JOURNAL OF BACTERIOLOGY, Nov. 2007, p. 8308–8320 Vol. 189, No. 22
0021-9193/07/$08.00+0 doi:10.1128/JB.00868-07

DivL Performs Critical Cell Cycle Functions in *Caulobacter crescentus* Independent of Kinase Activity▽

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Received 4 June 2007/Accepted 28 August 2007

The *Caulobacter* cell cycle is regulated by a network of two-component signal transduction proteins. Phosphorylation and stability of the master transcriptional regulator CtrA are controlled by the CckA-ChpT phosphorelay, and CckA activity is modulated by another response regulator, DivK. In a screen to identify suppressors of the cold-sensitive *divK341* mutant, we found point mutations in the essential gene *divL*. DivL is similar to histidine kinases but has a tyrosine instead of a histidine at the conserved phosphorylation site (Y550). Surprisingly, we found that the ATPase domain of DivL is not essential for *Caulobacter* viability. We show that DivL selectively affects CtrA phosphorylation but not CtrA proteolysis, indicating that DivL acts in a pathway independent of the CckA-ChpT phosphorelay. *divL* can be deleted in a strain overproducing the phosphomimetic protein CtrAD51E, but unlike Δ*ctrA* cells expressing CtrAD51E, this strain is profoundly impaired in the control of chromosome replication and cell division. Thus, DivL performs a second function in addition to promoting CtrA phosphorylation. DivL is required for bipolar DivK localization and positively regulates DivK phosphorylation. Our results show that DivL controls two key cell cycle regulators, CtrA and DivK, and that phosphoryl transfer is not DivL's essential cellular activity.

Several two-component signal transduction proteins have been identified in genetic screens for *Caulobacter crescentus* mutants with defects in cell cycle progression, cell division, and polar morphogenesis (reviewed in reference 38). The essential response regulator CtrA directly controls the expression of at least 95 genes (24) and promotes cell division, DNA methylation, and the biogenesis of polar organelles (22). However, CtrA also binds to five sites within the replication origin to inhibit the initiation of chromosome replication (33), and cells with unrestricted CtrA activity arrest in the G_1 phase of the cell cycle (6). CtrA activity must therefore be regulated tightly to allow the orderly progression of DNA replication, morphogenesis, and cell division.

The following three mechanisms control CtrA activity as a function of the cell cycle: (i) *ctrA* expression peaks after DNA replication has begun, due to the transcriptional effects of DnaA and GcrA (14); (ii) CtrA is activated by phosphorylation in swarmer cells, where DNA replication has not yet occurred (G_1), and in predivisional cells, where replication has already been initiated (17); and (iii) the CtrA protein is cleared from cells preparing to initiate DNA replication by the ATP-dependent protease ClpXP (6). Combined, these three mechanisms ensure that phosphorylated CtrA (CtrA~P) is present only early and late in the cell cycle, precisely when CtrA-dependent genes are activated or repressed. They also restrict CtrA phosphorylation and eliminate the protein itself just before S phase to free the origin for the initiation of DNA replication.

CtrA phosphorylation and proteolysis are coordinated by a phosphorelay containing the hybrid histidine kinase CckA and the histidine phosphotransferase ChpT (16). CckA autophosphorylates on a conserved histidine residue. The phosphoryl group is passed to a conserved aspartate in the receiver domain at the C terminus of CckA and from there to a histidine residue in ChpT. ChpT can pass the phosphoryl group to one of two response regulators, either CtrA or CpdR (3). Phosphorylation activates CtrA to regulate transcription and DNA replication, and phosphorylation of CpdR prevents it from promoting CtrA proteolysis (16). Thus, when the CckA-ChpT phosphorelay is active, CtrA is stable and activated, whereas when the phosphorelay is inactive, CtrA becomes dephosphorylated and proteolyzed.

Fluctuations in CtrA activity during the cell cycle are linked to changes in CckA activity, and CckA phosphorylation itself is cell cycle dependent (17). The only factor known to modulate CckA activity is the essential single-domain response regulator DivK. When phosphorylated, DivK downregulates the activity of the CckA-ChpT pathway. In the cold-sensitive *divK341* mutant (41) grown at the nonpermissive temperature, excess CckA~P and CtrA~P are present (3), CtrA is no longer proteolyzed, and the net result is G_1 cell cycle arrest (15). Conversely, DivK overexpression causes a sharp decrease in the phosphorylation of CckA and an accumulation of chromosomal DNA (3). The mechanism by which DivK affects the activity of CckA is currently unknown.

DivK is implicated not only in cell cycle regulation but also in the generation of cellular asymmetry. Each *Caulobacter* cell division produces two distinct cell types with different morphologies, protein contents, and cell fates (reviewed in refer-

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▽ Published ahead of print on 7 September 2007.

ence 7). The swarmer progeny is motile, contains CtrA~P, and cannot initiate DNA replication, while the stalked progeny is sessile, lacks CtrA, and can immediately begin a new round of DNA replication. DivJ, a histidine kinase that phosphorylates DivK, resides at the stalked pole of the predivisional cell, while PleC, a histidine kinase that dephosphorylates DivK, is positioned at the swarmer or flagellated pole (48). Once a cytoplasmic barrier is created in the late predivisional cell (21), phosphorylated DivK (DivK~P) is thought to accumulate in the stalked compartment due to the action of DivJ, triggering the downregulation of CckA and the degradation of CtrA. In the swarmer compartment, DivK is dephosphorylated by PleC, which maintains the activity of the CckA-ChpT phosphorelay and preserves CtrA function (27).

To identify components that act in concert with or in parallel to DivK, we performed a screen for temperature-sensitive suppressors of the cold-sensitive *divK341* mutant. Since *divK341* cells at the nonpermissive temperature fail to eliminate CtrA activity, we expected to find mutations that compromise CtrA activity in *ctrA* itself and in genes that positively regulate CtrA. Three independent suppressor mutations were identified in *divL*, which encodes an essential kinase that regulates the *Caulobacter* cell cycle (51). Depletion of the DivL protein from *Caulobacter* cells results in cell filamentation and accumulation of additional copies of the chromosome (36). DivL is unusual in that its dimerization and histidine phosphotransfer (DHp) domain contains a tyrosine residue (Y550) at the conserved phosphorylation site. DivL was shown to autophosphorylate on Y550 in vitro and to pass the phosphoryl group to CtrA (51). Conditional *divL* mutants also contain reduced levels of phosphorylated CtrA (31), indicating that DivL plays a role in CtrA activation in vivo.

Where does DivL fit into the network of two-component signaling proteins that regulate the *Caulobacter* cell cycle? One possibility is that DivL acts in a pathway parallel to CckA and ChpT to phosphorylate CtrA. However, *cckA* mutants lack detectable CtrA~P (17, 18), so DivL and CckA may act in the same signaling pathway. Some results indicate that DivL could interact with DivK and thereby modulate the CckA pathway. First, *divL* mutations can suppress phenotypic defects in *pleC* and *divJ* strains, which contain altered levels of active DivK (31, 41). Second, fragments of DivL were identified along with those of DivJ and PleC in a yeast two-hybrid screen for peptides that physically interact with DivK (30).

Here we show that DivL positively regulates CtrA phosphorylation without affecting CpdR phosphorylation or the rate of CtrA proteolysis, suggesting that DivL acts selectively on CtrA rather than modulating the CckA-ChpT phosphorelay. DivL also promotes DivK phosphorylation and localization of DivK to the cell poles. Surprisingly, *divL* alleles that produce proteins incapable of autophosphorylation or phosphoryl transfer can substitute for the wild-type *divL* gene, causing only moderate defects in cell division. DivL is therefore one of the few histidine kinase homologs known to perform a key cellular function distinct from phosphoryl transfer to a response regulator.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used for this study are listed in Table 1. All experiments were performed using derivatives of *Caulobacter crescentus* strain CB15N grown to mid-logarithmic phase. CB15N strains were grown in peptone-yeast extract (PYE; complex medium), M2G (minimal medium) (9), or M5G [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7, 1 mM NaCl, 1 mM KCl, 0.05% NH_4Cl, 0.01 mM Fe-EDTA, 0.2% glucose, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 0.1 mM phosphate] at the indicated temperatures. Solid and liquid media were supplemented with 3% sucrose, kanamycin (25 μg/ml and 5 μg/ml for solid and liquid media, respectively), chloramphenicol (1 μg/ml), naladixic acid (20 μg/ml), oxytetracyline (2 μg/ml and 1 μg/ml, respectively), or spectinomycin (100 μg/ml and 25 μg/ml, respectively), as required. *Escherichia coli* strains were grown in Luria broth at 37°C, and solid and liquid media were supplemented with carbenicillin (100 μg/ml and 50 μg/ml, respectively), chloramphenicol (30 μg/ml and 20 μg/ml, respectively), kanamycin (50 μg/ml and 30 μg/ml, respectively), tetracycline (12 μg/ml), or spectinomycin (50 μg/ml), as required.

Site-directed mutagenesis of *divL* to create *divL*(*Y550F*) was carried out using the QuikChange protocol (Stratagene), with pMR20-*divL* as the template. Truncated *divL* alleles were generated by PCR, using pKR170 as the template. The 3×FLAG epitope in pSK127 and pSK137 was constructed using an oligonucleotide linker and added the amino acid residues DYKDHDGDYKDHDIDYKD DDDK to the C termini of CpdR and DivL539. For the DivL-enhanced yellow fluorescent protein (DivL-EYFP) fusion, we used PCR to generate an NcoI site at the 3′ end of *divL* and cloned an ~880-bp BamHI-NcoI fragment of *divL* into pEYFP (Clontech). We integrated the *divL-eyfp* gene into the chromosomal *divL* locus using the suicide vector pNPTS138. The plasmid carrying *divL510*::*eyfp* was generated by site-directed mutagenesis of the *divL-eyfp* fusion gene on pKR196 to introduce the L740P mutation, creating integration plasmid pSK170. For the DivJ-YFP fusion, we used PCR to amplify *divJ* from genomic DNA and then performed a three-piece ligation of PCR-amplified *divJ* digested with SacI and XhoI, pNPTS-cc1063-YFP digested with XhoI and SphI, and pUC19 digested with SacI and SphI. The resulting plasmid, with full-length DivJ fused to YFP, was digested with SacI and HindIII, and the fragment was moved into pMR20 to generate pSK158. For the integration plasmid carrying *divK*::*egfp*, pMR20divK-EGFP was digested with SpeI, and the insert was ligated into pNPT228 to generate pSK171. All plasmids were mobilized from *E. coli* to *C. crescentus* by conjugation using *E. coli* strain S17-1 (9). Sequences of all primers used for amplification or mutagenesis are available upon request.

Isolation of suppressors of *divK341*. A CB15N strain containing a cold-sensitive mutation in the *divK* gene (41) was mutagenized with UV radiation and plated in PYE containing 0.3% agar at 20°C. Large swarms were selected from these plates, and the double mutants were screened for the inability to swarm in 0.3% agar at 37°C to identify *divK* suppressors that conferred a temperature-sensitive phenotype. We complemented the 37°C swarm defect in each suppressor strain using a cosmid library (2). Complementing cosmids were isolated, and the insert ends were sequenced. The complementing cosmid for suppressor strain KR510 included CC3484, encoding the essential tyrosine kinase DivL. A low-copy-number plasmid containing only the wild-type *divL* gene (pMR20-*divL*) also complemented KR510. A kanamycin resistance gene was integrated at 3.75 Mb in the genome of KR510, near *divL* (29, 47). The *divL* allele in strain KR510 (*divL510*) was moved into CB15N by cotransduction with the kanamycin resistance gene, using phage ΦCr30 (10).

Two-step gene replacement. Arms of homology flanking *divL* were generated by PCR amplification of the regions approximately 1,000 bp upstream and downstream of *divL*. The left arm included the first three codons of *divL*, and the right arm included the last three codons of *divL*. A streptomycin/spectinomycin resistance gene flanked by transcriptional and translational stop signals (32) from pHP45Ω was digested with BamHI and ligated between the two PCR-generated homology arms in pNPTS138 to generate plasmid pSK149. After pSK149 was mobilized into strains carrying various *divL* alleles on pMR20, first integrants were selected by plating on PYE containing streptomycin, oxytetracycline, and nalidixic acid. Two colonies from each conjugation were inoculated into separate cultures containing PYE with oxytetracycline and streptomycin. After overnight growth, the two cultures for each strain were combined, and serial dilutions were plated for counterselection on PYE containing 3% sucrose, oxytetracycline, and streptomycin. Fifty to 100 of the sucrose-resistant colonies were screened for kanamycin sensitivity and resistance to streptomycin and oxytetracycline to identify colonies in which the wild-type *divL* allele had been replaced by the Δ*divL* mutation. Gene replacements were verified by two PCR tests. First, to verify insertion at the *divL* locus, we used a forward primer in the streptomycin resistance cassette and a reverse primer placed downstream of the sequence included in the *divL* integration construct. A second PCR was performed using primers within the *sacB* gene of pNPTS138 to verify that sucrose resistance resulted from the second recombination event rather than *sacB* inactivation.

To generate the strain Δ*divL* Δ*ctrA*/pCtrAD51E, we amplified the Tet^r cassette

TABLE 1. Strains and plasmids

Strain or plasmid	Description or construction	Source or reference
Strains		
C. crescentus strains		
KR684	CB15N, from which all *Caulobacter* strains were derived	11
CJ396	Δ*cckA* Δ*ctrA*/pCtrAD51E	17
LS2716	Δ*ctrA*/pCtrAD51E	17
LS3196	Δ*divJ*	48
LS3570	*divK341*	15
NR664	*divL*::Tn*5* (inserted in codon 636)	This study
UJ506	Δ*pleC*	1
KR510	*divK341 divL510*	This study
KR635	*divL510*	This study
KR648	*divL510*/pMR20-*divL*	This study
KR649	*divL510*/pMR20	This study
KR674	*divL*::*eyfp*	This study
KR748	Δ*pleC*/pMR20divK-EGFP	This study
KR1609	CB15N/pMR20-*cpdR*::3×FLAG	This study
KR1612	*divL510*/pMR20-*cpdR*::3×FLAG	This study
KR1773	Δ*divL*/pMR20-*divL*	This study
KR1775	Δ*divL*/pMR20-*divL*(*Y550F*)	This study
KR1776	Δ*divL*/pMR20-*divL657*	This study
KR1777	Δ*divL*/pMR20-*divL635*	This study
KR1808	*divL510*/pMR20-*divJ*::*yfp*	This study
KR1809	CB15N/pMR20-*divJ*::*yfp*	This study
KR1841	*divK341 divL*::*eyfp*	This study
KR1843	*divL*::*eyfp* P_{xyl}-*divK*	This study
KR1846	CB15N/pMR20divK-EGFP	19
KR1848	*divL510*/pMR20divK-EGFP	This study
KR1985	Δ-*pleC*/pMR20-*divJ*::*yfp*	This study
KR2044	*divL510*::*eyfp*	This study
KR2045	Δ*divL* Δ*ctrA*/pCtrAD51E	This study
KR2046	*divL*::Tn*5* (NR664) *divK*::*egfp*	This study
KR2047	*divK*::*egfp*	This study
E. coli strains		
TOP10	Cloning strain	Invitrogen
S17-1	RP4-2; Tc::Mu Km::Tn*7*	37
CS191	S17-1/pLW126	47
Plasmids		
pMR20	Broad-host-range, low-copy-number vector	34
pNPTS138	Kan^r; *sacB*-containing integration vector	42
pNPT228	Kan^r integration vector	M. R. K. Alley, unpublished data
pJC52	Vector for fusions to the N terminus of ECFP	4
pHPV465	Spec/$Strep^r$ suicide vector	45
pHP45Ω	Plasmid containing Ω spectinomycin/streptomycin resistance cassette	32
pEYFP	Vector for fusions to the N terminus of EYFP	Clontech
pCtrA	pJS14-*ctrA*, pSAL14	6
pCtrAD51E	pJS14-*ctrAD51E*, pCTD14	6
pMR20divK-EGFP		19
pHXM-*divK*	P_{xyl}-*divK* in high-copy-number vector pJS71	3
pNPTS-cc1063-YFP	pNPTS138-*divJ*::*yfp*	M. Laub, unpublished data
pKOC3	Contains *frt*-flanked Tet^r cassette	40
pLW126	Vector for inserting Km^r cassette at 3.75 Mb	47
pKR174	pMR20-*divL*	This study
pKR196	pNPTS138-*divL*::*eyfp*	This study
pKR320	pNPTS138-*divL*::*tet*	This study
pSK54	pMR20-*divL*(*Y550F*)	This study
pSK118	pMR20-*divL657*	This study
pSK123	pMR20-*divL635*	This study
pSK128	pMR20-*cpdR*::3×FLAG	This study
pSK137	pMR20-*divL540*::3×FLAG	This study
pSK149	pNPTS138-*divL*::*spec*	This study
pSK158	pMR20-*divJ*::*yfp*	This study
pSK170	pNPTS-*divL510*::*eyfp*	This study
pSK171	pNPT228-*divK*::*egfp*	This study

from pKOC3 (39), using primers that added a BamHI site to each end. We digested this fragment with BamHI and ligated it into the BglII site between the left and right *divL* homology arms in pNPTS138 to create pKR320. After mobilizing pKR320 into Δ*ctrA*/pCtrAD51E (17), we selected first integrants on PYE-oxytetracycline-nalidixic acid plates. Individual colonies were selected and grown overnight in liquid PYE-oxytetracycline, after which they were plated onto PYE-oxytetracycline containing 3% sucrose for counterselection against the pNPTS138 vector backbone. Sucrose-resistant colonies were screened for kanamycin sensitivity, oxytetracycline resistance, and spectinomycin resistance to identify colonies in which the wild-type *divL* gene had been deleted. We performed a PCR using primers within the *sacB* gene of pNPTS138 to verify that sucrose resistance resulted from the second recombination event rather than from *sacB* inactivation.

Isolation of *pleC* suppressors. *divL*::Tn*5* insertions emerged from a genetic screen devised to isolate suppressors of the *pleC* mutant phenotype that will be published elsewhere (S. K. Rhadakrishnan, S. Pritchard, and P. H. Viollier, in preparation). Briefly, a promoterless *nptII* gene conferring resistance to kanamycin was transcriptionally fused to the promoter of the *pilA* gene (P*pilA*), a promoter that is dependent on PleC for optimal activity (43). The P*pilA-nptII* transcriptional reporter was integrated at the chromosomal *pilA* locus in wild-type CB15N and the Δ*pleC* mutant derivative (5). Of the two resulting reporter strains, the former was resistant to kanamycin (20 μg/ml), while the latter was sensitive. The Δ*pleC* P*pilA-nptII* strain was subsequently mutagenized with a Tn*5* derivative conferring resistance to tetracycline (20) to isolate mutants that were able to grow in the presence of kanamycin and tetracycline. The six Tn*5* insertions that were mapped to *divL* all occurred downstream of the codon for Y550. The Tn*5* elements in strains NR525, NR664, NR1190, NR1193, NR1244, and NR1245 were found to be inserted in the codons for D653, G636, A658, G636, I572, and D604, respectively.

Flow cytometry. Samples for flow cytometry were fixed at a final concentration of 70% ethanol, stored at 4°C for 1 day to 2 weeks, stained with SYTOX green nucleic acid stain as described previously (35), and analyzed using an Epics XL-MCL analyzer (Beckman-Coulter).

In vivo phosphorylation, pulse-chase, and immunoblot assays. In vivo phosphorylation experiments were performed as previously described (6), with the following modifications. Cells to be labeled were grown in M2G overnight, diluted the next day in M5G, diluted after 8 hours of growth in M5G, and grown overnight to an optical density at 660 nm of 0.3. One milliliter of culture was harvested for Western analysis, and another milliliter of culture was labeled for 4 min at 28°C, using 30 μCi [γ-^{32}P]ATP. Each sample was immunoprecipitated with 2.5 μl of anti-CtrA serum (6) along with either 2.5 μl of anti-FLAG serum (Sigma) or 2.5 μl anti-DivK serum (19). Radiolabeled proteins were resolved in sodium dodecyl sulfate (SDS)-polyacrylamide gels and quantified using a Typhoon phosphorimager (Molecular Dynamics). For pulse-chase experiments, CB15N strains grown at 28°C in M2G were shifted to 37°C for 4 h and then pulse labeled with 10 μCi/ml [^{35}S]methionine per ml of culture for 5 min, followed by a chase with 1 mM unlabeled methionine and 0.3% Casamino Acids. Samples (1 ml) were withdrawn from cultures every 15 min for 2 hours and centrifuged to pellet the cells. Cells were lysed using 50 μl SDS buffer (10 mM Tris-Cl, pH 8, 1% SDS, 1 mM EDTA) and diluted with 800 μl chilled wash buffer (50 mM Tris-Cl, pH 8, 150 mM NaCl, 0.5% Triton X-100). After preclearing of the sample with protein A-agarose, anti-CtrA serum (1.5 μl) and protein A-agarose (Roche) were added to each sample and incubated for 1 h at 4°C with rocking. Immunoprecipitates were washed three times with wash buffer and eluted from protein A-agarose using 15 μl 2× Laemmli sample buffer. Samples were separated in 12% SDS-polyacrylamide gels and analyzed using a Typhoon phosphorimager (Molecular Dynamics). Immunoblotting was performed using anti-CtrA serum at 1:10,000 and anti-FLAG serum at 1:5,000.

Differential interference contrast (DIC) and fluorescence microscopy. Log-phase cells were immobilized on 1% (wt/vol) agarose pads. Images were acquired using a Nikon Eclipse 80i microscope with a PlanApo 100×, 1.40-numerical-aperture objective and a Cascade 512B camera (Roper Scientific). Enhanced green fluorescent protein (EGFP) and EYFP were imaged using Chroma filter sets 41001 and 41028, respectively. Images were acquired using Metavue software (Universal Imaging).

RESULTS

A conditional *divL* mutation suppresses the phenotype of the *divK341* mutant. To identify proteins that participate in *Caulobacter* cell cycle regulation, we screened for mutations that restored swarming to the cold-sensitive *divK341* mutant (41) at 20°C. Suppressors were then screened for those that conferred a new growth or motility defect in swarm agar at 37°C. Some temperature-sensitive mutants were complemented by providing the *ctrA* gene on a low-copy-number plasmid, which was expected because one *ctrA* mutant previously suppressed the phenotype of the *divK341* mutant (50). Other mutants had temperature-sensitive phenotypes not complemented by *ctrA*, so we attempted to complement each one using a cosmid library containing *Caulobacter* genomic DNA (2). Three strains were complemented by cosmids containing *divL* and also by the *divL* gene alone on a low-copy-number plasmid (pMR20-*divL*). We integrated a kanamycin resistance gene at a chromosomal locus near *divL* (3.75 Mb) (29, 47) and transduced one temperature-sensitive allele (*divL510*) into the wild-type strain CB15N to examine its phenotype in isolation. In contrast to wild-type cells (Fig. 1A), *divL510* cells had a range of lengths at 28°C (Fig. 1B). At 37°C, *divL510* cells became more filamentous and accumulated DNA in excess of one or two chromosomes (Fig. 1E and H). These mutant cells resembled cells depleted of the DivL protein (36), and their phenotype was complemented by pMR20-*divL* (Fig. 1C, F, and I), indicating that *divL510* is a recessive loss-of-function allele. The *divL510* allele contains two point mutations, leading to the amino acid changes L740P and A140V, but the L740P mutation alone is sufficient to cause the observed phenotypes (data not shown).

DivL promotes CtrA phosphorylation without affecting the CckA-ChpT phosphorelay. The amount of CtrA∼P is reduced in a temperature-sensitive *divL* mutant (31), and the C-terminal kinase domain of DivL was shown to phosphorylate CtrA in vitro (51). However, the *cckATS1* strain contains undetectable levels of CtrA∼P (18), suggesting that no other kinase can generate a significant amount of cellular CtrA∼P independent of CckA. To account for these data, DivL could act between DivK and CckA, blocking DivK-mediated downregulation of the CckA-ChpT phosphorelay. In this model, DivL and CckA both affect CtrA, but each is essential for viability because they act in the same signaling pathway.

To determine if DivL acts upstream of CckA, we measured the phosphorylation of CtrA and CpdR simultaneously in strain CB15N and in *cckATS1* (18) and *divL510* mutants. We labeled cultures of each strain with [γ-^{32}P]ATP and immunoprecipitated CtrA and CpdR-3×FLAG, using anti-CtrA (6) and anti-FLAG (Sigma) antibodies, respectively. In agreement with previous results (16, 18), *cckATS1* cells contained undetectable levels of CtrA∼P and CpdR∼P, although both proteins were present (data not shown). CtrA∼P was reduced in *divL510* cells to ∼40% of the wild-type level (Fig. 2A, B, and D). Surprisingly, however, *divL510* cells contained a wild-type level of phosphorylated CpdR (Fig. 2A, C, and D). These data suggest that DivL specifically affects the amount of phosphorylated CtrA rather than modulating the activity of the entire CckA-ChpT phosphorelay.

To confirm that normal CpdR∼P levels are maintained in *divL510* cells, we measured the half-life of the CtrA protein in wild-type and *divL510* cultures grown at the nonpermissive temperature (37°C). By phosphorylating CpdR, the CckA-ChpT phosphorelay regulates CtrA proteolysis, limiting it to specific times in the cell cycle. In previous studies, the half-life of CtrA was reduced 2.3-fold in a mutant lacking CckA (17)

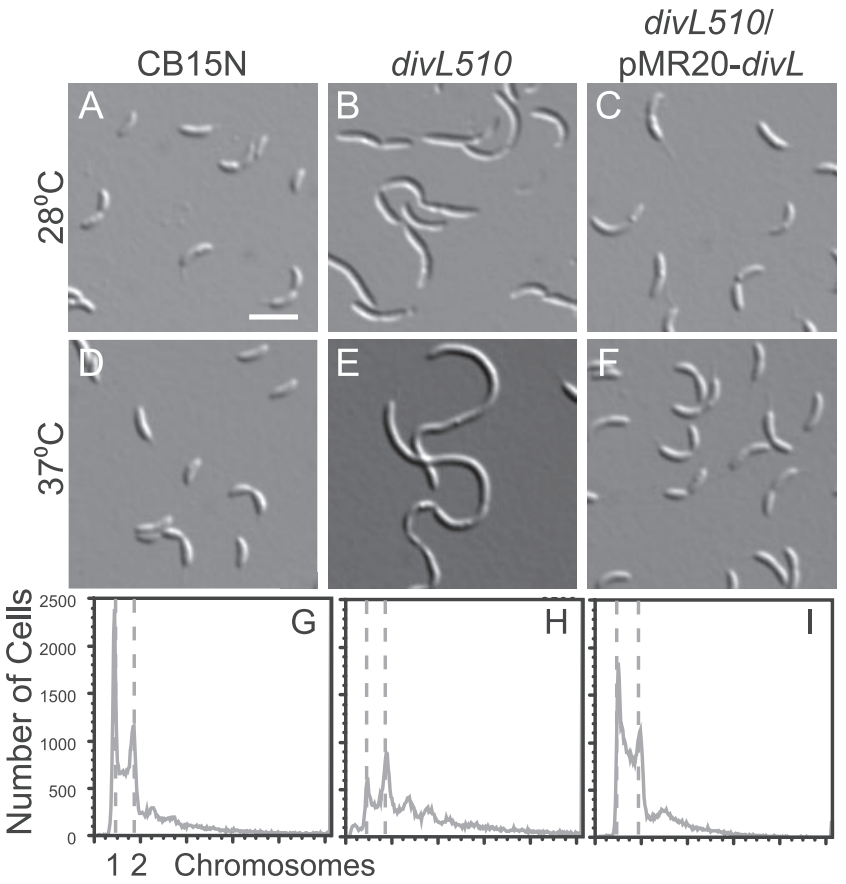


FIG. 1. The *divL510* mutant is impaired in cell division and regulation of chromosome replication. DIC images of CB15N (A and D), *divL510* (B and E), and *divL510*/pMR20-*divL* (C and F) cells grown at 28°C or 37°C for 4 h are shown. Bar, 2 μm. The DNA contents of CB15N (G), the *divL510* mutant (H), and the *divL510*/pMR20-*divL* mutant (I) grown at 37°C for 4 h were measured by flow cytometry of SYTOX green-stained cells. The two dashed lines represent DNA contents of one (left) and two (right) chromosomes.

and 4-fold in a *cpdR*(*D51A*) mutant, in which CpdR is present but cannot be phosphorylated (16). In the *divL510* strain, the half-life of CtrA was 25 ± 3 min, compared with 18 ± 3 min for the wild-type strain CB15N (Fig. 2E); thus, the rate of CtrA proteolysis is not increased in *divL510* cells. Because CpdR phosphorylation is not reduced and CtrA proteolysis is not increased in the *divL510* mutant, we infer that DivL selectively affects CtrA phosphorylation.

DivL performs a critical function other than regulating the level of CtrA~P. DivL clearly affects the cellular level of CtrA~P, regardless of the molecular mechanism. Is DivL's effect upon CtrA phosphorylation its only function in *Caulobacter*? To answer this question, we examined the phenotypes of three mutant strains, the Δ*ctrA*, Δ*divL* Δ*ctrA*, and Δ*cckA* Δ*ctrA* mutants, with each carrying the high-copy-number plasmid pCtrAD51E (6). When overexpressed, the phosphomimetic protein CtrAD51E can replace wild-type CtrA, yielding a mild defect in cell division (17). If the only role of DivL is to regulate CtrA phosphorylation, then we would expect the Δ*divL* Δ*ctrA*/pCtrAD51E strain to have a phenotype similar to that of the Δ*ctrA*/pCtrAD51E strain.

The *divL* gene could be deleted in a strain overexpressing CtrAD51E but not in a strain harboring pCtrA (6), which overexpresses the wild-type CtrA protein (data not shown). We deleted the wild-type *divL* gene in the Δ*ctrA*/pCtrAD51E strain to generate the Δ*divL* Δ*ctrA*/pCtrAD51E strain, but the resulting cells were filamentous and stalkless and contained excess chromosomal DNA (Fig. 3C and D). This phenotype is much more severe than that of the Δ*ctrA*/pCtrAD51E strain (Fig. 3A and B), indicating that the phosphomimetic protein CtrAD51E can rescue the lethality of the Δ*divL* mutant but cannot fully compensate for the absence of the DivL protein. A similar result was observed for the histidine kinase CckA (17), where the Δ*cckA* Δ*ctrA*/pCtrAD51E strain (Fig. 3E and F) was much more impaired in morphology and DNA content than the Δ*ctrA*/pCtrAD51E strain (Fig. 3A and B). CckA was subsequently found to regulate CtrA stability via the response regulator CpdR, in addition to phosphorylating CtrA via ChpT (17). In the case of DivL, however, the half-life of CtrA was not reduced in the *divL510* mutant, and CpdR was phosphorylated normally (Fig. 2). We therefore propose that DivL performs a second function critical for *Caulobacter* cell cycle progression in addition to promoting CtrA phosphorylation.

DivL is required for DivK localization. Although DivL seems not to regulate CckA, the isolation of *divK* suppressors in *divL* mutants suggests that DivL could still function downstream of DivK in a different signal transduction pathway. In this case, DivL localization may be altered in *divK* mutants. We

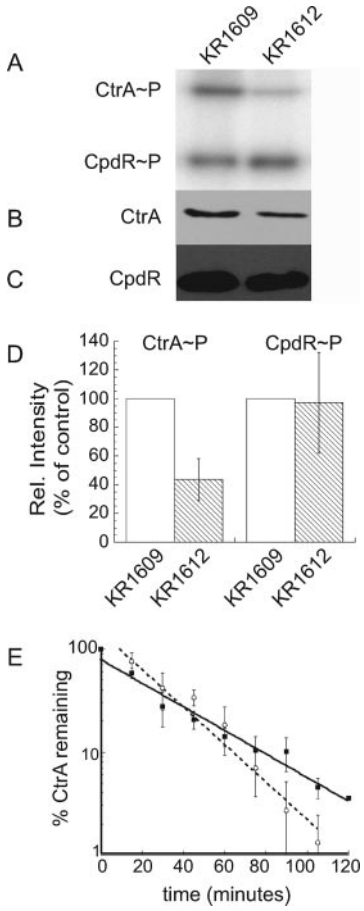


FIG. 2. DivL regulates the phosphorylation of CtrA but does not affect CpdR phosphorylation or CtrA stability. (A) In vivo phosphorylation was measured by immunoprecipitation of CtrA and CpdR from cells grown at 28°C and labeled for 4 min with [γ-^{32}P]ATP. CtrA (B) and CpdR (C) protein levels were determined by Western blotting. For panels A to C, anti-CtrA and anti-FLAG sera were used to recognize CtrA and CpdR, respectively. Lane 1, KR1609 (CB15N/pMR20-*cpdR*::3×FLAG); lane 2, KR1612 (*divL510*/pMR20-*cpdR*::3×FLAG). (D) Ratios of phosphorylated to total protein were calculated for three independent experiments with strains KR1609 and KR1612 and normalized to the wild-type strain. Error bars represent standard deviations. (E) CB15N (open circles) and *divL510* (closed squares) cells were grown for 4 h at 37°C, labeled for 5 min with [^{35}S]methionine, and chased with excess methionine and Casamino Acids. Samples were withdrawn at the indicated times for immunoprecipitation of CtrA, followed by SDS-polyacrylamide gel electrophoresis and phosphorimager analysis. Three independent experiments were performed for each strain, and error bars represent standard deviations.

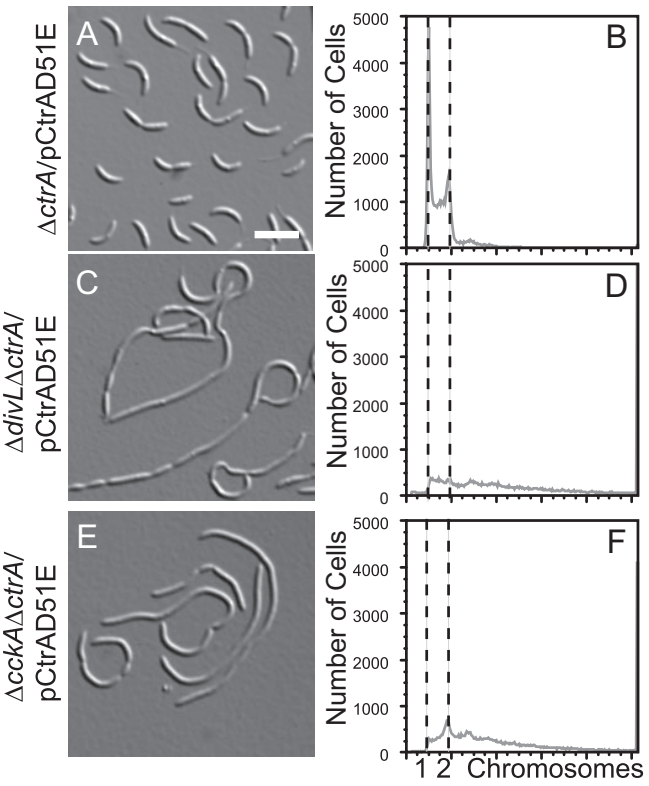


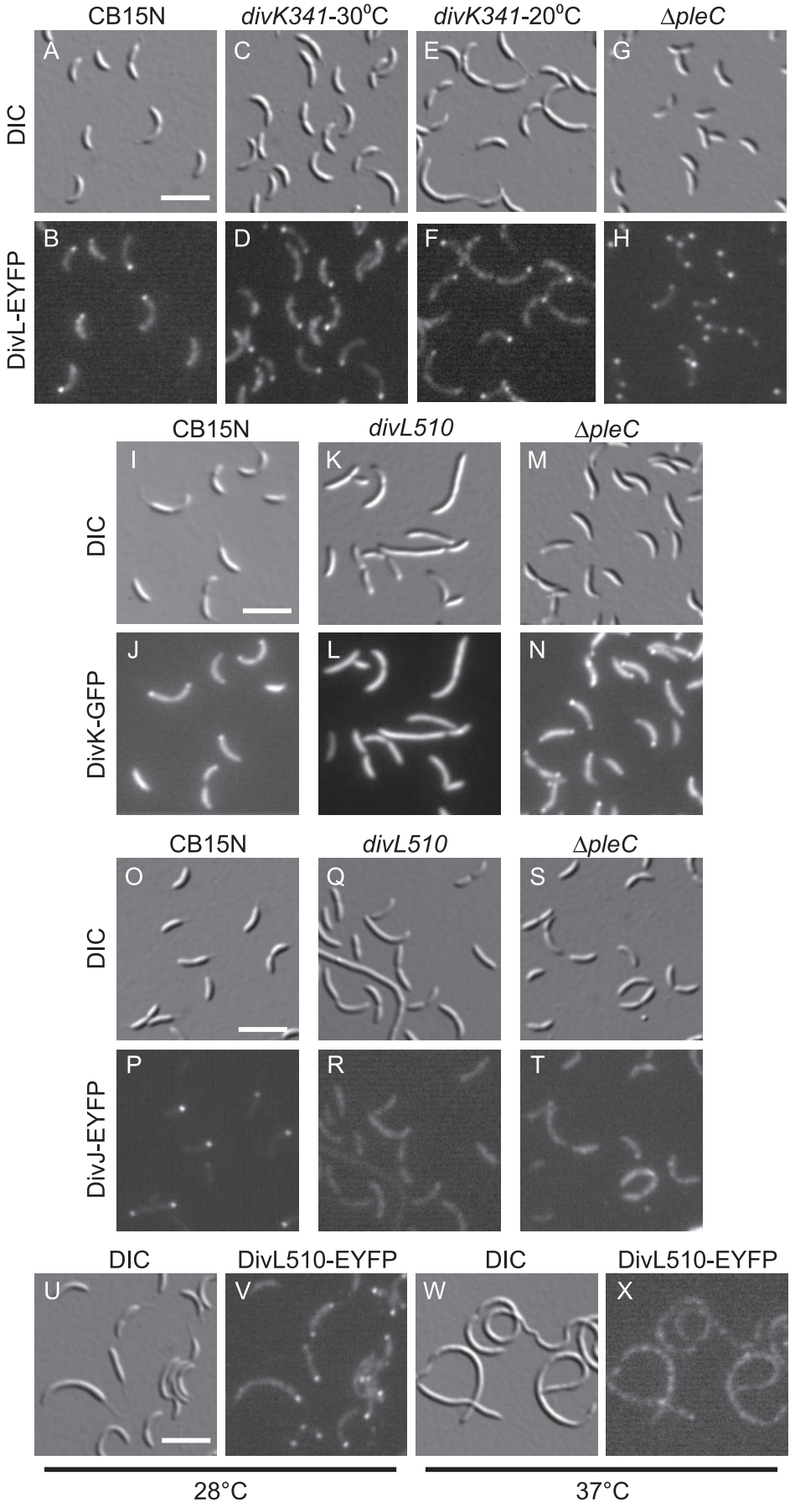
FIG. 3. DivL performs a critical function in addition to regulating CtrA phosphorylation. DIC images and DNA contents of SYTOX green-labeled cells of the Δ*ctrA*/pCtrAD51E (A and B), Δ*divL* Δ*ctrA*/pCtrAD51E (C and D), and Δ*cckA* Δ*ctrA*/pCtrAD51E (E and F) strains are shown. In panels B, D, and F, the two dashed lines represent DNA contents of one (left) and two (right) chromosomes. Bar, 5 μm.

fused the 3′ end of the *divL* coding region to the gene for EYFP (Clontech) and integrated this construct at the *divL* chromosomal locus so that *divL*::*eyfp* was the only source of DivL protein in the cell. As previously observed in wild-type cells (36), DivL-EYFP was located at a single pole or delocalized in swarmer cells and was located at the pole opposite the stalk in stalked and predivisional cells (Fig. 4A and B). In some predivisional cells, a second, dimmer focus of DivL-EYFP was visible at the stalked pole.

In the *divK341* mutant grown at either the permissive temperature (30°C) (Fig. 4C and D) or the nonpermissive temperature (20°C) (Fig. 4E and F), DivL-EYFP localization was unchanged, with foci occurring primarily at the cell pole opposite the stalk. To determine the effect of DivK overexpression, we transformed the P$_{xyl}$-*divK* plasmid into the strain producing DivL-EYFP. When the cells were grown in medium containing either 0.1% dextrose or 0.3% xylose, the location of DivL-EYFP was unchanged (data not shown). Therefore, the localization of DivL does not depend on DivK.

Because a yeast two-hybrid screen indicated that DivL may interact physically with DivK (30) and because we saw no change in DivL localization in strains with altered DivK activity, we examined the location of DivK-EGFP expressed from pMR20divK-EGFP (19) in wild-type and *divL510* cells. In the wild-type strain, DivK shuttles back and forth between the cell poles (27), so all cells contain some diffuse DivK-EGFP. In addition, stalked cells contain a focus of DivK-EGFP at the stalked pole, and predivisional cells contain foci at both poles (19). In contrast, DivK-EGFP was completely delocalized, even at the permissive temperature (28°C), in *divL510* cells (Fig. 4K and L). These data suggest that for purposes of localization, DivL is functionally upstream of DivK.

DivL promotes DivK phosphorylation and may act as a polar binding site for DivK. DivK must be phosphorylated to localize at the *Caulobacter* cell poles, and localization at the stalked pole specifically requires the histidine kinase DivJ (23). In the *divL510* mutant, DivK may be delocalized because it is


CB15N
divK341-30°C
divK341-20°C
ΔpleC
DIC
DivL-EYFP
A
B
C
D
E
F
G
H
CB15N
divL510
ΔpleC
DIC
DivK-GFP
I
J
K
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M
N
CB15N
divL510
ΔpleC
DIC
DivJ-EYFP
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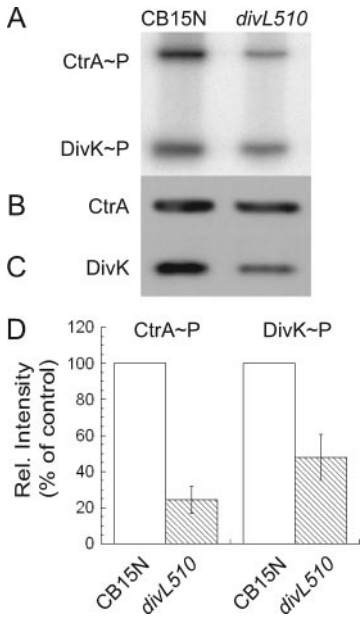

FIG. 5. DivL promotes DivK phosphorylation. (A) In vivo phosphorylation was measured by immunoprecipitation of CtrA and DivK from cells grown at 28°C and labeled for 4 min with [γ-^{32}P]ATP. CtrA (B) and DivK (C) protein levels were determined by Western blotting. For panels A to C, anti-CtrA and anti-DivK sera were used to recognize CtrA and DivK, respectively. Lane 1, KR684 (CB15N); lane 2, KR635 (*divL510*). (D) Ratios of phosphorylated to total protein were calculated for three independent experiments with KR684 and KR635 and were normalized to the wild-type strain. Error bars represent standard deviations.

dephosphorylated, because polar binding proteins for DivK are nonfunctional, or both. To determine how DivL participates in DivK localization, we first measured DivK and CtrA phosphorylation simultaneously in wild-type and *divL510* cells. In the *divL510* mutant, DivK~P was reduced to ~50% of its wild-type level, and CtrA~P was reduced to ~25% of its wild-type level (Fig. 5A to D).

We next examined the location of DivJ, a known polar binding protein for DivK, in the *divL510* mutant. In wild-type cells, DivJ-YFP expressed from a low-copy-number plasmid was delocalized in swarmer cells and was located at the stalked pole of stalked and predivisional cells (Fig. 4O and P). In *divL510* cells at the permissive temperature (28°C), DivJ-YFP was delocalized (Fig. 4Q and R). Wild-type DivL may therefore promote the polar localization of DivK by increasing cellular levels of DivK~P and by promoting DivJ localization.

Although DivJ is known to recruit DivK to the stalked pole, DivK binding proteins at the pole opposite the stalk have not been identified. The histidine kinase PleC dephosphorylates DivK (27, 48), is located at the pole opposite the stalk (48), and is required to release DivK from that pole following cell division (19). In a Δ*pleC* mutant, the proportion of cells with bipolar DivK-EGFP was increased (Fig. 4M and N) (19), but DivJ was delocalized (Fig. 4S and T) (48), suggesting that proteins other than DivJ and PleC can interact with DivK at the cell poles. DivL-GFP expressed from a plasmid was reported to localize normally in the *pleC301*::Tn*5* mutant (36). In contrast, we found that Δ*pleC* cells contained bipolar DivL-EYFP (Fig. 4G and H), while wild-type cells chiefly contained a single focus of DivL-EYFP at the pole opposite the stalk (Fig. 4A and B). The bipolar localization of DivK and DivL in Δ*pleC* cells, where DivK phosphorylation is increased, PleC is absent, and DivJ is delocalized, suggests that DivL recruits DivK to both poles in this mutant and may bind DivK to the pole opposite the stalk in wild-type cells.

Since DivK is delocalized in *divL510* cells, we fused the DivL510 protein to EYFP at the chromosomal *divL* locus and verified that the resulting strain was temperature sensitive. In contrast to the wild-type protein, DivL510-EYFP at the permissive temperature (28°C) was often located at both cell poles or at the stalked pole only (Fig. 4U and V) rather than at the pole opposite the stalk (Fig. 4A and B). At the nonpermissive temperature (37°C), DivL510-EYFP was located in patches throughout the cell. Thus, if DivL acts as a localization factor for DivK, then for DivL510 at the permissive temperature this function must be perturbed, delocalizing DivK while still allowing DivL510 to reach one or both cell poles.

The phosphorylation site and kinase domain of DivL are not essential for viability. Since DivL promotes the phosphorylation of CtrA and DivK in vivo and the kinase domain of DivL phosphorylates CtrA in vitro (51), we performed several experiments to determine if the essential function of DivL is its kinase activity. In two previous studies, the role of the phosphorylation site residue Y550 was investigated by replacing this amino acid with phenylalanine [*divL*(*Y550F*)] and determining whether this allele complements the growth and cell division defects of the temperature-sensitive *divL356* strain (41). Both studies concluded that DivLY550F could not complement the filamentation of the *divL356* mutant, but one study found that DivLY550F partially complemented the growth defect of the *divL356* mutant at 37°C (51). Histidine kinases dimerize, and each monomer within the dimer phosphorylates the conserved histidine residue of the other monomer (reviewed in reference 46). Partial complementation in the latter experiment could have resulted from heterodimers in which the functional kinase domain of DivLY550F phosphorylated Y550 of the DivL356 protein.

To resolve this issue, we examined the ability of *divL*(*Y550F*) to complement a deletion of *divL* rather than a conditional allele. In CB15N, we expressed DivLY550F from a low-copy-number plasmid using the *divL* promoter (pMR20-*divLY550F*) and then attempted to replace the chromosomal copy of *divL*

FIG. 4. DivL is required for polar localization of DivK. DIC and fluorescence images of DivL-EYFP expressed from the chromosomal *divL* locus in CB15N (A and B), *divK341* (4 h at 30°C) (C and D), *divK341* (4 h at 20°C) (E and F), and Δ*pleC* (G and H) cells are shown at the top. DIC and fluorescence images of DivK-EGFP expressed from pMR20divK-EGFP in CB15N (I and J), *divL510* (K and L), and Δ*pleC* (M and N) cells are shown next. DIC and fluorescence images of DivJ-YFP expressed from pMR20-*divJ*::*yfp* in CB15N (O and P), *divL510* (Q and R), and Δ*pleC* (S and T) cells are shown below these. DIC and fluorescence images of DivL510-EYFP expressed from the chromosomal *divL* locus in cells grown for 4 h at 28°C (U and V) or 37°C (W and X) are shown at the bottom. Bar, 2 μm.

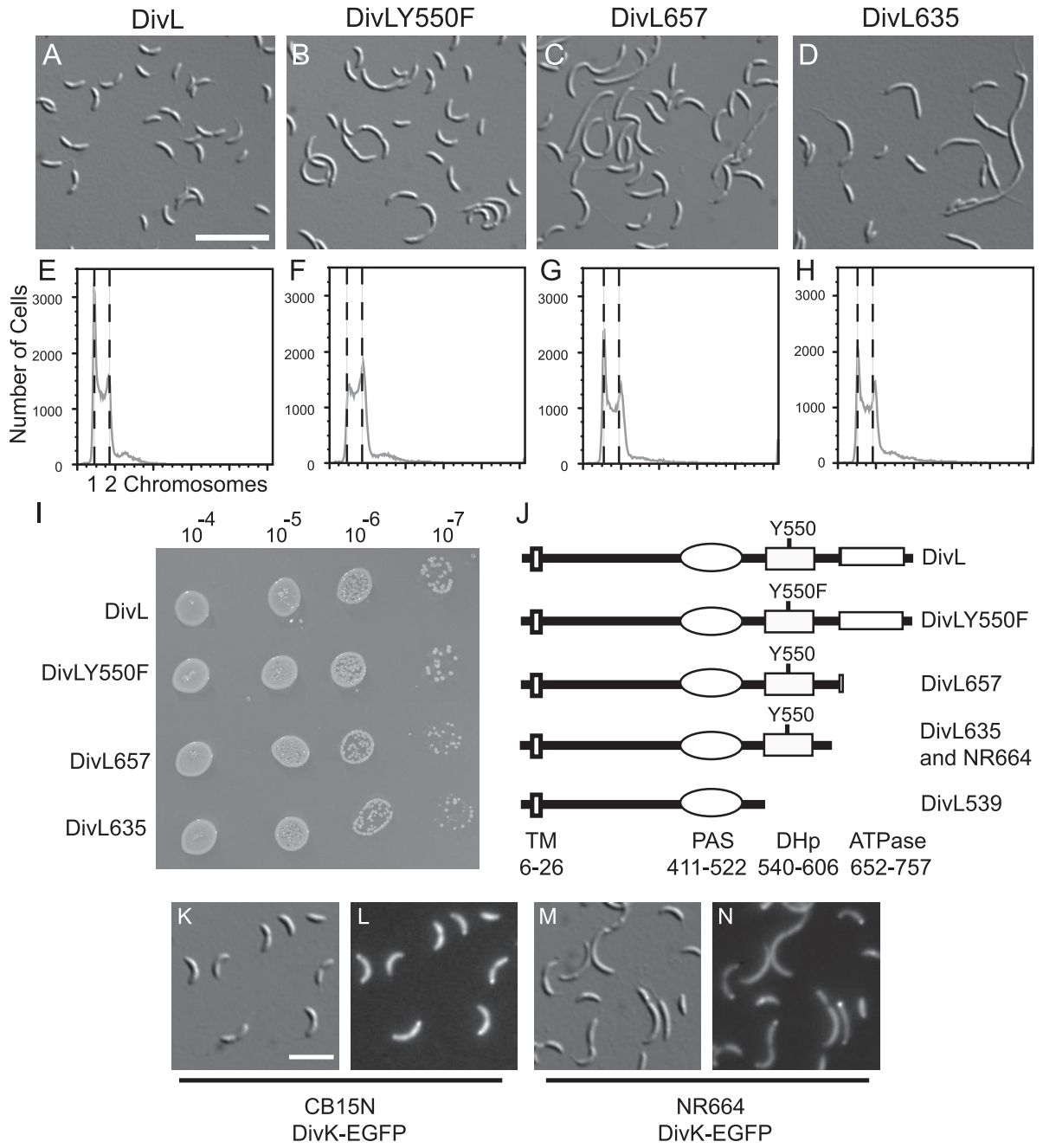

FIG. 6. DivL variants impaired in kinase activity or phosphoryl transfer support *Caulobacter* viability and DivK localization. DIC images (A to D) and DNA contents of SYTOX green-stained cells (E to H) are shown for Δ*divL* strains expressing the following proteins from pMR20: DivL (KR1773) (A and E), DivLY550F (KR1775) (B and F), DivL657 (KR1776) (C and G), and DivL635 (KR1777) (D and H). (I) Growth of Δ*divL* strains expressing the indicated proteins from pMR20. Serial dilutions of exponential-growth-phase cultures at an optical density at 660 nm of 0.25 were made, and 5 microliters of each indicated dilution was spotted onto a PYE plate and incubated for 2 days at 30°C. Three independent experiments were performed, and a representative plate is shown. (J) Diagram of the DivL protein, including the predicted transmembrane (TM), Per-ARNT-Sim (PAS), DHp, and catalytic (ATPase) domains. Y550 represents the site of phosphorylation, and numbers indicate amino acid residues. Mutated and truncated DivL proteins used in this study are depicted, and the DivL635 protein is truncated at the site of the Tn*5* insertion in strain NR664. (K to N) DIC and fluorescence images of DivK-EGFP expressed from the chromosomal *divK* locus in CB15N (KR2047) (K and L) and NR664 (KR2046) (M and N). Bar, 5 μm (A to D) or 2 μm (K to N).

with a spectinomycin/streptomycin resistance cassette (Δ*divL*), using a two-step recombination protocol. In a control strain containing the empty vector pMR20, we were unable to replace the wild-type *divL* allele with a Δ*divL* mutation, in agreement with previous reports showing that *divL* is essential for *Caulobacter* viability (51). However, we successfully replaced the chromosomal *divL* gene in a strain containing pMR20-*divLY550F* (Fig. 6B). The resulting cells were impaired in cell

division, with an average length two to three times greater than that for a control population containing pMR20-*divL* (Fig. 6A), and were often stalkless. Flow cytometry experiments measuring chromosomal DNA content revealed that cells of both strains contained primarily one or two chromosomes (Fig. 6E and F). In contrast, when cells are depleted of the DivL protein by making its transcription dependent upon a xylose-inducible promoter, the cells become filamentous and accumulate additional DNA (36). Thus, a tyrosine residue at position 550 is necessary for normal cell division and stalk biogenesis but is not strictly required for *Caulobacter* viability.

Conditional alleles of *divL* have been isolated in screens for point mutations that suppress the motility defect of *pleC* mutants (41). Since *divL* is essential for viability, however, we were surprised to obtain motile *pleC* suppressors containing transposon insertions in the *divL* open reading frame in a separate screen (see Materials and Methods). In all cases, the transposon insertions interrupted the catalytic domain of DivL downstream of the Y550 phosphorylation site. Spurred by these results, we constructed three *divL* truncations, expressed each truncated protein from pMR20, and attempted to delete the chromosomal *divL* gene in these strains. Two of the truncations, *divL635* and *divL657*, corresponded to transposon insertion sites, while the third truncation, *divL539*, was designed to remove both the DHp and ATPase domains of DivL (Fig. 6J).

We isolated viable strains containing the Δ*divL* mutation and expressing the truncated proteins DivL657 and DivL635, but not DivL539. DivL539 contains a C-terminal 3×FLAG tag which can be detected using an anti-FLAG antibody on Western blots (8). CB15N cells containing pMR20-*divL539* produced a FLAG-tagged protein of the correct size (data not shown), suggesting that this protein is produced in *Caulobacter* but is unable to support life in the absence of the full-length *divL* gene. The *divL657* and *divL635* mutants supported viability, but each truncation yielded slower-growing cells (Fig. 6I) with a mixture of lengths (Fig. 6C and D). While Δ*divL*/pMR20-*divLY550F* cells were stalkless (Fig. 6B), Δ*divL*/pMR20-*divL635* cells often had elongated stalks (Fig. 6D). Again, despite the morphology defects, cells expressing each truncated DivL protein had normal DNA contents, containing primarily one or two chromosomes (Fig. 6G and H).

These findings are at odds with a previous study (36) in which a DivL-GFP fusion protein lacking the C-terminal 23 amino acids of DivL was incapable of complementing the temperature-sensitive lethality of the *divL346* mutant. Our experiment is different in that the truncated DivL proteins tested were not GFP fusions, and we assayed complementation of a *divL* deletion rather than a temperature-sensitive mutation. We are confident that the ATPase domain of DivL is dispensable for viability, however, because two separate techniques, transposon mutagenesis and deletion analysis, gave similar results. The phenotypes of our *divL* alleles indicate that DivL catalytic activity and phosphorylation at Y550 are not required for viability. Thus, DivL performs an essential function other than autophosphorylation or phosphoryl transfer to a response regulator.

Since DivL participates in localizing DivK to the cell poles, we assayed DivK-EGFP localization in the mutant NR664, which contains a Tn*5* element inserted in *divL* after codon 635 and which was the basis for the DivL635 truncation. In the wild-type background, DivK-EGFP expressed from the chromosomal *divK* locus was delocalized in swarmer cells and present at the stalked pole (31%) or at both poles (65%) of cells with a polar stalk ($n = 250$) (Fig. 6K and L), in agreement with previous results using DivK-EGFP expressed from a low-copy-number plasmid (19). In the *divL*::Tn*5* mutant NR664, DivK-EGFP was located at the stalked pole (54%) or both poles (34%) of cells that possessed a polar stalk ($n = 160$) (Fig. 6M and N). Since localization of DivK was shifted toward the stalked pole in NR664 but was not abolished, we concluded that the catalytic domain of DivL, and therefore DivL kinase activity, is not strictly required for polar localization of DivK.

DISCUSSION

Our studies have revealed unexpected phenotypes of *divL* mutants that prompt reconsideration of the biochemical function of DivL and indicate that DivL performs more than one role in *Caulobacter* cell cycle regulation. Although *divL* is essential for viability (51) and depletion of DivL causes chromosome accumulation (36), removal of the DivL catalytic domain or replacement of its phosphorylation site tyrosine residue with phenylalanine (Y550F) caused moderate effects on cell morphology and had no effect on chromosome content. In contrast, replacement of the phosphorylation site histidine residue in PleC or DivJ with alanine mimics the effect of deleting *pleC* or *divJ*, respectively (23, 44). DivL is therefore unusual in that it performs a critical function other than phosphoryl transfer to or from a response regulator. The DivL variants that cannot autophosphorylate or participate in phosphoryl transfer are not perfect substitutes for wild-type DivL. At present, we cannot distinguish between two models to explain these results. DivL may phosphorylate CtrA or another response regulator at a low level required for precise control of cell cycle progression, or the catalytic domain and Y550 residue may be required to create an optimal conformation for interaction with other proteins.

Two other systems provide examples of histidine kinases that function solely or partly via protein-protein interactions. First, the NifL protein regulates the expression of genes for nitrogen fixation in response to redox and fixed nitrogen status (reviewed in reference 26). NifL contains the conserved DHp and catalytic domains of histidine kinases and binds adenine nucleotides (40), but amino acid substitutions at the conserved phosphorylation site, H304, do not disable NifL (49). Rather than phosphorylating its partner protein, the transcriptional regulator NifA, NifL blocks the transcription of genes for nitrogen fixation under the appropriate conditions by sequestering NifA in a complex (28). In the second example, the hybrid histidine kinase RpfC of *Xanthomonas campestris* performs two distinct functions, namely, regulating virulence factor production via phosphoryl transfer to the cognate response regulator RpfG and regulating synthesis of the cell-cell communication signal DSF by a protein-protein interaction between the RpfC receiver domain and the enzyme RpfF (12).

We show that the *divL510* mutation reduces CtrA phosphorylation but not CtrA stability, suggesting that DivL affects CtrA by a pathway independent of CckA-ChpT (Fig. 7). The last 299 amino acids of DivL, comprising the DHp and catalytic do-

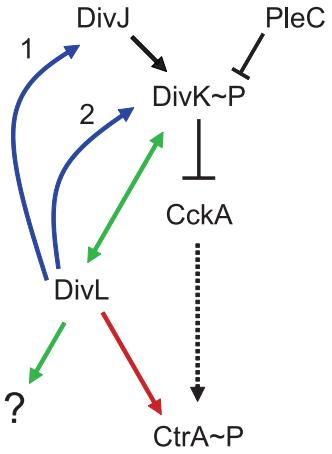

FIG. 7. Model of DivL and DivK activities in *Caulobacter* cell cycle regulation. Black connectors represent interactions shown in other studies. The dotted arrow connecting CckA and CtrA represents the branched phosphorelay including ChpT and CpdR. Colored connectors represent interactions inferred from this work. DivJ phosphorylates DivK and binds to DivK at the stalked pole of the cell. PleC resides at the flagellated pole of the cell and dephosphorylates DivK. When phosphorylated, DivK decreases cellular CtrA activity by downregulating the CckA-ChpT phosphorelay, leading to CtrA dephosphorylation and proteolysis. DivL positively regulates CtrA phosphorylation by a mechanism independent of CckA and ChpT (red arrow). DivL promotes DivK localization by regulating DivJ localization and activity (blue arrow 1) and/or by binding directly to DivK at the flagellated pole (blue arrow 2). In concert with DivK, DivL regulates cell cycle processes other than CtrA activity (green arrows).

mains, can phosphorylate CtrA in vitro (51), but the *cckATS1* (18) and *ctrAΔ3M2* Δ*cckA*/pCtrAD51E (17) strains contain no residual phosphorylation on the CtrA and CtrAΔ3M2 proteins, respectively. Furthermore, the Δ*cckA*/pCtrAD51E and Δ*ctrA* Δ*cckA*/pCtrAD51E strains are equally impaired in cell division, chromosome content, and gene expression, suggesting that in the absence of CckA, the wild-type CtrA protein in the former strain is not phosphorylated to provide additional CtrA function (17). Thus, DivL may provide a low level of CtrA phosphorylation that is below the limit of detection in vivo, but we favor a model in which DivL regulates CtrA phosphorylation indirectly, perhaps by protecting CtrA~P from dephosphorylation. Regardless of the mechanism by which DivL promotes CtrA phosphorylation, this activity is not DivL's only function in *Caulobacter*, since the phosphomimetic protein CtrAD51E does not complement the Δ*divL* Δ*ctrA* double mutant as well as it complements the Δ*ctrA* mutant alone.

Our protein localization and in vivo phosphorylation results indicate that DivK function is also compromised in the *divL510* mutant. Even at the permissive temperature, DivK phosphorylation is reduced and DivK-EGFP is delocalized in *divL510* cells. DivL could affect DivK by a combination of mechanisms. First, it is formally possible that DivL phosphorylates DivK directly. In this scenario, the relative dephosphorylation of DivK in *divL510* cells causes its delocalization. However, in a previous in vitro study, the DivL kinase domain preferentially phosphorylated CtrA over DivK in reaction mixtures containing all three proteins (51). Furthermore, since the ATPase domain of DivL is not absolutely required for DivK localization but phosphorylation of DivK is itself needed for localization (23), it is unlikely that DivL is a significant source of DivK~P in the cell. Second, DivL could modulate DivK function by promoting the polar localization and activity of the DivJ histidine kinase (Fig. 7, blue arrow 1). DivJ is normally located at the stalked pole but is delocalized in *divL510* cells. Since DivJ phosphorylates DivK and recruits DivK to the stalked pole (23, 48), its impaired function could account for the observed effects on DivK phosphorylation and, thus, localization. At present, however, we cannot determine if phosphoryl transfer from DivJ to DivK is impaired in *divL510* cells. Finally, DivL may function as a binding site for DivK at the pole opposite the stalk (Fig. 7, blue arrow 2). In wild-type predivisional cells, foci of DivK are present at both poles. DivJ is thought to bind DivK at the stalked pole (24), but a comparable DivK binding protein at the flagellated pole has not been identified. DivL could perform this function, since it is located at the flagellated pole. In our model, DivK does not concentrate at the flagellated pole of *divL510* cells because its interaction with DivL510 is impaired, nor is DivK sequestered at the stalked pole because DivJ is also delocalized. Consistent with this idea, in Δ*pleC* cells, where DivK~P is elevated and DivJ is delocalized (48), bipolar DivK localization may be mediated by DivL, whose location is shifted from the flagellated pole to both poles.

How does the *divL510* mutation suppress the temperature-sensitive growth and motility defects of *divK341* cells? We infer that *divK341* is a loss-of-function mutation because it is recessive (13) and its G_1 cell cycle arrest at the nonpermissive temperature (15) is distinct from the chromosome accumulation caused by *divK* overexpression (3). Since DivK activity is also reduced in *divL510* cells, the *divL510* mutation is not likely to suppress *divK341* by a direct action on DivK. Instead, the *divL510* mutation could suppress *divK341* by reducing the amount of CtrA~P in the cell, acting parallel to DivK (Fig. 7, red arrow).

It is paradoxical that DivL promotes DivK phosphorylation and localization yet does not appear to modulate the CckA-ChpT phosphorelay. How can DivL alter DivK activity without affecting downstream events? We propose a branch point in the signal transduction network, at which DivK can either downregulate the CckA-ChpT pathway (Fig. 7, black bar) or act in concert with DivL to affect other cellular processes (Fig. 7, green arrows). Alternatively, it is possible that the reduction in DivK~P in *divL510* cells is too modest to affect CckA.

Finally, it is unusual that mutations in *divL* have been isolated as suppressors of both *divJ* and *pleC* mutants (31, 41; this work), when DivJ and PleC have opposing effects on DivK phosphorylation. One model to explain these results is that distinct *divL* mutations increase or decrease DivL's propensity to interact with DivK~P. Histidine kinase dimers are believed to cycle between two states to accommodate dual activities, i.e., autophosphorylation and phosphoryl transfer (25). During autophosphorylation, the DHp domain (including the phosphorylation site histidine residue) of one monomer interacts with the ATPase domain of the other monomer. During phosphoryl transfer, the ATPase domain must move to allow interaction between the phosphorylated histidine and a response regulator receiver domain. Since the DHp domain of DivL specifically interacted with DivK in a yeast two-hybrid screen (30), various *divL* mutations could promote or block an interaction between

this domain and DivK. This model predicts that *divL* mutants isolated as *pleC* suppressors will not suppress *divJ* and *divK* mutant phenotypes, and vice versa. It also predicts that purified DivL proteins with different amino acid substitutions or truncations will have different affinities for DivK in vitro. We are pursuing studies to characterize the interaction between DivL and DivK and to identify additional processes regulated by these essential signal transduction proteins.

ACKNOWLEDGMENTS

We thank Michael Laub and Jeff Skerker for pNPTS-cc1063-YFP, helpful discussions, and critical readings of the manuscript, and we thank Christine Jacobs-Wagner for DivK antiserum.

This work was supported by NIH grant GM032506 to Lucy Shapiro, by startup funds provided by Case Western Reserve University School of Medicine and the Mount Sinai Health Care Foundation to P.V., and by NSF grant 0543801 to K.R.

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