Polar Localization of the CckA Histidine Kinase and Cell Cycle Periodicity of the Essential Master Regulator CtrA in Caulobacter crescentus

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The phosphorylated form of the response regulator CtrA represses DNA replication initiation and regulates the transcription of about 100 cell cycle-regulated genes in Caulobacter crescentus. CtrA activity fluctuates during the cell cycle, and its periodicity is a key element of the engine that drives cell cycle progression. The histidine kinase CckA controls the phosphorylation not only of CtrA but also of CpdR, whose unphosphorylated form promotes CtrA proteolysis. Thus, CckA has a central role in establishing the cell cycle periodicity of CtrA activity by controlling both its phosphorylation and stability. Evidence suggests that the polar localization of CckA during the cell cycle plays a role in CckA function. However, the exact pattern of CckA localization remains controversial. Here, we describe a thorough, quantitative analysis of the spatiotemporal distribution of CckA during the cell cycle plays a role in CckA function. We also identify two cis-acting regions in CckA that are important for its proper localization and function. The disruption of a PAS-like motif in the sensor domain affects the stability of CckA accumulation at the poles. This is accompanied by a partial loss in CckA function. Shortening an extended linker between β-sheets within the CckA catalysis-assisting ATP-binding domain has a more severe effect on CckA polar localization and function. This mutant strain exhibits a dramatic cell-to-cell variability in CpdR levels and CtrA cell cycle periodicity, suggesting that the cell cycle-coordinated polar localization of CckA may be important for the robustness of signal transduction and cell cycle progression.

The alphaproteobacterium Caulobacter crescentus provides a model system that has been particularly useful for unraveling the bacterial cell cycle (2, 5, 7, 16, 20, 42). Its life cycle is characterized by an asymmetric cell division that yields two distinct daughter cells, the motile swarmer cell and the sessile stalked cell. The swarmer cell grows into a stalked cell, during which time DNA replication is initiated. The stalked cell then proceeds with cell elongation, polar morphogenesis, cell division, and daughter cell separation (Fig. 1A).

His-Asp phosphorylase systems, which are one of the predominant stimulus response systems in prokaryotic cells (50), play an essential role in coordinating cell cycle progression in C. crescentus (2, 5, 42). The membrane-bound hybrid histidine kinase CckA is involved in two interconnected phosphorylases that regulate the DNA-binding activity of the CtrA response regulator (Fig. 1B). In its active phosphorylated form, CtrA~P inhibits the initiation of DNA replication while regulating the expression of about 100 cell cycle genes, including genes involved in cell division and flagellar and pilus biosynthesis (9, 34, 43, 44). In the first phosphorylase, CckA, via the single-domain histidine phosphotransferase ChpT (1), mediates the phosphorylation of CtrA (25, 26). In the second phosphorylase, CckA, again through the intermediate ChpT, mediates the phosphorylation and thereby the inactivation of the single-domain response regulator CpdR (Fig. 1B) (1, 23). In its unphosphorylated, active form, CpdR promotes the polar localization of the ClpXP protease, leading to the regulated proteolysis of CtrA (23, 28, 38, 45). Thus, the activation of CckA through autophosphorylation both activates CtrA through phosphorylation and prevents the targeted degradation of CtrA through the phosphorylation of CpdR.

The response regulator CtrA is a key factor in driving the C. crescentus cell cycle, and as such its activity is tightly regulated at the transcriptional level and at the posttranslational level (2, 5), leading to the periodicity of active CtrA~P during the cell cycle (Fig. 1A). CtrA~P is present in swarmer cells (8, 25), where it binds to five sites within the chromosomal origin region to inhibit the initiation of DNA replication (44). This inhibition is relieved later through the dephosphorylation and ClpXP-dependent proteolysis of CtrA~P during the swarmer-to-stalked cell transition (8, 28). The proteolysis of CtrA is triggered by multiple proteins and polar events (10, 38, 41), including the localization of unphosphorylated CpdR to the stalked pole, where it helps recruit the ClpXP protease (23). The essential single-domain response regulator DivK also has been proposed to negatively regulate CtrA activity during this swarmer-to-stalked cell transition by affecting CckA and CpdR function (1, 22, 24, 56). CtrA~P reaccumulates in predivisional cells through de novo transcription and then phosphotransfer
CckA signal transduction pathway and its role in cell cycle regulation in C. crescentus. (A) The C. crescentus cell cycle is driven by the periodicity of CtrA-P. When CtrA-P is present, the initiation of DNA replication is inhibited. During the swarmer-to-stalked (SW-ST) cell transition, CtrA is recruited to the old pole and degraded, allowing DNA replication to initiate. CtrA-P reaccumulates later in the ST and predivisional (PD) cell stages due to transcriptional activation and ensuing phosphorylation. The black circle represents the chromosome, while the asterisk shows the late PD stage when cells are compartmentalized due to cytokinesis preceding daughter cell separation. (B) The CckA signal transduction pathway regulates cell cycle progression and polar morphogenesis. Curved arrows between proteins indicate the flow of phosphoryl groups, while straight lines indicate activation or inhibition.

Critical for our understanding of cell cycle regulation in C. crescentus. For instance, a recent report proposes that the polar localization of CckA may be correlated with the activation of CtrA-P and the subsequent inhibition of DNA replication initiation, and conversely the dispersion of CckA from the pole is accompanied by the inactivation of CtrA-P and the subsequent initiation of DNA replication (1). This led to a model in which DivK-P causes the delocalization and inactivation of CckA from the old pole, and this displacement causes CtrA-P inactivation (1). However, this sequence of cell cycle events is incompatible with the original description of the CckA localization pattern during the cell cycle (26).

Here, we report a quantitative analysis of the spatial distribution of CckA during the cell cycle that affects the current models of cell cycle regulation. We also identify two regions within CckA that contribute to the proper polar localization of CckA and show that these regions are important for protein function. Finally, we present data suggesting that the proper polar localization of CckA may be critical for the cell cycle periodicity of CtrA levels, which drives cell cycle progression and polar morphogenesis.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. C. crescentus strains were grown in peptone-yeast extract (PYE complex medium), M2G (minimal medium), M2G+ (minimal medium supplemented with 1% PYE), or M5GG (low-phosphate M5G medium supplemented with 1 mM glutamate) (13, 27, 33) with kanamycin (5 μg/ml), oxytetracycline (1 μg/ml), spectinomycin-streptomycin (50 and 5 μg/ml, respectively), gentamicin (5 μg/ml), glucose (0.2%), or xylose (0.3%) when appropriate. Motility was determined by the ability to form swarm colonies in softagar PYE plates. Sensitivity to PhCk caulophages was determined by spotting PhCk caulophages onto a PYE agar plate inoculated with a C. crescentus culture by the spread-plate method. Plasmids were mobilized from Escherichia coli strain S17-1 into C. crescentus by bacterial conjugation or transformed into C. crescentus by electroporation (13). Strains and plasmid construction methods can be found in the supplemental material.

Synchronized populations of swarmers were obtained using a modification of the technique described by Evinger and Agabian (14). Swarmer cells were isolated from exponentially growing cultures (optical density at 600 nm [OD600] =0.2 to 0.3). Sixteen-milliliter polypropylene polycarbonate round-bottom centrifuge tubes (Nalgene Labware) containing 12 ml culture mixed with 3 ml LUDOX AS-40 colloidal silica (Sigma-Aldrich), adjusted to pH 7 with 1 M HCl, were centrifuged for 30 min at 9,700 × g and 4°C. The swarmer cell bands (the lower of the two bands) were isolated, combined, resuspended with cooled 1× M2 salt buffer (6.15 mM Na2HPO4, 3.9 mM KH2PO4, 9.35 mM NH4Cl) in a 16-ml centrifuge tube, and centrifuged at 4°C for 10 min at 7,700 × g. The pellet containing the swarmer cells was recovered and washed three times with cooled 1× M2 salt buffer and microcentrifuged at 6,000 × g. The swarmer cells were resuspended in the appropriate medium.

Microscopy. Cells were mounted on slides layered with a 1 or 1.5% agarose pad containing M2G or M2G+ (33). Samples for population or time-course images were observed on a Nikon E1000 microscope equipped with a CFI Plan Apochromat 100×/H differential interference contrast (DIC) objective lens (Nikon Instruments, Inc.) or a CFI Plan Apochromat DM 100×/H phase-contrast objective lens (Nikon Instruments, Inc.) and an Orca-ER LCD camera (Hamamatsu Photonics). Samples for time-lapse images were observed on a Nikon E800 microscope equipped with a CFI Plan Apochromat DM 100×/H phase-contrast objective lens (Nikon Instruments, Inc.) and an iXon+ EMCCD camera (Andor Technology). Both microscope systems were driven by MetaMorph software (MDS Analytical Technologies).

Image analysis. Cell identification and fluorescence intensity profile quantification were performed using a custom-made, MATLAB-based program (cell-Tracker). Briefly, this program was used to identify, outline, and determine the centerline of the individual cells in phase-contrast images using bandpass two-dimensional Fourier filtering, morphological opening, and thresholding for the initial guess, followed by a variant of the active contour (snake) model (31). For time-lapse experiments, the contour from the previous frame was used as the...
initial guess of the cell shape. A cell mesh was created that split individual cells into pixel-wide segments along the centerline. The average background fluorescence intensity of cell-free areas was subtracted from the corresponding fluorescence image, and the cell mesh was then applied to the corrected image. The integrated fluorescence signal inside each segment of the mesh was divided by the segment area; since the images were two-dimensional, cell thickness was considered similar among cells and constant along the cell body.

For time-lapse experiments with Cjw2638 cells, the mesh orientation of a cell was manually designated using the localization of DivJ-timer2 as an old-pole marker (55). For time-lapse experiments with Cjw2085 and Cjw2857 cells, the mesh orientation was manually designated using the stalk as an old-pole marker. Cell cycle time-lapse images were analyzed from the start of cell synchronization to the time point when daughter cell separation was discernible (i.e., when daughter cells each possessed two visually distinguishable cell poles in the phase-contrast image).

For examining CckA-monomericcyan fluorescent protein (Ccka-mCFP) in predivisional cells,Cjw2079 cell populations were synchronized, resuspended in M2G +/ medium plus oxytetracycline, and incubated in a 30°C shaking water bath for ~70 min. Cell samples were concentrated by microcentrifugation at 6,000 g and immobilized on M2G +/agarose pads for tissue. For time-lapse experiments, images were acquired every 2 min for 2 h to allow time for cytokinesis and cell separation. cellTracker was used to create separate kymographs of Ccka-mCFP and DivK-myan fluor protein (DivK-mYFP) localizations as described above, using the stalk as an old-pole marker to orient the cells. Polar foci of Ccka-mCFP and DivK-mYFP were identified using another custom-built MATLAB-based program named spotFinder (P. Montero-Llopis et al., unpublished data). Only the spots above a certain threshold were considered foci, and the spot-sizes and locations were selected manually for each imaging condition and each protein species to be within the intensity of the protein foci under the conditions of known localization and the largest noise spots within cells. For examining Ccka-mCFP localization in late predivisional cells, images in multiple fields of view were acquired. The cytoplasmic compartmentalization of late predivisional cells was determined by examining the localization of DivK-mYFP, since cytokinesis causes the delocalization of DivK from the new pole (27, 37). The new-pole localization of Ccka-mCFP was quantified using spotFinder.

For the quantification of the CpdR-YFP fluorescence cell concentration, the cell length and average cellular fluorescence intensity were computed for each cell. Cell length was defined as the length of the mesh centerline. The comparative histograms of average CpdR-YFP fluorescence intensity and the scatter plot of average CpdR-YFP fluorescence intensity versus cell length were produced using standard MATLAB routines.

For tracking YFP-CtRA levels during the cell cycle, strains Cjw2029, Cjw2159, and Cjw2171 were cultured in M2G + supplemented with 0.3% xylose for 2 h and immobilized on M2G +/agarose pads supplemented with 0.3% xylose. Images were acquired every 15 min for 12 h. MetaMorph was used to create 3 × 3-pixel square regions within cells to sample the fluorescence intensity. Data collection was initiated after cells completed one round of division, noted by the cell body. To ensure that both new-poles and old-pole cell cycle lineages were tracked from beginning to end, the data then were exported to Microsoft Excel for analysis.

Immunoblotting. Samples for immunoblot analysis were normalized by the OD_{660}. One milliliter of each normalized culture was microcentrifuged at 14,000 g, and cell pellets were boiled in 60 μl sample buffer (60 mM Tris-HCl [pH 6.7], 10% glycerol, 2% SDS, 715 mM β-mercaptoethanol). Samples (10 μl) were separated by SDS-PAGE (8% for Ccka and Ccka fusions, 15% for CtRA, and 18% for CpdR) and then transferred to an Immobilon-P transfer membrane (Millipore) using a Trans-blot SD semi-dry transfer cell (Bio-Rad). Ccka, CtRA, and CpdR were identified using anti-Ccka (1:1,000), anti-CtRA (1:10,000), and anti-CpdR (1:1,000) sera, respectively. Green fluorescent protein (GFP), monomeric GFP (mGFP), and mCFP fusion proteins were identified using a 1:1,000 anti-CpdR serum preincubated with Protein A-agarose (Roche Diagnostics) and/or the expression conditions of the Ccka-mCFP.

RESULTS

Quantitative analysis of Ccka localization during the cell cycle using a functional fluorescent fusion. The ambiguity of the cell cycle pattern of Ccka spatial distribution may come from different sources, such as the dynamic behavior of Ccka localization, cell-to-cell variability, poor temporal resolution, and/or the expression conditions of the Ccka-GFP fusions. The functionality of GFP fusions also has been raised as a potential issue (1). It therefore was critical to determine an accurate representation of Ccka subcellular distribution dur-
ing the cell cycle. For this, we generated genetically encoded fluorescent constructs that maintain native conditions, rigorously tested and verified the functionality of the fluorescent fusions, and used an automated and unbiased method for the quantitative analysis of CckA distribution in time and space.

The fluorescent cckA fusion constructs were improved over previously used constructs in several ways. In previous CckA fluorescent fusions, the last two amino acids were missing. In the new constructs, the full length of CckA was preserved. The 3’ end of cckA was tagged with either monomeric gfp (mgfp) or mcfp instead of the older versions, which can potentially dimerize and cause artificial protein clustering. The tagged constructs were introduced at the native locus under the control of the cckA promoter in place of cckA, and unlike previous constructs, all plasmid sequences were removed, thereby maintaining native 5’ and 3’ genetic environments and the physiological level of expression. The functionality of the fluorescent fusions was thoroughly verified using multiple tests (e.g., cell morphology, motility, phage sensitivity, and the localization of the DivK polar marker) in both wild-type and sensitized genetic backgrounds (see the supplemental text and Fig. S1).

Next, for quantitative and unbiased analysis, we used a MATLAB-based software tool that identifies cells in phase-contrast images and automatically extracts the spatial distribution of fluorescently tagged molecules from corresponding fluorescence images. We used a red fluorescent fusion of DivI (DivJ-timer2, produced from the native divI locus) as an old-pole marker to automatically orient the cells along the long axis. To examine the distribution of CckA-mGFP during the cell cycle, we performed a series of time-lapse microscopy experiments starting with a synchronized population of swarmer cells. Images were acquired every 2 min. Kymographs of 99 cells were constructed to show the average fluorescence intensity along the cell body over time. Three aspects of CckA-mGFP localization were largely consistent among the 99 cells (Fig. 2A).

First, in the swarmer cell stage, the CckA-mGFP signal was patchy and very dynamic, showing, on average, no preferential localization. Second, CckA-mGFP nearly always accumulated at the new pole during the stalked/early predivisional cell stage, and this new-pole accumulation remained stable throughout most of the predivisional stage. Third, before the separation of the daughter cells became discernible, CckA-mGFP delocalized from the new pole.

Most cells also displayed an accumulation of CckA-mGFP at the old pole, but the timing and stability of this accumulation varied substantially from cell to cell. Fifty-one percent of cells exhibited old-pole localization of CckA-GFP during the swarmer cell stage and retained this localization pattern for chromosomally produced CckA-mGFP. The remaining 38% of cells had stable, old-pole localization of plasmid-produced CckA-GFP during the swarmer cell stage and retained this localization pattern throughout the cell cycle. Our data showed that 74% of CJW2638 cells (n = 113) displayed no polar localization of CckA-GFP (Fig. 3A, top left). Only a minority of cells showed an accumulation at the old pole (21%) (Fig. 3A, bottom left), at both poles (3%) (Fig. 3A, top right), or at the new pole (2%) (Fig. 3A, bottom right) that lasted 3 min or longer. Even then, these polar accumulations were transient. These data are inconsistent with the idea that the old-pole localization of CckA is a prerequisite for the repression of DNA replication initiation in swarmer cells.

CckA is released specifically from the new pole after cytokinesis and before daughter cell separation. Our data showed
FIG. 2. Quantitative analysis of CckA localization during the cell cycle by time-lapse microscopy. (A) Kymographs of CckA-mGFP signal along the cell body as a function of time after the synchronization of CJW2638 swarmer cells that produce CckA-mGFP from the chromosome showing three characteristic patterns. Images were acquired every 2 min. The top left panel shows a representative cell with discontinuous, transient, and weak old-pole localization during the stalked and predivisional cell stages. The top right panel shows a cell with continuous old-pole localization during the stalked and predivisional cell stages. The bottom panel illustrates a cell with no old-pole localization during the cell cycle. (B) Kymographs like those described for panel A, except that CckA localization was examined using a plasmid-produced CckA-GFP fusion (strain CJW2085). The left panel shows a cell with continuous old-pole localization during the stalked and predivisional cell stages. The right panel shows an example of a cell with continuous old-pole localization during the entire cell cycle.
that CckA, whether produced from the chromosome or a low-copy-number plasmid, delocalizes from the new pole before the swarmer and stalked daughter cells are clearly separated from each other (Fig. 2A and B). Because the length of the cell cycle varies among growing cells (even within the same agarose-padded slide), it remained unclear if delocalization preceded or followed cytokinesis (i.e., the cytoplasmic compartmentalization of the predivisional cell), a key cell cycle event that determines the fate of daughter cells (2, 5, 7, 16, 20, 42). Cytokinesis triggers many changes in the cell, including the release of DivK from the new pole into the swarmer cell compartment, while DivK remains localized at the old pole in the stalked cell compartment (27, 37). DivK delocalization from the new pole thus can be used as a temporal marker of cyto-

FIG. 3. Analysis of CckA localization in swarmer and late predivisional stages. (A) Kymographs of CckA-mGFP signal along the cell body as a function of time following the synchronization of CJW2638 swarmer cells producing CckA-mGFP from its endogenous chromosomal locus. Images were acquired every 30 s for 30 min. CckA localization was considered polar if a polar focus was present by both the visual analysis of raw data and kymograph analysis for a period of at least 3 min (six consecutive images). (B) CckA-mCFP delocalizes from the new pole between the time of the cytoplasmic compartmentalization of the predivisional cell and cell separation. Images of predivisional cells producing CckA-mCFP and DivK-mYFP (CJW2079) were acquired every 2 min for 2 h. Selected images are shown. The black arrow denotes the old pole. The arrowhead denotes the first frame with observable daughter cell separation. White arrows show the delocalization of DivK (red in the cartoon) or CckA (purple in the cartoon) from the new pole. DivK delocalization from the new pole marks cytokinesis and the compartmentalization of the cytoplasm. Scale bar, 2 μm.
kinases. Time-lapse imaging of predivisional CJW2079 cells expressing cckA-mCFP and divK-mYFP showed the delocalization of CckA-mCFP from the new pole following DivK-mYFP release with some delay (n = 57) (Fig. 3B), indicating that the new-pole delocalization of CckA occurs after cytokinesis. This, together with time-lapse data presented in Fig. 2A, indicates that CckA delocalizes from the new pole some time after cytoplasmic compartmentalization (i.e., cytokinesis) and before cell separation.

In summary, our quantitative analysis indicates that CckA is largely delocalized in the swarmer cell stage, localizes at the new pole in the stalked/early predivisional cell stage, and retains its accumulation at the new pole during the predivisional stage until it loses this new-pole accumulation in the late predivisional cell stage after cytokinesis but before cell separation. This sequence of events, which is consistent with a previous report (26), seems the most relevant cytologically, since it is highly reproducible within the cell population. Conversely, the old-pole accumulation is variable in occurrence and timing among cells. Furthermore, an approximately fivefold overproduction of CckA (through the use of a low-copy-number plasmid; see Fig. S1A in the supplemental material) does not affect the new-pole localization sequence but results in an increase in old-pole accumulation, suggesting that the old pole may serve as a depot for excess CckA.

Phosphorylation of CckA is required for function but does not appear to be important for polar localization. With the cell cycle localization of CckA determined, we next examined the possible relationship between the activity of CckA and its polar localization. We initiated a mutagenesis study and asked whether the phosphorylation of CckA affects the localization of the protein and vice versa. The CckA hybrid histidine kinase has a modular architecture with a sensor domain (SD) with two adjacent transmembrane (TM)-spanning segments, a dimerization/histidine phosphotransfer (DHP) domain carrying the phosphorylatable His322, a catalysis-assisting ATP-binding (CA) domain of the GHKL family (11), and a receiver domain (RD) containing the phosphorylatable Asp623 (Fig. 4A).

First, we tested whether the phosphorylation of H322 and/or D623 was important for localization by replacing these residues with alanine independently and in combination. Each mutant fused to gfp was expressed in trans on the pMR20 low-copy-number plasmid in an otherwise wild-type strain to support viability in case the phosphorylation of CckA is necessary for function. Because of the plasmid copy number per cell, the expression of cckA-gfp from pMR20 results in an approximately fivefold overproduction compared to expression from the chromosome (see Fig. S1A in the supplemental material). Therefore, the expression of CckA-GFP mutants from pMR20 should favor the formation of CckA-GFP mutant homodimers relative to heterodimers of mutant CckA-GFP and wild-type CckA; we thus assume that most of the GFP signal reflects the localization of the CckA-GFP mutant homodimers.

The D623A mutation had no discernible effect on protein localization (Fig. 4B, D623A) compared to that of the isogenic strain producing plasmid-borne CckA-GFP (Fig. 4B, WT). The phage transduction of a cckA-null allele (ΔcckA::aacC1) was not possible when the D623A mutant was produced in trans, while it occurred at a high frequency with wild-type CckA-GFP produced from the same plasmid (data not shown). Thus, the phosphorylation of D623 is required for protein function.

Unexpectedly, the single H322A and double H322A D623A point mutants and truncations were transcriptionally fused to gfp and cloned into low-copy-number plasmid pMR20, and the resulting constructs were introduced into wild-type CJW51N to prevent cell death in case the cckA mutants could not support viability. The localization of the GFP fusions to wild-type CckA (WT; CJW407) or mutant CckA—D623A (CJW603), H322A (CJW802), H322A D623A (CJW803), ΔRD (CJW416), ΔCA ΔRD (CJW1156), SD (CJW2853), and TM (CJW419)—were examined by fluorescence microscopy. White arrows indicate the old pole. Scale bar, 2 μm.
each allele was placed under the control of the xylose-inducible promoter (P_{xyl}) on pMR20, and the resulting plasmids were introduced separately into the wild-type strain in the presence of the glucose repressor to prevent the detrimental expression of cckA_{H322A-D623A-GFP} or cckA_{H322A-D623A-GFP}. Two hours of growth in the presence of the xylose inducer resulted in the synthesis and polar localization of CckA_{H322A-CA-GFP} and CckA_{H322A-D623A-GFP} in cells that still maintained a relatively normal morphology (Fig. 4B, H322A and H322A D623A). After 4 h of induction, cells expressing either allele stopped dividing and became filamentous (see Fig. S2A in the supplemental material). From immunoblots of the H322A mutant (see Fig. S2B in the supplemental material), we estimated that the levels of GFP-labeled mutant proteins are about five-fold higher than that of endogenous CckA after 2 and 4 h of induction. Not surprisingly, neither cckA_{H322A-GFP} nor cckA_{H322A-D623A-GFP} could support viability on its own, as determined by phage transduction experiments with the cckA-null allele (data not shown). Collectively, these results suggest that while essential for function, the proper phosphorylation of CckA does not appear to be necessary for the polar localization of the protein.

The PAS-like motif of the SD and linker 4 of the CA domain are involved in the polar localization of CckA. We generated a series of C-terminal truncations to identify the regions in CckA that are required for polar localization. We used plasmid-produced GFP fusions in an otherwise wild-type background to preserve cell viability and to facilitate the rapid and crude screening of the domains that may contain motif(s) important for polar localization. The stability of each construct was verified by immunoblotting (data not shown). The deletion of the CckA RD had no effect on polar localization (Fig. 4B, 3RD). The additional removal of the CA domain caused a strong reduction in the new-pole localization of the fusion protein (Fig. 4B, ΔCA 3RD), suggesting that elements of the CA domain are important for new-pole localization. A larger truncation, leaving only the SD, weakened the old-pole localization signal (Fig. 4B, SD) as determined by time-lapse imaging (data not shown). A short N-terminal fragment (residues 1 to 108) encompassing the TM-spanning region fused to GFP failed to localize at the poles (Fig. 4B, TM), indicating that while required for polar localization (26), the TM region is not sufficient for this function. This mutagenesis study indicated that the CA domain and the linker region of the SD, which connects the DHp domain to the membrane, both are important for CckA polar localization.

We first searched the SD linker for known structural elements and found a very weak PAS-like motif (residues 80 to 149) with an E value of 8.97e-10 according to the SMART algorithm (36, 47). This PAS-like motif carries the structural elements of PAS domains except for the β-scaffold (52). A CckA-mGFP fusion lacking this PAS-like motif (CckA_{APAS-mGFP}) was able to support viability even when expressed from the native chromosomal cckA locus (CJW2857 strain) as the only cckA copy in the cells. However, these cells varied in length (Fig. 5A). This phenotype was accompanied by an impairment of the polar localization of CckA_{APAS-mGFP} (Fig. 5A). While cells still displayed some polar localization, the new-pole localization tended to be weaker and more transient (see Fig. S3 in the supplemental material) than that of wild-type CckA-mGFP (Fig. 2A), indicating that the PAS-like motif in the SD is important for the stable accumulation of CckA at the new pole.

The CA domain, which binds ATP and assists in catalyzing its hydrolysis, is conserved among histidine kinases (50). The alignment of the CA domain of CckA with that of other histidine kinases from various bacteria revealed an additional stretch of residues in the CckA sequence (highlighted in black in Fig. 5B). The modeling of the CckA CA domain using the nuclear magnetic resonance structure of the EnvZ CA domain (51) showed that the additional sequence results in an extension of the linker 4 between β3 and β4 (see Fig. S3 in the supplemental material). When CckA_{LD-mGFP} was produced from the plasmid, new-pole localization was virtually abrogated, whereas the protein retained old-pole localization (see Fig. S5 in the supplemental material). When CckA_{LD-mGFP} was produced from the chromosomal locus (CJW2856 strain), but in both cases, the cells exhibited a severe cell division defect (Fig. 5A; also see Fig. S5 in the supplemental material). When CckA_{LD-mGFP} was produced from the plasmid, new-pole localization was virtually abrogated, whereas the protein retained old-pole localization (see Fig. S5 in the supplemental material). This is consistent with the localization pattern of the plasmid-produced CckA_{ΔCA 3RD}-GFP fusion that lacks the CA domain (Fig. 4B, ΔCA 3RD). The new-pole localization of plasmid-produced CckA_{LD-mGFP} also was significantly reduced in normal-sized cells carrying wild-type cckA on the chromosome (data not shown), indicating that the localization defect occurs independently of cell size. When CckA_{LD-mGFP} was produced from the chromosome at native expression levels in place of wild-type CckA, localization at either pole was abolished (Fig. 5A). Thus, the removal of the L4 linker extension dramatically affects CckA localization and cell function.

Disruption of polar localization is accompanied by defects in CckA function. While the cckA_{APAS} mutant retained the ability to localize in a polar fashion, the localization was weak and unstable (Fig. 5A; also see Fig. S3 in the supplemental material). This was accompanied by a variable cell division defect (Fig. 5A). The L4r mutation in the CA domain caused a more severe polar localization disruption and a correspondingly more dramatic division defect (Fig. 5A). Growth rates of the cckA_{APAS} and cckA_{LD} mutants were proportionally decreased, with a doubling time of 1.9 and 2.4 h, respectively. The rate was 1.6 h for the isogenic wild-type cckA strain. CckA regulates the transcription of pilus and flagellar genes via CtrA activity (25, 26). Both cckA_{APAS} and cckA_{LD} mutants caused a mild decrease in sensitivity to the pilus-binding PhbK phage and a more severely reduced motility, especially for the cckA_{LD} mutant, although this may reflect exacerbation by its filamentous cell morphology (Fig. 5C).

The function of the CckA histidine kinase relies on autokinase and phosphotransfer activities. Therefore, in vivo phosphorylation experiments were performed to examine the autokinase activity of CckA and the ability of CtrA and CpdR,
the cognate response regulators of CckA (1, 23, 25), to become phosphorylated. Cultures with similar OD660s were pulsed with \([32P]\)orthophosphoric acid, lysed, and immunoprecipitated with the proper antibody. Immunoprecipitates were separated by SDS-PAGE, and then the gel was dried and exposed to a phosphorimager. A sample from each prepulsed culture also was obtained for immunoblot analysis to compare the total amount of each protein between strains. Although the morphology of the strains differs significantly (Fig. 5A), Bio-Rad protein assays showed that a similar OD660 between cultures of each strain corresponded to a similar total protein amount (data not shown), validating the use of the OD660 as a reference.

The results of these in vivo phosphorylation experiments were difficult to interpret, largely because of the considerable data variability observed for the mutant strains (Fig. 5D), even though the morphology of the strains differs significantly (Fig. 5A). Bio-Rad protein assays showed that a similar OD660 between cultures of each strain corresponded to a similar total protein amount (data not shown), validating the use of the OD660 as a reference.

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though these experiments were repeated up to six times. Interpretation also was complicated by the existence of multiple positive and negative feedback loops within the regulatory network (24, 35). The levels of phosphorylated CckA\textsubscript{ΔPAS}\textsuperscript{-mGFP} and CckA\textsubscript{L4r}\textsuperscript{-mGFP} were similar or slightly reduced, but since their total protein levels (which include both phosphorylated and unphosphorylated forms) were even more reduced (Fig. 5D), one could argue that the autokinase activity of the mutants was slightly enhanced. The measurements in protein and phosphorylation levels were, however, population averages. There might be wide differences at the single-cell level. This possibility was suggested by the large variability in phosphorylation and/or amounts of CckA, CtrA, and CpdR proteins (shown by the large standard deviations in Fig. 5D), which was highly unusual from our experience.

The level of CpdR protein accumulation and its cell-to-cell variability are greatly increased in the cckA\textsubscript{L4r} mutant. Immunoblot analysis (Fig. 5D) revealed an increased level of CpdR proteins in the cckA\textsubscript{L4r-mGFP} mutant, which likely reflects the CtrA-controlled expression of cpdR (24, 39). Since, as mentioned above, immunoblots only give population averages of protein levels, we used a plasmid-produced CpdR-YFP fusion and quantitative fluorescence microscopy to examine cell-to-cell variability. While there was little variability of CpdR-YFP signal in wild-type cckA and cckA\textsubscript{ΔPAS} backgrounds (Fig. 6A and B, left), the cellular concentration of CpdR-YFP was highly variable in the cckA\textsubscript{L4r} background (Fig. 6A), as quantitatively shown by its large distribution (Fig. 6B, right). In addition, a large fraction of the cckA\textsubscript{L4r} cell population had elevated concentrations of CpdR-YFP (Fig. 6B, right), in agreement with the immunoblot results (Fig. 5D). The wide distribution in cellular CpdR-YFP concentrations among cells appeared to be independent of cell size (data not shown), excluding a secondary effect induced by cell filamentation.

CtrA periodicity is disrupted in the cckA\textsubscript{L4r} mutant. In both cckA\textsubscript{ΔPAS} and cckA\textsubscript{L4r} mutants, the average CtrA protein levels were slightly decreased at the cell population level according to immunoblot analysis (Fig. 5D). Pulse-chase [\textsuperscript{35}S]methionine labeling in cell populations revealed that CtrA turnover changed only slightly in the cckA\textsubscript{ΔPAS} and cckA\textsubscript{L4r} mutants (CJW2857 and CJW2856 strains), with half-lives of 20.1 \pm 0.3 and 29.5 \pm 1.7 min, respectively, than in the corresponding isogenic wild-type strain (CJW1329; 35.4 \pm 3.4 min \(n = 3\)). β-Galactosidase assays established that ctra transcription also was affected in the cckA mutants containing a plasmid carrying the full ctra promoter region fused to the β-galactosidase-encoding gene, lacZ. Transcriptional activity in the cckA\textsubscript{ΔPAS} (CJW2635 strain) and cckA\textsubscript{L4r} (CJW2632 strain) mutants were 36% \(\pm 16%\) and 40% \(\pm 14%\) (\(n = 3\)), respectively, of the activity in the isogenic wild-type strain (CJW2629 strain). These differences in CtrA synthesis and turnover likely account for the overall decrease in CtrA protein levels in both cckA mutant populations.

Since the periodicity of CtrA is a central element to the control of the cell cycle (2, 5), we decided to examine the concentration of CtrA across cell lineages at the single-cell level. Using time-lapse fluorescence microscopy, we monitored the level of a YFP-CtrA fusion constitutively expressed from P\textsubscript{lacZ} to alleviate the transcriptional activity defects of P\textsubscript{cckA} in the cckA mutants and because the cell cycle-regulated prote-
FIG. 7. Analysis of the cell cycle periodicity of YFP-CtrA in cckAΔPAS and cckAΔL4r. (A) Schematic representation of the new-pole (blue) and old-pole (red) cell cycle lineages. Each forking arrow represents a cell division event. (B) The schematics depict the wild-type periodicity of YFP-CtrA for a swarmer (new-pole) cell (left) and a stalked (old-pole) cell (right). The blue and red squares, for the new-pole and old-pole cell cycles, respectively, represent the relative position sampled to determine the average YFP-CtrA level. Time-lapse experiments were performed with strains CJW2029 (the wild type [WT]), CJW2159 (ΔPAS), and CJW2171 (L4r). Each row depicts an independent lineage tracked from a single dividing cell. A representative WT lineage shows correct periodicity of YFP-CtrA in both new-pole cell and old-pole cell generations. The majority of ΔPAS cells had the correct YFP-CtrA periodicity. The two representative L4r lineages shown here lose YFP-CtrA periodicity, although correct periodicity can occur occasionally during certain cycles.
cckA periodicity appeared mostly normal in the of single-cell analyses over population averages. The YFP-CtrA periodicity appeared mostly normal in the cckAΔpAS background (Fig. 7B, ΔpAS), but it was aberrant or lost in the cckAΔ550 background (Fig. 7B, L4r). In this mutant, the pattern of cell divisions was, by and large, no longer in sync with fluctuations of CtrA levels.

**DISCUSSION**

We report here a comprehensive and quantitative analysis of CckA spatial distribution during the cell cycle using a fully functional fluorescent fusion of CckA produced under native conditions. There were three consistent aspects of CckA subcellular distribution during the cell cycle. First, CckA is dynamically distributed in the membrane of swarmer cells, showing no preferential localization to the poles (Fig. 2A and 3A). Second, CckA is localized to the new pole during the stalked/early predivisional cell stage and most of the predivisional stage (Fig. 2A). Third, CckA-mGFP disperses from the new pole at some point between the compartmentalization of the predivisional cell and daughter cell separation (Fig. 2A and 3B). This new-pole localization pattern of CckA during the cell cycle is consistent with the original qualitative description (26). Additionally, CckA can localize to the old (stalked) pole during the stalked and predivisional cell stages, but this localization is variable among the cell population, suggesting that it is less important for cellular function. This work provides a high temporal resolution of CckA localization patterns, and it identifies the consistent and variable elements of this pattern. This highlights the importance of using quantitative, unbiased analysis to determine with temporal accuracy the localization of proteins with complex, dynamic spatial behaviors. It should be noted that all of our imaging experiments were done with cultures in early log phase. It was shown recently that CckA localization changes with the growth phase of the culture as swarmer cells from stationary-phase cultures exhibit a stable polar localization that is not apparent in exponentially growing cell cultures (M. Laub, personal communication).

It has been proposed that CckA localizes at the old pole in swarmer cells, and that its delocalization from that pole during the swarmer-to-stalked cell transition is correlated with the initiation of DNA replication through the inactivation of CtrA→P (1). The cell cycle localization of chromosome-or plasmid-encoded CckA (Fig. 2 and 3A) is incompatible with this notion. It also is inconsistent with the idea that an increase in DivK→P levels during the swarmer-to-stalked cell transition displaces CckA from the pole. Moreover, using purified components, we found that the autokinase activity of the soluble kinase moiety of CckA (CckA-HK) was not significantly affected by the presence of DivK under the experimental conditions tested (see Fig. S6 in the supplemental material). Similarly, DivK→P, obtained by the precubation of DivK with a PleC kinase fragment (18), had no major effect on CckA-HK autophosphorylation (see Fig. S6 in the supplemental material). Taken together, our results suggest that DivK→P does not affect CckA polar localization or autokinase activity in a direct manner, at least not under the conditions tested. Therefore, DivK→P likely plays an indirect role in the regulation of CckA function (1). There also is recent evidence that DivK→P promotes the accumulation of unphosphorylated CpdR (24), which leads to the regulated proteolysis of CtrA via CpdR-recruited ClpXP protease complex (23, 28, 38, 45).

Using site-directed mutagenesis, we showed that similarly to other polarly localized histidine kinases (4, 17, 32, 54), the phosphorylation of the hybrid kinase CckA on the conserved histidine and/or aspartic acid residue(s) does not appear to be necessary for polar localization. Both phosphorylation sites were, however, required for CckA function and cell viability. Interestingly, the CckAH322A and CckAH322AD623A mutants cause a dominant-negative cell division (and eventually lethal) phenotype. Several histidine kinases have been shown to harbor phosphatase activity that does not require the His residue of the DHP domain (15, 21, 37). The preliminary analysis of *in vivo* phosphorylation experiments showed a decreased level of CtrA→P when CckAH322A was present in addition to wild-type CckA (data not shown), suggesting that the dominant-negative phenotype associated with the production of CckAH322A is due to increased CckA phosphatase activity. This is supported by recent *in vitro* studies (6). It also is possible that heterodimerization with the wild-type protein contributes to the dominant-negative effect.

How CckA accumulates at the poles and how the new-pole accumulation is spatially and temporally regulated are unclear. It recently has been shown that the multimeric pole-organizing factor PopZ is necessary for CckA polar localization, although the interaction between PopZ and CckA may be indirect (12). PopZ localizes at the old pole throughout the entire cell cycle, and additionally it localizes at the new pole in the stalked cell stage around the time the segregation of the replicated chromosomal origins is completed (3, 12). Unlike PopZ, CckA does not exhibit a stable polar localization in the swarmer cell and delocalizes from the new pole following the cytoplasmic compartmentalization of the late predivisional cell. Thus, our results concur with the proposal that additional factors and/or physical cues must exist to regulate the temporal and spatial association of CckA with the PopZ complex (12).

While the nature of these putative factors or cues remains unknown, we identified two *cis*-acting regions in CckA that are important for its proper localization and function. The first one was a weak PAS-like motif in the sensor domain of CckA. Its deletion resulted in an unstable polar localization and a mild cell division defect. PAS motifs in prokaryotes often are found in the sensor domain of histidine kinases, where they are involved in sensory functions (e.g., sensing changes in the cellular amount of oxygen, redox potential, and energy metabolism) or protein-protein interactions that regulate the activity of the signal transduction system (52). The second region that is important for both polar localization and function is the 15-residue linker 4 between β-sheets D and E of the CA domain. Its replacement by a two-residue linker 4 reminiscent of the EnvZ linker (creating CckAΔ550) disrupts the polar localization of CckA and has a severe effect on the regulation of CpdR levels and CtrA periodicity. Whereas the activity of the downstream effectors CpdR and CtrA is tightly regulated and reproducible in wild-type cells, their activity is highly variable in the cckAΔ550 mutant populations, which provides an explanation for the various CtrA-dependent cellular defects observed in this mutant, including the defect in the timing of cell division. It is tempting to speculate that the cell cycle-dependent polar
localization of CckA is important for the robustness of the CckA signal transduction pathway and consequently for the robustness of the C. crescentus cell cycle. Identifying the factor(s) or cue(s) that interacts with the PAS-like motif and linker 4 of the CA domain of CckA will be important for understanding the underlying mechanisms.

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