ_S cleavage and release from the membrane during the swarmer-to-stalked cell transition (Chen *et al*, 2005). Notably, the CtrA master regulator is also cleared from the cell during this period of the *Caulobacter* cell cycle (Domian *et al*, 1997). Proteolytic removal of CtrA should stop further expression of *perP*, which is positively regulated by CtrA. Ending production of the PerP protease during the swarmer-to-stalked transition would enable PodJ_L to accumulate when it is synthesized in the early predivisional cell, beginning the cycle again. Therefore, our results demonstrate that regulated, sequential proteolysis contributes to the cell cycle-dependent variations in PodJ_L and PodJ_S levels.

Cell cycle-regulated expression of *perP* is critical for PodJ_L to perform its functions, because constitutive, untimely *perP* expression prevents PodJ_L accumulation and abolishes pili formation. However, the absence of an observable phenotype associated with the Δ *perP* mutation is puzzling. Clearly, PerP-mediated proteolysis contributes to the maintenance of polarity at the molecular level: PodJ loses its asymmetric distribution in the absence of PerP. There are at least two possible explanations for why this loss of molecular asymmetry does not translate into an overt physiological defect. First, although significantly less efficient, some degradation of PodJ does occur when PerP or MmpA (Chen *et al*, 2005) is absent. Residual degradation by other proteases may prevent sufficient PodJ accumulation for interference of cellular functions. The second and more likely explanation is that redundant mechanisms regulate PodJ activity, as is the case for CtrA. For example, inhibition of CtrA proteolysis alone does not prevent cell cycle progression; constitutive phosphorylation of the CtrA response regulator is also needed to have an adverse effect on cell viability (Domian *et al*, 1997). Similarly, post-translational modification of the PodJ protein, other than proteolysis, may modulate its function. PodJ activity may also be regulated via its interacting partners. PodJ requires other proteins, such as PleC and the CpaE pili

Figure 8 Model of PodJ_L processing includes a regulatory loop: (1) PodJ_L localizes PleC to the incipient swarmer pole; (2) compartmentalization, monitored in part by PleC, activates PerP expression; and (3) PerP cleaves PodJ_L.

assembly protein, to effect downstream functions (Viollier *et al*, 2002a), and those interacting proteins may be active or available only at specific times during the cell cycle. The full significance of sequential PodJ proteolysis may be masked until additional mechanisms that regulate PodJ and its downstream effectors are elucidated. However, the elaborate cell cycle-dependent mechanism that controls the sequential proteolysis of the PodJ polarity determinant provides a striking example of the integration of spatial and temporal elements to yield asymmetry.

Materials and methods

Bacterial strains, growth conditions, and molecular biology procedures

C. crescentus CB15, NA1000, and their derivatives were grown in PYE or M2 minimal media with appropriate supplements, as described previously (Chen *et al*, 2005). Unless noted otherwise, cultures were grown to mid-exponential phase at 30°C for subsequent analysis. Standard protocols were used for synchronization, electroporation, bacteriophage Φ Cr30-mediated transduction, and plasmid mobilization from *E. coli* by conjugation (Ely, 1991). Assays to examine chemotaxis ability on semisolid media, pili biogenesis using bacteriophage Φ CbK, and holdfast synthesis by rosette formation have all been reported (Wang *et al*, 1993; Skerker and Shapiro, 2000; Viollier *et al*, 2002a; Chen *et al*, 2005). Standard techniques were used for the cloning, manipulation, and analysis of DNA (Ausubel *et al*, 1988). Detailed descriptions of strains and plasmids are provided in Supplementary Tables S1 and S2.

Immunoblots and pulse-chase analysis

Immunoblotting was performed using standard procedures (Ausubel *et al*, 1988): samples were collected at appropriate times, resolved by SDS-PAGE, and transferred to PVDF membrane for detection. Antibodies that detect CtrA, McpA, FlhF, PleC, CpaE, PilA, GFP, or different domains of PodJ were used as described (Viollier *et al*, 2002a,b; Chen *et al*, 2005). Pulse-chase analysis of PodJ stability was performed according to Chen *et al* (2005), except that the cells were pulse-labeled for 7.5 min and chased with 1 mM methionine and 2 mg/ml casamino acids.

Microarray analysis and β -galactosidase assay

RNA preparation and hybridization to microarrays were performed as described (Holtzendorff *et al*, 2004). Total RNA extracted from

asynchronous cultures of CB15 Δ pleC, Δ pleC_{H610A}, Δ podJ, Δ divJ, or Δ divJ::Tn5 strains were compared to that of wild-type CB15 grown in PYE. For the Δ divK_{D90G} mutant, swarmer cells were isolated, grown in M2G at the restrictive temperature (18°C), and then shifted to the permissive temperature (33°C). Samples from various time points were compared to reference RNA from an asynchronous culture of CB15N grown at 30°C in M2G. Data were filtered and normalized as described by Hottes *et al* (2004) and visualized using Cluster and Treeview (Eisen *et al*, 1998). Supplementary data contains the complete data sets and additional details about data processing. Assays for β -galactosidase were performed according to standard protocol (Ausubel *et al*, 1988), except that incubation with chromogenic substrate was at 25°C.

Microscopy

Live cells expressing YFP-PodJ were placed on 1% agarose pads and examined by differential interference contrast and fluorescence microscopy, as described previously (Viollier *et al*, 2002a; Chen *et al*, 2005).

Cleavage assays

The periplasmic domain of PodJ (residues 675–974) was purified from soluble *E. coli* cell extract as described previously (Viollier *et al*, 2002a). The periplasmic domain of PerP (residues 29–172) was tagged with His₆ and T7 epitope at the N-terminus and with His₆ at its C-terminus, overexpressed in *E. coli*, purified from inclusion bodies under standard denaturing conditions (Qiagen), and dialyzed into 50 mM Na₂HPO₄ (pH 8), 300 mM NaCl, and 5% glycerol. Cleavage reactions occurred at 37°C in 50 mM Na₂HPO₄ (pH 8), 180 mM NaCl, 60 mM KCl, 16.7 mM imidazole, 60 μ M EDTA, and 7.5% glycerol. Each reaction contained 10 μ g of PerP_{ALS} and/or 3 μ g of PodJ_{PERI}. Samples were resolved using 12 or 15% SDS-PAGE and visualized by Coomassie brilliant blue or silver staining (Bio-Rad).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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