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Allosteric Regulation of Histidine Kinases by Their Cognate Response Regulator Determines Cell Fate

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SUMMARY

The two-component phosphorylation network is of critical importance for bacterial growth and physiology. Here, we address plasticity and interconnection of distinct signal transduction pathways within this network. In *Caulobacter crescentus* antagonistic activities of the PleC phosphatase and DivJ kinase localized at opposite cell poles control the phosphorylation state and subcellular localization of the cell fate determinator protein DivK. We show that DivK functions as an allosteric regulator that switches PleC from a phosphatase into an autokinase state and thereby mediates a cyclic di-GMP-dependent morphogenetic program. Through allosteric activation of the DivJ autokinase, DivK also stimulates its own phosphorylation and polar localization. These data suggest that DivK is the central effector of an integrated circuit that operates via spatially organized feedback loops to control asymmetry and cell fate determination in *C. crescentus*. Thus, single domain response regulators can facilitate crosstalk, feedback control, and long-range communication among members of the two-component network.

INTRODUCTION

Asymmetric cell division underlies the fundamental basis for the developmental evolution of organisms. It refers to the capability of stem cells to simultaneously produce a continuous output of differentiated cells and to maintain their own population of undifferentiated cells. The regulation of asymmetric division is achieved by the controlled segregation of basally localized cell fate determinants, which leads to the polarization of the stem cell along its axis (Betschinger and Knoblich, 2004). In the bacterium *Caulobacter crescentus*, asymmetry is established by members of the two-component signaling systems, which control various aspects of bacterial physiology including cell differentiation and virulence. Two sensor histidine kinases, DivJ and PleC, are positioned to opposing poles of the *Caulobacter* predivisional cell (McAdams and Shapiro, 2003). A cylindrical extension of the cell body, the stalk, and an adhesive holdfast occupy the DivJ-marked pole, while the PleC-occupied pole bears a rotating flagellum and adhesive pili (Figure 7A). Upon division the stalked cell re-enters S-phase immediately, whereas the motile-swarmer cell takes advantage of the replication inert G1 phase to spread out before it undergoes reprogramming into a surface adherent stalked cell.

C. crescentus cell fate is implemented by the essential single domain response regulator DivK (Hecht et al., 1995; Matroule et al., 2004). DivK localizes to both poles of the predivisional cell in a phosphorylation-dependent manner, but is released from the flagellated pole after completion of cytokinesis (Figure 7A) (Jacobs et al., 2001). While the DivJ autokinase is the main phosphodonor for DivK and responsible for its sequestration to the cell poles (Lam et al., 2003), the PleC phosphatase activity displaces DivK from the flagellated pole by maintaining DivK∼P levels low in the swarmer cell (Lam et al., 2003; Matroule et al., 2004). Compartmentalization of the DivJ kinase and the PleC phosphatase during cell division results in the sudden reduction of DivK∼P levels in the swarmer cell and the initiation of the swarmer-specific developmental program (Matroule et al., 2004). Conversely, a rapid DivJ-mediated increase of DivK phosphorylation is critical for G1-to-S transition and cell differentiation (Hung and Shapiro, 2002; Jacobs et al., 2001; Wu et al., 1998). Activated DivK has recently been proposed to control cell cycle progression and development via the CckA-ChpT pathway, which regulates the activity of the master cell cycle regulator CtrA (Biondi et al., 2006).

Although it is clear that DivK phosphorylation by DivJ and dephosphorylation by PleC are vital for *C. crescentus* cell cycle control and development, the significance of the spatial behavior of DivK and its molecular role remain unclear. Here we propose that DivK together with PleC and DivJ form the core of an integrated regulatory circuitry that operates via spatially organized cellular feedback loops. We show that DivK directly interacts with both polar proteins to strongly boost their kinase activities. By switching PleC from the phosphatase into the autokinase mode and by forming a strong positive feedback loop with the

DivJ autokinase, DivK effectively and robustly mediates G1-to-S transition.

One of the readouts of the DivK-driven network is the synthesis of the second messenger cyclic di-GMP via the activation of the response regulator PleD (Aldridge et al., 2003; Paul et al., 2004). PleD phosphorylation results in dimerization-based activation of the C-terminal diguanylate cyclase domain and sequestration of the regulator to the differentiating pole where it directs flagellar ejection, holdfast biogenesis, and stalk formation (Levi and Jenal, 2006; Paul et al., 2007, 2004). Genetic experiments indicated that DivJ, PleC, and DivK are upstream components required for the activation of the PleD diguanylate cyclase (Aldridge et al., 2003; Sommer and Newton, 1991). Consistent with this, in vivo and in vitro experiments had shown a direct role for PleC and DivJ in modulating phosphorylation, diguanylate cyclase activity, and polar localization of PleD (Aldridge et al., 2003; Paul et al., 2004). This indicated that PleC, in addition to functioning as phosphatase in swarmer cells, also plays a role as autokinase and contributes to the stalked cell-specific program via PleD activation. We present in vitro and in vivo evidence that DivK together with the DivJ and PleC kinases serves to activate and sequester the PleD diguanylate cyclase during the G1-to-S transition. DivK contributes to the specific phosphorylation of PleD by acting as a specific and effective enhancer of the PleC and DivJ autokinases. Our findings propose a regulatory role for single domain response regulators in two component signal transduction pathways as diffusible modulators of their cognate sensor histidine kinases.

RESULTS

DivK Stimulates the PleD Diguanylate Cyclase Activity in a PleC-Dependent Manner

To explore the regulatory link between PleD, PleC, and DivK, activation of PleD diguanylate cyclase activity was assayed in vitro in the presence of PleC and DivK. In line with earlier results (Paul et al., 2004) PleC alone only marginally stimulated PleD activity (Figure 1A). Surprisingly, in the presence of DivK or $DivK_{D53N}$, a mutant that cannot be phosphorylated because it lacks the phosphoryl acceptor site, PleD diguanylate cyclase activity was dramatically stimulated (Figures 1A and 1B). Stimulation by DivK wild-type was less effective, presumably because DivK itself can use PleC∼P as phosphodonor (Hecht et al., 1995) and thus can sequester some of the available phosphoryl groups. DivK-dependent stimulation of PleD activity required ATP, PleC autokinase activity, and the PleD phosphoryl acceptor site Asp53 (Figure 1B). In particular, purified $PleC_{F778L}$, a mutant that lacks autokinase activity but shows normal phosphatase activity (Matroule et al., 2004) failed to support DivK-dependent stimulation of PleD. Because PleD is activated by dimerization (Paul et al., 2007), residual diguanylate cyclase activity can be detected at this protein concentration (5 μM) even in the absence of phosphorylation (the K_d for dimerization of nonactivated PleD is 100 μM [Wassmann et al., 2007]) (Figure 1B). It is interesting to note that when all three proteins were present PleD diguanylate cyclase activity decreased below the basal level of nonactivated PleD under conditions that did not allow phosphorylation (no ATP, no PleC autokinase activity) (Figure 1B). Altogether, these experiments demonstrate that the single domain response regulator DivK is able to efficiently stimulate the in vitro activity of PleD and that this activation requires an active histidine protein kinase PleC.

DivK Stimulates PleC Autokinase but Not Phosphatase Activity

The above experiments suggested that DivK activates PleD by interfering with PleC autokinase activity. To test this, PleC autophosphorylation activity was monitored in the presence and absence of DivK. Because PleC readily phosphorylates DivK in vitro (Hecht et al., 1995; Wu et al., 1998), we used $DivK_{D53N}$ to avoid a reduction of PleC∼P by phosphotransfer to DivK. As shown in Figure 2A and Figure S1A (available online), $DivK_{D53N}$ stimulated levels of PleC∼P in a concentration dependent manner. Because the stability of PleC∼P was not affected by DivK (Figure S1B), $DivK_{D53N}$ seems to specifically stimulate PleC autophosphorylation activity. As a consequence of this stimulation, phosphotransfer from PleC to PleD (Figure 2B) as well as PleD dimerization (Figure S2) was increased when both response regulators were present in the reaction. This is consistent with the observed increase in c-di-GMP synthesis in reactions containing PleD, PleC and DivK (Figure 1). Likewise, phosphotransfer from PleC∼P to DivK was stimulated in the presence of $DivK_{D53N}$ (Figure 3A). Stimulation of PleC autokinase activity did not result from a DivK-mediated in vitro artifact (e.g., through DivK assisted folding of PleC), as solubility, quarternary structure, and activity of PleC preincubated with or without $DivK_{D53N}$ was indistinguishable (Figure S3). Finally, we tested if $DivK_{D53N}$ was able to stimulate PleC phosphatase activity. Purified DivK∼P was mixed with PleC in the presence or absence of $DivK_{D53N}$ to assay dephosphorylation rates. In contrast to PleC mediated phosphorylation of DivK, dephosphorylation of DivK was not increased in the presence of $DivK_{D53N}$ (Figures 3A–3C). Rather, the rate of DivK∼P dephosphorylation was reduced in the presence of $DivK_{D53N}$. In conclusion, these experiments provide strong evidence that the response regulator DivK is able to selectively stimulate the kinase but not the phosphatase activity of its cognate histidine kinase PleC.

DivK and PleD Compete for Phosphorylation by the DivJ Kinase

Because DivK and PleD also interact with DivJ, we tested if the response regulators also showed some synergistic behavior with respect to DivJ or if they would compete for DivJ kinase. As shown in Figure 1C, the PleD diguanylate cyclase was activated by DivJ, but the addition of DivK efficiently blocked PleD activation. PleD activation was restored only when DivK was diluted below a molar ratio of 1:10. $DivK_{D53N}$ also reduced DivJ-mediated PleD activation but was less efficient than DivK wild-type (Figure 1C). These results suggested that PleD and DivK compete for the phosphodonor DivJ∼P. This was confirmed by monitoring phosphotransfer from DivJ∼P to the two response regulators. Although DivJ∼P readily served as phosphodonor for PleD in the absence of DivK, phosphoryl groups were transferred exclusively to DivK when both response regulators were present in the reaction mixture (Figure 2D). Altogether, these experiments show that DivK and PleD compete for phosphorylation by the stalked pole specific kinase DivJ, and that in vitro DivJ∼P prefers to transfers phosphate to DivK.

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