Productive interaction between the chromosome partitioning proteins, ParA and ParB, is required for the progression of the cell cycle in *Caulobacter crescentus*

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Summary

In *Caulobacter crescentus* the partitioning proteins ParA and ParB operate a molecular switch that couples chromosome partitioning to cytokinesis. Homologues of these proteins have been shown to be important for the stable inheritance of F-plasmids and the prophage form of bacteriophage P1. In *C. crescentus*, ParB binds to sequences adjacent to the origin of replication and is required for the initiation of cell division. Additionally, ParB influences the nucleotide-bound state of ParA by acting as a nucleotide exchange factor. Here we have performed a genetic analysis of the chromosome partitioning protein ParB. We show that *C. crescentus* ParB, like its plasmid homologues, is composed of three domains: a carboxyl-terminal dimerization domain; a central DNA-binding, helix–turn–helix domain; and an amino-terminal domain required for the interaction with ParA. *In vivo* expression of amino-terminally deleted parB alleles has a dominant lethal effect resulting in the inhibition of cell division. Fluorescent *in situ* hybridization experiments indicate that this phenotype is not caused by a chromosome partitioning defect, but by the reversal of the amounts of ATP-versus ADP-bound ParA inside the cell. We present evidence suggesting that amino-terminally truncated and full-length, wild-type ParB form heterodimers which fail to interact with ParA, thereby reversing the intracellular ParA-ATP to ParA-ADP ratio. We hypothesize that the amino-terminus of ParB is required to regulate the nucleotide exchange of ParA which, in turn, regulates the initiation of cell division.

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Introduction

How sister chromosomes partition to opposite ends of the predivisional cell is a central and unresolved aspect of the bacterial cell cycle (reviewed in Gordon and Wright, 2000; Draper and Gober, 2002). For many years chromosome partitioning was thought to be a passive process relying on zonal membrane growth between two chromosome attachment sites. Recent work, however, using *in situ* hybridization (Niki and Hiraga, 1998; Jensen and Shapiro, 1999) and fluorescence microscopy (Gordon et al., 1997; Lewis and Errington, 1997; Webb et al., 1997; 1998) demonstrated that chromosomes move actively between well-defined subcellular positions. This is especially evident for the origin of replication (oriC) region, where rapid movement away from the cell centre towards the poles has been observed in several organisms. A combination of different mechanisms such as DNA replication and chromosome condensation have been proposed to facilitate chromosome movement (Lemon and Grossman, 1998; 2000; 2001). A central role in this process has been attributed to the partitioning proteins, ParA and ParB (reviewed in Gordon and Wright, 2000). Homologues of these were originally shown to play an important role in the stable inheritance of prophage P1 and F-factor plasmids, and are proposed to function in orienting the oriC toward the cell poles. In plasmids, ParA and ParB (SopA and SopB in F-factor), and a downstream cis-acting centromere-like site, parS (sopC in F-factor), are required for segregation (reviewed in Gordon and Wright, 2000). ParB is a *parS*-specific, DNA-binding protein that is composed of three different domains: an amino-terminal ParA interaction domain (Radnedge et al., 1998); a central DNA-binding domain; and a carboxyl-terminal dimerization domain (Lobocka and Yarmolinsky, 1996). ParA possesses an ATPase activity that can be stimulated by ParB (Davis et al., 1992; Watanabe et al., 1992; Davey and Funnell, 1994). ParA (SopA) binds to operator sequences upstream of the *parAB* (sopAB) operon and represses transcription (Friedman and Austin, 1988; Davey and Funnell, 1994; Hayes et al., 1994; Hirano et al., 1998). In its ADP-bound form, ParA is a repressor of the *parAB* operon, whereas ATP-bound ParA interacts with the ParB-*parS* nuc-
Homologues of \textit{parA} and \textit{parB} have been identified in at least 29 different eubacterial species (Yamaichi and Niki, 2000). In \textit{Bacillus subtilis}, the \textit{parA} and \textit{parB} homologues, \textit{soj} and \textit{spo0J}, have been shown to be important for chromosome partitioning during both vegetative growth and sporulation (Ireton \textit{et al.}, 1994). The \textit{spo0J} gene was first discovered as a sporulation deficient mutant (Mysliwiec \textit{et al.}, 1991), a phenotype that could be suppressed by a mutation in \textit{soj} (Ireton \textit{et al.}, 1994). In vegetative cells, the lack of \textit{Spo0J} increases the number of anucleate cells indicating a defect in chromosome partitioning (Ireton \textit{et al.}, 1994). \textit{Spo0J} binds to at least 10 \textit{parS} sequences that are located in the origin-proximal 20\% of the chromosome (Lin and Grossman, 1998). Furthermore, subcellular localization experiments have demonstrated that \textit{Spo0J} forms bipolar foci at locations coincident with \textit{oriC} (Glaser \textit{et al.}, 1997; Lewis and Errington, 1997; Lin \textit{et al.}, 1997). \textit{spo0J} has also been shown to be required for maintaining the proper orientation of the chromosome entering the prespore compartment (Sharpe and Errington, 1996). These experiments suggest that \textit{Spo0J} may be important for chromosome partitioning or the positioning of the \textit{oriC} region. The sporulation deficiency in \textit{soj} mutant strains indicates an additional regulatory role for these proteins. Mutations in \textit{soj} restore the expression of early (\textit{spolII}) sporulation genes in \textit{spo0J} mutants. \textit{Soj} has been shown to repress these early sporulation genes by binding to single-stranded DNA in the open transcription complex (Cervin \textit{et al.}, 1998; Quisel \textit{et al.}, 1999; Quisel and Grossman, 2000). \textit{Spo0J} bound to the \textit{oriC} regions of partitioned chromosomes at the cell poles relieves \textit{Soj}-mediated repression. Therefore, \textit{Soj}/\textit{Spo0J} operate a simple signal transduction system that couples chromosome partitioning to the initiation of sporulation. \textit{Soj} apparently ‘senses’ the presence of bipolar \textit{Spo0J} foci by transiently localizing at one pole of the cell, and then the other (Marston and Errington, 1999; Quisel \textit{et al.}, 1999; Autret \textit{et al.}, 2001).

Experiments with the \textit{ParA} and \textit{ParB} homologues of \textit{Caulobacter crescentus} have shown that \textit{ParB} regulates \textit{ParA} activity by modulating its nucleotide-bound state (Easter and Gober, 2002). \textit{Caulobacter crescentus} \textit{ParB} also binds to several \textit{parS} sites close to the origin and localizes to the poles upon the completion of DNA replication (Mohl and Gober, 1997). In contrast to \textit{B. subtilis}, where inactivation of \textit{soj}/\textit{spo0J} results only in a mild chromosome partitioning defect, these two genes are essential for the viability of \textit{C. crescentus}. Depletion of \textit{ParB} inhibits the formation of stable \textit{FtsZ} rings which in turn results in long smooth filamentous cells (Mohl \textit{et al.}, 2001) (Fig. 1). Furthermore, overexpression of \textit{ParA} results in an almost identical filamentous phenotype. Thus, proper maintenance of the ratio of \textit{ParA} to \textit{ParB} within the cell is required for cell division. It is hypothesized that these proteins operate a checkpoint coupling chromosome partitioning to cell division (Fig. 1). Biochemical experiments have shown that these two proteins represent a regulatory pair. \textit{ParA} can stably exist in either an ADP- or ATP-bound form (Easter and Gober, 2002). The ADP-bound form binds to single-stranded DNA and presumably represses the expression of a critical cell division gene. The ATP-bound form dissociates \textit{ParB} from its \textit{parS} binding sites.

**Fig. 1.** Model depicting the coupling of chromosome partitioning to cytokinesis in \textit{C. crescentus}.

A. Shown is a schematic of a \textit{C. crescentus} swarmer cell type with a single circular chromosome attached at the pole containing the flagellum. At this early stage in the cell cycle, there exists a single partitioning complex (i.e. single origin of replication, \textit{Cor}) which contains \textit{ParB} bound to \textit{parS} sequences (Mohl and Gober, 1997). It is thought that a fraction of \textit{ParA} in the cell interacts with the partitioning complex and the remainder is distributed throughout the cell where it functions to inhibit cell division, possibly by acting as a transcriptional repressor. In this model, the formation of a second partitioning complex (\textit{ParB}–\textit{parS}) as a result of DNA replication, recruits additional \textit{ParA}, which then relieves repression, resulting in the formation of an \textit{FtsZ} ring and cell division.

B. Under conditions of \textit{ParB} depletion, all of the \textit{ParA} in the cell is free, and thus active, to inhibit division (Mohl \textit{et al.}, 2001). Increasing, the intracellular concentration of \textit{ParA} relative to that of \textit{ParB} results in a phenotype that is indistinguishable form \textit{ParB}-depleted cells, supporting the idea that two partitioning complexes are required to convert \textit{ParA} into a non-repressive form. \textit{ParA} possesses two distinct biochemical activities depending on the bound form of adenine nucleotide. \textit{ParA}-\textit{ADP} possesses a single-stranded DNA binding activity. Interaction with \textit{ParB}, which would be localized to the cell poles as a consequence of chromosome partitioning, results in the rapid exchange of ADP for ATP.
an activity which would influence the temporal transcription of the operon containing parA and parB (Easter and Gober, 2002). ParB regulates the switch between these forms by promoting nucleotide exchange. Kinetic experiments have demonstrated that following ATP hydrolysis, ParA is unable to release ADP, unless ParB is present. ParB stimulates a rapid exchange of ADP for ATP, and the cycle repeats. An increased ratio of ParA to ParB, results in fractionally more ADP-bound ParA in the cell and an inhibition of cell division (Easter and Gober, 2002).

Here we present a genetic analysis of a chromosomally encoded ParB protein. We show that ParB from C. crescentus possesses three distinct domains: (i) a carboxyl-terminal dimerization domain; (ii) a central DNA-binding, helix–turn–helix domain; and (iii) an amino-terminal domain required for the interaction with ParA. Interestingly, low level expression of some ParB alleles containing amino-terminal deletions, inhibited cell division even though full-length ParB was present, but did not result in a partitioning defect. We demonstrate that an amino-terminally truncated ParB allele fails to interact with ParA and distorts the ratio of ADP- to ATP-bound ParA inside the cell. These findings indicate that productive interaction of ParA and ParB are required for the proper regulation of cell division in C. crescentus.

**Results**

**ParB contains a carboxyl-terminal dimerization domain and a central DNA-binding domain**

ParB proteins of the prophage P1 and the F-plasmid possess at least three distinct functional domains. These individual domains interact with ParA (Radnedge et al., 1998), bind parS DNA sequences, and participate in dimerization (Lobocka and Yarmolinsky, 1996). For the chromosomally encoded partitioning proteins of C. crescentus, DNA-binding and dimerization, interaction between ParA and ParB have been demonstrated (Mohl and Gober, 1997; Easter and Gober, 2002) but the corresponding functional domains have not been defined. We wanted to identify the regions within C. crescentus ParB required for DNA-binding and dimerization. In order to accomplish this, we constructed several amino-terminal and carboxyl-terminal deletion mutants of ParB and tested these with respect to DNA-binding and dimerization (Fig. 2).

Dimerization activity was assayed in vivo in Escherichia coli using a one-hybrid technique. Full-length ParB and truncated ParB alleles were fused to an E. coli LexA mutant that lacks its dimerization domain (Porte et al., 1995; Dmitrova et al., 1998). If the test protein fusions can form dimers, the LexA portion of the fusion will bind to its binding site and repress transcription of a reporter lacZ gene. Thus, strong dimerization capacity of the test protein would result in decreasing the levels of β-galactosidase activity. For example, a control plasmid without insert DNA, resulted in relatively robust expression of the lacZ reporter, generating 827 units of β-galactosidase activity (Fig. 3A). Fusion of wild-type ParB to LexA resulted in a marked decrease in lacZ expression (52 units) (Fig. 3A). This reduction by a factor of 16 indicates that full length ParB fused to LexA can form dimers. Similarly, all amino-terminal deletions of ParB fused to LexA were equally capable of reducing lacZ transcription, indicating that the amino-terminal domain of ParB is not required for dimerization. Interestingly, some amino-terminal deletions of ParB even slightly improved the dimerization capacities of the fusion proteins, reducing the lacZ level to as low as 27 units when 172 amino acids were deleted (ParBON172) (Fig. 3A). The deletion of 172 amino acids at the amino-terminus also removes central domain residues, which contains the putative helix–turn–helix DNA binding motif (see Fig. 2). In contrast to the amino-terminal truncations, deletions at the carboxyl-terminus permitted substantial expression of lacZ. In the case of the mutant ParBΔC72 the β-galactosidase activity (1026 units) even surpassed the levels generated from the expression of LexA alone (827 units) (Fig. 3A). Deletions of 21 and 41 amino acids (ParBΔC21 and ParBΔC41, respectively) also resulted in generating significant expression of the lacZ reporter gene (Fig. 3A). These results suggest that these carboxyl-terminal sequences of ParB are required for the dimerization of the protein. To rule out that the differences in lacZ expression were

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**Fig. 2.** Dimerization and DNA binding properties of ParB deletion mutants. In full length (FL) ParB the amino-terminal domain required for interaction with ParA (light grey), the putative helix–turn–helix motif (black) and the carboxyl-terminal dimerization domain (dark grey) are indicated. Dimerization of truncated ParB proteins was assayed by a LexA-based one hybrid system (Dmitrova et al., 1998) (see Fig. 3A). For the determination of DNA-binding activity, overexpressed ParB and truncated ParB was used in gel shift analyses (see Fig. 3B). (+): dimerizes/binds DNA (−): does not dimerize/bind DNA, n.d. not determined.

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caused by the differing stability of the fusion proteins, we examined expression levels by immunoblot using ParB-specific antibody. All fusions showed approximately equal expression levels with the exception of ParB\textsubscript{D\textsubscript{N80}}, which could not be detected by immunoblot, and therefore was not included in the analysis (data not shown).

To investigate the role of dimerization in DNA-binding, purified His-tagged full-length ParB and His-tagged fusions of the deleted alleles were tested in gel mobility shift assays using a 74 bp DNA fragment with a \textit{parS} sequence from the \textit{gidABparAB} promoter region as substrate (see Experimental procedures). Full-length ParB readily formed a gel mobility shift complex with \textit{parS} DNA (Fig. 3B). Amino-terminal deletions of up to 107 amino acids (ParB\textsubscript{D\textsubscript{N107}}) significantly reduced, but did not completely abolish DNA binding. This result is consistent with the predicted position of the helix–turn–helix DNA binding motif, between amino acid residues 150 and 171, as determined using a previously described algorithm (Dodd and Egan, 1990). Mutant protein ParB\textsubscript{D\textsubscript{C72}} exhibited a complete loss of DNA-binding activity. Interestingly, however, a mutant ParB with a deletion of 21 amino acids (ParB\textsubscript{D\textsubscript{C21}}) at the carboxyl-terminus retained DNA binding activity (Fig. 3B). This deletion had a significant effect on dimerization, as determined by the two-hybrid assay (Fig. 3A), suggesting that strong dimer-
Expression of amino-terminal deletion mutants of ParB results in the inhibition of cell division

We next wanted to determine if these mutant parB deletion alleles could function in C. crescentus cells. To accomplish this, we tested whether expression of the truncated ParB proteins could complement the cell division defect in a ParB depletion strain (UC9031) (Mohl et al., 2001). In this strain, the sole copy of parB gene is under the control of an xylose inducible promoter. The wild-type and the deleted alleles of parB were placed downstream of the E. coli lac promoter in plasmid pMR4, and introduced into UC9031 and the cells were plated on media lacking xylose. In contrast to the plasmid containing full-length parB, all of the deletion mutants were unable to complement the lethality of ParB depletion. The deleted alleles, parBΔC21, parBΔC41, parBΔC72, parBΔN107, and parBΔN12, were expressed at much lower levels than endogenous parB when introduced into wild-type C. crescentus (data not shown), thus we were unable to determine whether these deletions were functional in vivo. During the course of these experiments, we found that mutants parBΔN40 and parBΔN80 on pMR4 could not be introduced into C. crescentus either by conjugation or transformation. To investigate this phenomenon we cloned these alleles behind the inducible xyiX promoter in the low-copy plasmid (pAA74) and introduced the corresponding plasmids into wild-type C. crescentus (LS107). In the absence of xylose, the cells containing both deleted alleles appeared morphologically similar to wild-type cells, but started to filament when xylose was added to a concentration of 0.125% (Fig. 4A). Following 6 h of induction, swarmer cells were nearly absent from mid-log phase cultures and the cells had grown to long smooth filaments, indicating that cell division was blocked at an early stage (Fig. 4A). In contrast, no change in cell morphology could be observed when wild-type parB (Fig. 4A) and other parB alleles were tested in this experiment (data not shown), suggesting that parBΔN40 and parBΔN80 were behaving as dominant negative alleles. One possible cause of this dominant negative phenotype was that parBΔN40 and parBΔN80 were expressed at higher levels than the other parB alleles. Previous experiments have shown that overexpression of wild-type parB can result in a relatively severe cell division defect (Mohl and Gober, 1997). Surprisingly, all of the deleted ParB mutant proteins were expressed at much lower levels than the endogenous wild-type ParB (Fig. 4B). This observation together with the fact that parBΔN40 and parBΔN80 when expressed from a lac promoter are lethal, indicate that these two alleles are detrimental for cell viability.

In E. coli, a critical concentration of FtsZ is required for the initiation of cell division (Dai and Lutkenhaus, 1991). We wanted to determine if the cell division defect observed during the expression of the amino-terminal dominant negative mutants of parB was caused by a decrease in the FtsZ concentration. We expressed parBΔN40 in wild-type C. crescentus and determined the cellular levels of FtsZ by immunoblot with FtsZ-specific antibody (Mohl et al., 2001) (Fig. 5). Samples were taken before induction (0 h) and every two hours after induction for a total of 10 h. During the course of this experiment, the FtsZ level remained nearly constant after 2 h of inducing parBΔN40 expression and decreased by almost 50%
over time (Fig. 5). We also determined if changes in the ratio of ParA and ParB concentrations changed upon the expression of parBΔN40. ParA and ParB concentrations have been shown to be critical for the initiation of cell division (Mohl et al., 2001; Easter and Gober, 2002). The mutant ParBΔN40 was detectable following 2 h of induction and remained at relatively the same level during the experiment. The concentration of wild-type ParB did not change upon induction of parBΔN40 (Fig. 5). Likewise, expression of parBΔN40 did not affect cellular ParA levels (Fig. 5). These results indicate that the observed cell division defect is not caused by changes in ParA and ParB concentrations, but may be attributable to the decrease in FtsZ levels. Because the expression of parBΔN40 and parBΔN80 resulted in a filamentous phenotype that is similar to that observed when ParB is depleted from cells (Mohl et al., 2001), we hypothesize that these mutant proteins interfere with the function of wild-type ParB.

Effect of ParB depletion and the expression of dominant parB alleles on the positioning of the origin of replication

Caulobacter crescentus exhibits a rapid partitioning of the origins of replication (Cori) toward the cell poles shortly following the initiation of DNA replication (Jensen and Shapiro, 1999). At least five ParB binding sites lie within 20 kb of the origin (J. Easter, R.M. Figge, and J.W. Gober, in preparation) and ParB forms a large nucleoprotein complex with the origin proximal region, suggesting that this complex may play a role in tethering the Cori to the cell poles. If this is the case, ParB movement to the poles should be coincident with the movement of the Cori region. In order to test this idea, we assayed the subcellular localization of the Cori region using fluorescence in situ hybridization (FISH), and simultaneously examined ParB localization using immunofluorescence in a synchronized population of wild-type C. crescentus cells. As previously reported (Jensen and Shapiro, 1999), in isolated swarmer cells, before the initiation of DNA replication, the cells contained one Cori localized at a pole of the cell (Fig. 6). We found that a focus of ParB almost always co-localized with the labelled Cori [97.7% of stained Cori had co-localized ParB (n = 211)] (data not shown) (Fig. 6). Following the initiation of DNA replication, the newly replicated Cori region was located at the opposite pole and ParB was observed to co-localize with both of the origins of replication during the entire cell cycle (Fig. 6). To assess whether ParB has a role in orienting the Cori region toward the poles, we investigated whether the movement or positioning of the origins was disturbed when cells were depleted of ParB. ParB depletion has been shown to cause a cell division block leading to filamentation (Mohl et al., 2001). In the long filaments of ParB-depleted cells, the origins were still moved apart but lacked a regular pattern of localization (Fig. 7A). This aberrant positioning could be the result of a mild partitioning defect resulting from ParB depletion. Filamentation also occurred upon expression of the dominant-negative deletion mutants of ParB (see Fig. 4A). In order to determine if this phenotype was attributable to an aberrantly positioned Cori region, we assayed Cori localization in a strain expressing the dominant parBΔN40 allele. Cells were induced for 2 h with xylose, synchronized and allowed to proceed through the cell cycle (Fig. 7B). As with the ParB depletion strain, some of the origins were mislocalized (Fig. 7B, last panel). The same result was obtained when ParA was overexpressed (data not shown). Therefore, we hypothesize that ParB activity may be important in the polar positioning of the origin of replication region.

A dominant-negative, amino-terminal deletion allele of ParB fails to interact with ParA

In bacteriophage P1, interaction of ParB and ParA involves the amino-terminal domain of ParB and is required for replicon partitioning (Radnedge et al., 1998). In the experiments presented here, the filamentous phenotype resulting from the expression of either ParBΔN40 or ParBΔN80 could be a consequence of the failure of these two mutant proteins to interact with ParA. In order to test this idea, we performed an in vitro assay for ParA interaction using purified ParBΔN40. In vitro experiments have demonstrated that ATP-bound ParA is capable of dissociating ParB from its parS DNA binding site (Easter and Gober, 2002). We determined whether ParA-ATP could abolish the DNA binding activity of ParBΔN40 using
Dominant-negative ParB alleles

a surface plasmon resonance experiment (SPR) with an oligonucleotide containing parS sequences immobilized to the sensor chip. In a control experiment, purified, wild-type ParB formed a stable complex with the immobilized parS DNA (Fig. 8A) ($t_{1/2}$ of the ParB-DNA complex = 11 min). However, when ParB was premixed with ParA-ATP, before application to the sensor chip, there was no apparent DNA binding activity (Fig. 8A). When purified ParBΔN40 was applied to the sensor chip, it bound to the immobilized parS oligonucleotide with slightly lower affinity as wild-type ParB ($t_{1/2}$ = 8 min) (Fig. 8B). This observation, together with the results of the dimerization and gel mobility shift assays presented above, indicates that the central DNA binding domain and the carboxyl-terminal dimerization domain are still functional in ParBΔN40. We next determined the effect of premixing of ParA-ATP with ParBΔN40 on its DNA binding properties. In contrast to the results obtained with wild-type ParB, the addition of ParA-ATP to ParBΔN40 did not reduce the amount of protein bound to the immobilized parS DNA (Fig. 8B). These results indicate that ParA-ATP is probably unable to productively interact with ParBΔN40.

The levels of ParBΔN40 and ParBΔN80 required to cause a severe filamentous cell phenotype are quite low compared to the amount of wild-type ParB present (see Fig. 4). This observation suggests that the low concentration of deleted ParB protein is interfering with function of the wild-type protein. Because the dimerization domain is still intact in these mutants, one plausible possibility is that the deleted ParB derivatives are forming non-functioning heterodimers with wild-type ParB. To test this idea, we performed a mixing experiment where equal concentrations of ParBΔN40 and wild-type ParB were combined and then applied to the sensor chip containing the immobilized parS DNA (Fig. 8C). The simultaneous addition of both ParBΔN40 and wild-type ParB to the sensor chip resulted in a stable complex with the immobilized DNA ($t_{1/2}$ = 13 min). Premixing these two proteins with ParA-ATP had no effect on their binding ($t_{1/2}$ = 13 min) (Fig. 8C). In order to determine whether lower amounts of ParBΔN40 relative to wild-type ParB could have a similar effect, we performed the same mixing experiment using three times more wild-type ParB than ParBΔN40 (Fig. 8C). In this case there was less total protein bound than when equal amounts of ParB and ParBΔN40 were mixed, however, the complex that was formed with the DNA was quite stable ($t_{1/2}$ = 15 min). This complex apparently binds to the immobilized DNA with significantly higher affinity than ParBΔN40 ($t_{1/2}$ = 15 min versus 8 min) suggesting that the species bound to the chip in this experiment is not solely ParBΔN40. Because wild-type ParB cannot bind DNA in this experiment, we hypothesize that the species bound here may be heterodimers of ParB and ParBΔN40. This result is consistent with the hypothesis that the presence...
of ParBΔN40 renders wild-type ParB DNA binding activity insensitive to ParA-ATP.

Previous experiments have demonstrated that ParB serves as a nucleotide exchange factor for ParA (Easter and Gober, 2002). Following ATP hydrolysis, ParB is required to stimulate the exchange of ADP for ATP. As the in vitro experiments presented above indicated that the presence of ParBΔN40 interfered with the interaction with ParA, we wanted to test whether this dominant negative mutant could influence the nucleotide bound state of ParA in living cells. Cultures were grown for six generations in the presence of $^{32}$PO$_4$-3. ParA-nucleotide complexes were immunoprecipitated from cell extracts and the bound radioactive nucleotides quantitated after separation by thin-layer chromatography. Cells expressing parBΔN40 had only 40% of the ParA in the ATP bound form (Fig. 8D). This is in contrast to wild-type cells grown with or without a control plasmid (pAA74l), in which 82% of ParA was in the ATP bound form (Fig. 8D). As the intracellular concentration of ParB does not change under these conditions (see above) this finding strongly supports our assumption that the expression of ParBΔN40 disturbs ParA/ParB interaction. This may be attributable to the formation of heterodimers that reduce the amount of functional ParB in the cell. Based on these results we conclude that interaction of ParA with the amino-terminus of ParB is required.

Fig. 7. Origin localization using FISH upon depletion of ParB and expression of ParBΔN40.
A. Localization of origins using FISH and FtsZ using immunofluorescence upon depletion of ParB. The strain UC9031, a conditional parB mutant (Mohl et al., 2001) was grown to mid-logarithmic phase in PYE supplemented with 0.125% xylose. Cells were harvested and washed three times with PYE before culturing them in PYE without xylose. A constant OD$_{600}$ = 0.3–0.6 was retained by diluting the culture during 10 h of growth. At the indicated time points cells were fixed and hybridized with a Cy3-origin probe (pink dots) before visualizing FtsZ localization using immunofluorescence staining with a secondary antibody labelled with Alexa-Flour 488 (green dots). The arrows indicate irregular origin spacing.
B. Cell cycle localization of origins by FISH and ParB by immunofluorescence upon expression of ParBΔN40. Cells containing the parBΔN40 allele on a multicopy plasmid were grown to an OD$_{600}$ = 0.5 in PYE. Xylose was added to 0.125% and cells continued to grow for 2 h. Swarmer cells were isolated, washed with M2 salts containing xylose and allowed to progress through the cell cycle. At the indicated time points cells were fixed and hybridized with a Cy3-labelled Cori probe and ParB was visualized using immunofluorescence staining using a secondary antibody labelled with Alexa-Flour 488. The arrow indicates irregular origin spacing. A schematic depiction of the cell cycle is shown. Chromosomes are represented as blue circles, the localization of the origin regions and ParB localization is shown in red and green respectively.
for nucleotide exchange and thus for the regulation of cell division.

Discussion

Evidence suggests that chromosomally encoded homologues of ParA (SopA) and ParB (SopB) originally found to be required for partitioning of prophage P1 and F factor plasmids may also play an important role in partitioning bacterial chromosomes (reviewed in Gordon and Wright, 2000; Draper and Gober, 2002). We have performed a detailed analysis of the ParB chromosome partitioning protein from *C. crescentus*. We have isolated mutants in the carboxyl-terminus that are deficient in dimerization, in the central region that have defects in DNA-binding, and amino-terminal deletions that disrupt interaction with

![Fig. 8](image.png)

Fig. 8. The dominant negative mutant ParBΔN40 fails to interact with ParA. (A-C) A biotinylated 26 bp double stranded oligonucleotide containing sequence of the ParB-binding site within the gidAparAB operon promoter region was immobilized on a streptavidin sensor chip (Easter and Gober, 2002). Surface plasmon resonance was used to investigate the effects of ATP-bound ParA on ParB DNA binding activity. Reaction components were preminixed immediately before injection at (5 μl, 25°C) with a flow rate of 5 μl min⁻¹. Proteins were at a final solution concentration of 1.5 μM before injection. A typical sensorgram is shown for injections of:

A. ParB and ParB premixed with ParA-ATP.

B. ParBΔN40 and ParBΔN40 premixed with ParA-ATP.

C. Equimolar amounts of ParBΔN40, ParB with and without ParA-ATP, and ParBΔN40, ParB with ParA-ATP premixed at a ratio of 0.75:0.25:1.0.

D. Effect of ParBΔN40 on the ParA nucleotide binding in vivo. The ratio of ParA-ATP/–ADP was determined in mid-logarithmic cultures expressing *parBΔN40*. Cultures of *C. crescentus* either with or without a xylose-inducible *parBΔN40* were grown for five generations in low phosphate media to mid-log phase (OD₆₀₀ = 0.35) in the presence of 32PO₄³⁻ (0.4 mCi ml⁻¹). The cells were harvested, washed with 1.5 M NaCl, lysed with 5 mg ml⁻¹ lysozyme (15 min, 4°C), and rapidly frozen in liquid nitrogen. ParA was immunoprecipitated by adding Protein A-agarose and anti-ParA antibody to the extracts. Radioactive nucleotides were recovered from immunoprecipitated ParA with perchloric acid, separated by TLC, and visualized by autoradiography. The developed nucleotides on the TLC plates were quantitated for the amount of radioactivity present by phosphorimager analysis. The percentages represent the mean values from at least three different experiments. The standard deviation in all cases was less than 4.0%. Intracellular ATP concentrations were determined from cell extracts using a bioluminescence kit as described (Easter and Gober, 2002).
ParA. Interestingly, expression of a ParB deletion mutant that fails to interact with ParA inhibits cell division. This phenotype is most likely not caused by a chromosome partitioning defect, but rather by the inability of the Par proteins to couple Cori positioning to cell division.

The biochemical functions of the three ParB domains
ParB of prophage P1 and SopB of F-plasmids have both been shown to form dimers in solution (Funnell, 1988) and when bound to DNA (Davis and Austin, 1988; Funnell and Garnier, 1993; Hayes and Austin, 1993). In order to assess the dimerization capability of C. crescentus ParB, we tested the capacity of deletion mutants to promote the dimerization of LexA lacking its cognate dimerization domain. Our results show that C. crescentus ParB forms dimers only if its carboxy-terminus is present (Fig. 3). Similarly, in P1, the carboxy-terminus of ParB is required for dimerization (Lobocka and Yarmolinsky, 1996; Surtees and Funnell, 1999). In contrast to P1 ParB, where a second dimerization domain was identified in the amino-terminus (Surtees and Funnell, 1999), amino-terminal deletions in C. crescentus ParB increased the dimerization capacity. In order to test whether dimerization was required for DNA-binding, we used purified ParB deletion mutant proteins in gel mobility shift analyses. Like ParB from P1, a portion of the carboxy-terminus was dispensable for DNA-binding (Surtees and Funnell, 2001). However, a deletion of 72 amino acids from the carboxy-terminus resulted in the complete absence of DNA-binding activity, which also correlated with the loss of the ability to dimerize (Fig. 3). Thus, dimerization capacity provided by the carboxy-terminus is crucial for DNA binding. Because the amino-terminus and part of the carboxy-terminus is dispensable for DNA-binding, the DNA binding domain of C. crescentus ParB must be localized within the central residues of the protein. This is consistent with a computer analysis (Dodd and Egan, 1990) that locates a putative helix–turn–helix motif between amino acids 150 and 171. For the B. subtilis homologue Spo0J, it has been demonstrated that point mutations within this central region abolish DNA binding activity (Aauret et al., 2001). Similarly experiments with P1 ParB have shown that DNA-binding activity is localized in the central domain (Lobocka and Yarmolinsky, 1996).

Previous experiments have demonstrated that C. crescentus ParA-ATP interacts with ParB and dissociates the ParB-parS nucleoprotein complex (Easter and Gober, 2002). The experiments presented here show that an amino-terminal deletion of 40 amino acids of ParB (ParB\textsubscript{N40}) is sufficient to suppress the interaction with ParA so that ParB\textsubscript{N40} cannot be removed from parS. We also assayed the effect of ParA when ParB\textsubscript{N40} and wild-type ParB were premixed. Surprisingly, we observed that the addition of ParA did not significantly reduce the amount of protein bound, indicating that most of ParB\textsubscript{N40} and ParB bound to DNA in the presence of ParA-ATP. Based on the fact that both ParB\textsubscript{N40} and wild-type ParB are capable of forming homodimers (see Fig. 1B), we hypothesize that mixing of the two ParB species leads to the formation of ParB\textsubscript{N40} and wild-type ParB heterodimers. These heterodimers probably cannot interact with ParA and therefore remain bound to DNA in the presence of ParA-ATP. We hypothesize that ParB/ParB\textsubscript{N40} heterodimer formation in vivo may reduce the amount of wild-type ParB that can interact with ParA–ATP. Interaction between ParB and ParA has been shown to regulate the transcription of the gid\textsubscript{AB}par\textsubscript{AB} operon in C. crescentus (Easter and Gober, 2002). ParB binds several sites upstream of gid\textsubscript{A} thereby repressing transcription (J. Easter and J. W. Gober, unpublished). ParA is capable of dissociating these ParB-DNA complexes: overexpression of ParA led to a sixfold increase in the activity of the gid\textsubscript{AB}par\textsubscript{AB} promoter (Easter and Gober, 2002). Even though ParB\textsubscript{N40} and ParB/ParB\textsubscript{N40} heterodimers are probably refractory to this feed back regulation, we did not see any changes in ParA and ParB concentrations upon the expression of ParB\textsubscript{N40}. This indicates that other factors such as proteolysis may also regulate the abundance of ParA and ParB.

Signalling or partitioning? The role of ParA and ParB in Caulobacter
We found that expression of low levels of ParB\textsubscript{N40} and ParB\textsubscript{N80} led to a relatively rapid inhibition of cell division. Initially we reasoned that this may result from the failure to partition chromosomes correctly. However, FISH experiments indicated that expression of ParB\textsubscript{N40} did not affect the separation of the origins of replication. Similar results were obtained in a ParB depletion strain demonstrating that presence of ParB is not required for origin movement. Nevertheless, we cannot exclude that ParB plays a role in the fine organization and localization of the chromosome. This seems plausible as in B. subtilis the transport of the chromosome into the forespore is significantly altered in the absence of ParA and ParB (Sharpe and Errington, 1996). Additionally, we observed that some filaments did not have a regular distribution of origins in ParB-depleted cells, which may be indicative of a subtle partitioning or chromosome organization defect.

Easter and Gober (2002) demonstrated that the intracellular ratio of ATP- versus ADP-bound ParA is reversed upon the overexpression of ParA, which results in filamentation. Because expression of ParB\textsubscript{N40} most likely leads to the formation of heterodimers and thus would sequester wild-type ParB from interacting with ParA, we investigated whether the ratio of ADP- to ATP-bound ParA would...
be altered. We found that the expression of ParB∆N40 led to a significant increase of ADP-bound ParA. This provides strong evidence that in vivo interaction between the two proteins is required for nucleotide exchange. This observation together with the finding that ParA and ParB levels inside the cell remain nearly unchanged upon expression of ParB∆N40 demonstrates that the ratio between ADP and ATP-bound ParA inside the cell is critical for the initiation of cell division.

In *B. subtilis* the ParA homologue (Soj) oscillates between the poles and this movement is dependent on the presence of polar SpoOJ (ParB) foci (Marston and Errington, 1999; Quisel et al., 1999; Autret et al., 2001). It was shown that some mutations in SpoOJ increase the oscillation frequency of Soj, indicating that the interaction between the two proteins is crucial for the movement of Soj (Autret et al., 2001). We have been unable to test whether *C. crescentus* ParA also oscillates from pole-to-pole owing to the lethality of ParA-Gfp fusions. However, we consider it likely that ParA in *C. crescentus* may also oscillate, as another highly related ATPase, MinD, has also been shown to behave in this fashion (Raskin and de Boer, 1999). Recent experiments have demonstrated that ParB stimulates nucleotide exchange in ParA in vitro (Easter and Gober, 2002). Increasing amounts of ParA-ADP probably lead to changes in the dynamic properties of ParA. Because *C. crescentus* lacks the MinCDE system that controls the positioning of cell division in *E. coli* and *B. subtilis*, it may be that *C. crescentus* ParA and ParB fulfill a similar role in division site selection that uses chromosome localization as the input signal. It will be interesting to see if ParA-ADP interacts directly with the cell division machinery while oscillating or functions as a transcription factor as was shown in *B. subtilis* (Cervin et al., 1998).

**Experimental procedures**

**Strains and plasmids**

All *C. crescentus* strains were derived from LS107, an ampicillin-sensitive, synchronizable strain (Stephens et al., 1997). *Caulobacter crescentus* strains were grown in peptone-yeast extract (PYE) (Poindexter, 1964) or M5-medium at 31°C supplemented with the appropriate antibiotics. For the construction of pAA74I a 834 bp fragment of the xylose promoter was amplified using polymerase chain reaction (PCR) (5'-ATCGATACCATGGTCTCGAACAGGGCCG-3' and 5'-AAGCTTAGATC-CCGGGACGCTACTTG-3') and cloned into the *EcoRI* site of pBluescript KSII (Stratagene). PCR-generated fragments were sequenced using the dideoxy chain termination method (Big dye, Perkin Elmer).

**Overproduction of ΔParB-his6 and ParB∆N40-GST**

Strain BL21(DE3) was transformed with each of the expression plasmids. Cultures (100 ml to 2 L depending on the expression levels) of the resulting strains were grown in LB medium until they reached an OD_{600} of 0.4–0.6 and induced by the addition of IPTG (1 mM final concentration). For the overproduction of wild-type ParB and ParB∆C72 cells were grown at 37°C and harvested after 1 h. ParB∆N40, ParB∆N107 was purified from non-induced stationary phase cultures grown at 25°C. ParB∆C21 was purified from cultures grown at 25°C after 12 h induction. All ParB proteins were purified using nickel affinity chromatography according to the Quiaexpressionist (Quiagen, Hilgen, Germany). Overproduction of ParB∆N40-GST was performed in BL21 (DE3). Cells were grown at 30°C and induced with 0.1 mM IPTG for 3 h. ParB∆N40 fusion protein was purified using glutathione-charged sepharose according to the instruction of the supplier (Amersham Pharmacia Biotech). Thrombin-digested ParB∆N40-GST was applied to a heparin-sepharose column, washed with buffer [10 mM Hepes (pH 7.4), 150 mM NaCl] and eluted with 10 mM Hepes (pH 7.4), 600 mM NaCl. For SPR experiments ParB∆N40 was dialysed against 50 mM Hepes (pH 7.5), 50 mM KCl, 10% (v/v) glycerol, 1 mM DTT. ParA was overproduced as described in Easter and Gober (2002).

**Gel mobility shift assays**

The EcoRI fragment harbouing parS was isolated from pAA45 and 32P-labelled using Klenow enzyme. Purified ParB protein (0.5–5 µg) was incubated in binding buffer [7.5 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, 50 mM NaCl, 0.1 mg ml⁻¹ poly dl/dc, 0.4 mg ml⁻¹ BSA, 10% glycerol] for 15 min at room temperature before the addition of 2000–5000 c.p.m. of the radiolabelled DNA. The assay was then...
resolved on a 5% polyacrylamide gel using 25 mM Tris (pH 8.3), 0.19 M glycine, 1 mM EDTA (pH 8.0) as the running buffer. Gels were dried and exposed to Phosphor storage screens (Molecular Dynamics), and visualized by a Phosphorimager (Molecular Dynamics).

Fluorescent in situ hybridization (FISH) and immunofluorescence microscopy

Fluorescent in situ hybridization was essentially performed as described in Jensen and Shapiro (1999). For the generation of an origin probe lacking the parB gene, plasmid pGM1342 (G. Marczynski, unpublished) was digested with EcoRI and BspHI and the 10 kb fragment subcloned into pNPTS129, generating pRF66. This plasmid served as the source of a 10 kb XbaI/EcoRI fragment that was isolated from agaroose gels using gene clean (Bio101, Vista, California) and subsequently labelled as described (Jensen and Shapiro, 1999). ParB and FtsZ localization was performed after FISH labelling as described previously (Mohl and Gober, 1997; Mohl et al., 2001).

DNA binding assays and interaction studies

All DNA binding experiments and ParA/ParB interaction experiments were done by surface plasmon resonance (SPR). Conditions were as described in Easter and Gober (2002). ParB5N40 was purified as a GST fusion (see above). In order to determine the half-life of ParB-DNA complexes in the SPR experiments, the rate of decrease in response units over time was determined after stabilization of the instrument following the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see above). To determine the half-life of ParB-DNA complexes in the SPR experiments, the rate of decrease in response units over time was determined after stabilization of the instrument following the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduction of wash buffer, usually between 150 and 200 s in the run (see Fig. 8). To assess the dimerization properties of ParB different LexA-ParB fusions were generated (see plasmids). Dimerization of LexA correlates with a reduction in LacZ levels which were measured in the introduc

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