Bacterial DNA segregation dynamics mediated by the polymerizing protein ParF

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Prokaryotic DNA segregation most commonly involves members of the Walker-type ParA superfamily. Here we show that the ParF partition protein specified by the TP228 plasmid is a ParA ATPase that assembles into extensive filaments *in vitro*. Polymerization is potentiated by ATP binding and does not require nucleotide hydrolysis. Analysis of mutations in conserved residues of the Walker A motif established a functional coupling between filament dynamics and DNA partitioning. The partner partition protein ParG plays two separable roles in the ParF polymerization process. ParF is unrelated to prokaryotic polymerizing proteins of the actin or tubulin families, but is a homologue of the MinD cell division protein, which also assembles into filaments. The ultrastructures of the ParF and MinD polymers are remarkably similar. This points to an evolutionary parallel between DNA segregation and cytokinesis in prokaryotic cells, and reveals a potential molecular mechanism for plasmid and chromosome segregation mediated by the ubiquitous ParA-type proteins.

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Introduction

The molecular events that promote accurate chromosome segregation in eukaryotes are well understood. In contrast, the processes involved in faithful distribution of bacterial genomes at cell division have been elaborated less thoroughly. Nevertheless, it is clear that bacterial plasmids and chromosomes are partitioned in an orderly fashion that requires the participation of dedicated segregation proteins and *cis*-acting DNA sequences (Draper and Gober, 2002; Surtees and Funnell, 2003).

Plasmid partition systems broadly are of two types, involving either an ATPase with a deviant Walker-type ATP-binding motif (generally denoted ParA), or an ATPase that is a member of the actin protein family (Motallebi-Veshareh *et al*, 1990; Bork *et al*, 1992). Proteins of the former class, which

are much more prevalent, are also specified by many bacterial chromosomes (Hayes, 2000). The ATPase activity of ParA proteins is required for successful DNA segregation (Davis *et al*, 1996; Libante *et al*, 2001). In ParA-type systems, a second protein (often termed ParB) specified by the partition operon binds to the *cis*-acting partition site located near to the operon (Surtees and Funnell, 2003). The ATPase does not directly contact partition site DNA, but is instead recruited into the partition nucleoprotein complex through interactions with ParB (Bouet and Funnell, 1999; Barillà and Hayes, 2003). For ParA-type systems, recent evidence suggests that plasmid copies align at midcell shortly before cell division and are propelled bidirectionally along the cell axis by the partition apparatus. Subsequent cell division compartmentalizes the plasmids within new daughter cells (Li and Austin, 2002). In contrast, the actin-type protein of plasmid R1 apparently promotes partition by generating filaments that direct replicated plasmids towards the cell poles (Møller-Jensen *et al*, 2002; van den Ent *et al*, 2002). It remains to be elucidated whether bacterial DNA segregations mediated by Walker- and actin-type ATPases are mechanistically distinct.

The multidrug resistance plasmid TP228 replicates at low copy number in *Escherichia coli*. The partition cassette of TP228 consists of the *parFG* genes and upstream noncoding sequences that harbour a series of repeat motifs. ParG (8.6 kDa), which is unrelated evolutionarily to ParB (Hayes, 2000), is a dimer and binds the upstream region (Barillà and Hayes, 2003). ParG consists of an unstructured N-terminal tail and a folded C-terminal domain that contains a ribbon-helix-helix motif that contacts DNA (Golovanov *et al*, 2003).

The ParF protein (22 kDa) interacts with ParG and modulates binding of the latter to the upstream DNA region (Barillà and Hayes, 2003). ParF is the prototype of a phylogenetically distinct subgroup within the ParA superfamily of Walker-type ATPases. In fact, ParF is more closely related to the MinD cell division site-selection protein than to well-characterized ParA partition proteins such as ParA, SopA and Soj specified by the P1 and F plasmids, and the *Bacillus subtilis* chromosome, respectively (Hayes, 2000). MinD, in conjunction with MinC, prevents placement of the cell division septum at all locations in *E. coli*. This inhibition is relieved specifically at midcell by the MinE protein thereby allowing proper cell division to proceed. Division inhibition by MinCD is mediated by their rapid oscillation between the cell poles in a MinE-dependent manner (Raskin and de Boer, 1999; Fu *et al*, 2001; Hu and Lutkenhaus, 2001). Oscillation apparently occurs along a permanent, but dynamic MinCDE spiral framework that extends along the entire cell length (Shih *et al*, 2003). This oscillation correlates with polymerization of MinD into long filaments *in vitro* modulated by ATP, phospholipids and MinE (Hu *et al*, 2002; Suefuji *et al*, 2002).

Here, we show that the purified ParF protein assembles into extensive filaments when incubated with ATP. These

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filaments are remarkably similar to those of the evolutionarily related MinD protein, but are quite distinct from those of the unrelated FtsZ tubulin homologue involved in cell division and the actin-type partition protein of plasmid R1. By analogy with the Min system, a smaller protein, ParG, modulates the polymerization of ParF as well as stimulating its ATPase activity. These results point to a possible mechanistic parallel between DNA segregation and cell division in prokaryotes, and suggest a molecular mechanism for DNA segregation by ParA-type proteins.

Results

ParF is a Walker-type ATPase of the ParA superfamily

The functionally diverse Walker superfamily of ATPases is characterized by the signature A motif GxxxxGKS/T that is involved in contacts with the triphosphate moiety of ATP (Leipe *et al*, 2002). A number of subgroups of the Walker family are distinguished by a deviant A box, GKGhGKS/T (Motallebi-Veshareh *et al*, 1990). ParF is a member of one of these subgroups, the ParA family, that mediates bacterial plasmid and chromosome partitioning (Hayes, 2000). The ATPase activity of ParF was assessed by thin-layer chromatography (TLC) (Figure 1B–E). The protein has a weak intrinsic

ATPase activity like other ParA family members (Davis *et al*, 1996; Fung *et al*, 2001; Libante *et al*, 2001). The $K_{0.5}$ for ATP is approximately 100 μ M, which is in the same range as the K_M values for other examined ParA proteins (Davey and Funnell, 1997; Easter and Gober, 2002).

ParF assembles into filaments, and polymerization is potentiated by ATP

Ultracentrifugation studies hinted that the ParF protein has a propensity to polymerize *in vitro*, and that ATP enhances this polymerization (Barillà and Hayes, 2003). This was investigated further by conducting sedimentation assays in which ParF (8 μ M) was incubated in the presence or absence of nucleotides for 10 min at 30°C, separated by centrifugation into pellet and supernatant fractions, and analysed by SDS-PAGE. If the protein assembles into filaments of sufficiently high molecular weight, it will enter the pellet, whereas unpolymerized or partially polymerized protein will remain in the supernatant. In the absence of nucleotides, ~30% of ParF was recovered in the pellet (Figure 2A). In contrast, ~60% of ParF was pelleted after incubation with ATP, indicating that the protein had formed an increased quantity of polymeric species. To assess whether ATP binding was sufficient for polymerization, ParF was incubated with the

Figure 1 ParF harbours a deviant Walker A motif and exhibits ATPase activity. (A) Schematic representation of the ParF protein displaying the Walker A and B motifs with an alignment of Walker A boxes of ParF and other ParA superfamily members. Amino-acid residues subjected to mutagenesis are numbered and indicated by asterisks. (B, C) ATP hydrolysis plotted as a function of high (B) and low (C) protein concentration with ATP at 250 μ M. (D, E) ATPase experiments performed with proteins (4 μ M) at 50–1000 μ M (D) and 0.05–1.0 μ M (E) ATP concentration.

Figure 2 ParF polymerizes and mutations in the Walker A box perturb the polymerization profile. ParF (**A**), ParFK15Q (**B**) and ParFG11V (**C**) were incubated in the absence (–) or presence of nucleotides and the reactions were then centrifuged. In all, 100 and 33%, respectively, of the pellet (P) and supernatant (S) fractions were resolved on a 12% SDS gel and stained with Coomassie blue. The percentages of ParF protein detected in the pellet fractions are shown. (**D**) ATP stimulates ParF assembly into polymeric structures. ParF polymerization was detected by DLS. The bottom panels illustrate the increase in light scattering intensity, expressed as kct/s, upon nucleotide addition (arrow). The top panels show the corresponding augmentation in polymer average size (nm). Black, no nucleotide added; green, ADP (500 μ M); blue, ATP (500 μ M); red, ATP γ S (500 μ M). (**E**, **F**) ParFK15Q and ParFG11V analysed as described for ParF in panel D. Note the difference in vertical scale in the three panels.

nonhydrolysable analogue adenosine-5-*O*-(3-thiotriphosphate) (ATP γ S). Virtually all of the input ParF protein ($\sim$ 95%) sedimented, showing that ATP γ S efficiently promotes ParF sedimentation, but that nucleotide hydrolysis is unnecessary for polymerization. Interestingly, the amount of ParF in the pellet was reduced to $\sim$ 15% in the presence of ADP, which suggests that this nucleotide antagonized polymerization and perhaps even reversed it (Figure 2A). A corollary of these studies is that the binding of di- or triphosphate nucleotides is likely either to mediate significant conformational changes in ParF or to modify the monomer-monomer interface.

The critical concentration required for ParF assembly in the presence of 2 mM ATP was determined to be $0.34 \pm 0.05 \mu$ M at 14 000 r.p.m. This value was unaltered at 50 000 r.p.m. (Figure 3). As determined from the slopes of these lines, equivalent amounts of the protein enter the pellet fractions at both centrifugation speeds, indicating that $\sim$ 60% of ParF assembles into polymers under these reaction conditions. Similarly, a survey of centrifugation speeds showed that, at concentrations above the critical value, $\sim$ 60% of ParF entered the pellet fraction at 14 000 r.p.m. This percentage progressively diminished as the speed was reduced, declining to $\sim$ 13% at 1000 r.p.m.

Dynamic light scattering (DLS), which allows an assessment of both particle abundance and size, was used to further analyse the polymerization of ParF. DLS differs from static light scattering in that it measures the hydrodynamic radius of a protein. The average hydrodynamic size is the diameter of a sphere that has the same diffusion coefficient as that of the particle being measured. In the absence of nucleotides at

30°C, ParF protein (2.16 μ M) remained stable for $\sim$ 12 min with an average intensity of 70–80 kilocounts/second (kct/s) and a particle size of $\sim$ 25 nm (Figure 2D). This average size is likely to represent an oligomeric, prefilamentous form of the protein, which correlates with previous results (Barillà and Hayes, 2003). Subsequently, the intensity of light scattering increased steadily, reaching a final value of $\sim$ 2000 kct/s after 40 min (Figure 2D, bottom). In parallel, the size of the particles increased to $\sim$ 450 nm (Figure 2D, top). These results indicate that ParF has an intrinsic tendency to polymerize.

When ATP (500 μ M) was added to ParF, the count rate immediately elevated from 70 to $\sim$ 1000 kct/s and then gradually increased to $\sim$ 2500 kct/s (Figure 2D, bottom). This was accompanied by an increase in particle size from $\sim$ 20 to $\sim$ 800 nm (Figure 2D, top). Therefore, ATP stimulates ParF polymerization. In contrast, addition of ADP (500 μ M) not only failed to induce polymerization but entirely inhibited it: the intensity of light scattering and particle size remained constant for 40 min (Figure 2D). This result correlates with the outcome of pelleting assays in which ADP decreased the amount of ParF in the pellet fraction (Figure 2A) and indicates that polymerization requires an appropriate nucleotide configuration.

The response of the protein to ATP γ S was also analysed by DLS (Figure 2D). The addition of ATP γ S (500 μ M) to ParF triggered instantaneous and extensive polymerization as reflected by a pronounced increase in light scattering intensity from $\sim$ 60 to 12 000 kct/s. In parallel, particle size increased 80-fold (Figure 2D, top). Therefore, ATP γ S has a more dramatic impact on ParF polymerization compared to

Figure 3 Critical concentration for ParF polymerization. Different protein concentrations were analysed by sedimentation assays at 14 000 r.p.m. (diamonds) or 50 000 r.p.m. (squares) and the amount of ParF in the pellet fractions was plotted against total ParF in the reaction. Extrapolating the Y value to 0 gives the critical concentration for ParF assembly into polymers.

ATP. The DLS results mirror those obtained by the pelleting assay: in the presence of ATP γ S, virtually all of ParF assembles into stable and numerous filaments, probably because they are irreversibly locked into a polymeric state as ATP hydrolysis cannot occur. In contrast, only a portion of ParF is recovered in the pellet in the presence of ATP and the intensity of light scattering is comparably lower, presumably because filamentation is more dynamic with polymerization and depolymerization occurring simultaneously. In summary, the sedimentation and DLS data conclusively demonstrate that extensive polymerization of ParF occurs in response to ATP.

Ultrastructure of ParF polymers

The stages of ParF polymerization were investigated by negative-stain electron microscopy (EM). In the absence of ATP, purified precentrifuged ParF (2.16 μ M) appeared as globular particles whose size (10–20 nm) is consistent with that of small oligomers (Figure 4A). This reflects the tendency of the protein to assemble into higher order structures even in the absence of exogenous nucleotide (Barillà and Hayes, 2003). ParF was next incubated with ATP (2 mM) at 30°C and aliquots were withdrawn at intervals and applied to EM grids. After 5 min, needle-like projections (~100 nm long) were visible (Figure 4A). These structures, which are likely to represent an early morphological stage in filament accretion, appeared longer after 10 min. Within 20 min, the fibres had elongated into extensive filament bundles (Figure 4A). These fibres were 400–650 nm in length and 30–70 nm wide. One end of many of the polymers had an irregular, frayed appearance, whereas the opposite end was more compact. Higher magnification images revealed a multistranded ultrastructure of parallel protofilaments (Figure 4B). Each protofilament appeared as a chain of bead-like particles with a cross-sectional diameter of ~2.5 nm. These results confirm that ParF assembles into filaments extensively in response to ATP. The ParF fibres induced by ATP γ S were morphologically identical to those produced with ATP, confirming that nucleotide binding is sufficient for polymerization.

Figure 4 Ultrastructure of ParF filaments observed by EM. (A) ParF was examined before and after addition of ATP (2 mM) at the indicated time points. In the 'no ATP' panel, arrowheads point to globular structures likely to be nucleation seeds (bar = 100 nm). In the following panels, the scale bar is 500 nm. (B) Higher magnification, reverse contrast image highlighting details of ParF fibres. Bar = 100 nm.

Mutations in conserved residues of the Walker motif A in ParF abolish proper plasmid segregation

The pivotal role of ATP in ParF polymerization and plasmid segregation was investigated further by constructing mutations in critical residues of the ATP-binding domain of ParF.

Residues 10–17 of ParF correspond to the variant Walker A motif. A conserved glycine residue in this motif was changed to valine (G11V) and a conserved lysine to glutamine (K15Q) (Figure 1A). The residue equivalent to G11 in other Walker A proteins is thought to be involved in nucleotide hydrolysis: the glycine-to-valine substitution in Ha-ras-p21 induces perturbations of the catalytic site, resulting in impaired GTPase activity and insensitivity to GTPase-activating protein (GAP) stimulation (Vogel *et al*, 1988; Maegly *et al*, 1996). The lysine residue corresponding to K15 in ParF is almost fully conserved among Walker family ATPases (Figure 1A): its side chain forms hydrogen bonds to the β - and γ -phosphate oxygens of ATP (Hayashi *et al*, 2001). Mutation of this lysine residue into glutamine has been investigated *in vivo* in a number of ParA family members. For example, both *E. coli* MinDK16Q and *B. subtilis* SojK16Q have lost their canonical cell pole localization and are dispersed throughout the cell (Quisel *et al*, 1999; Hu *et al*, 2002). The effects exerted by the G11V and K15Q changes in ParF were first assessed *in vivo* by partition assays. Both mutations entirely abrogated ParF-mediated plasmid segregation: the level of plasmid retention after ~25 generations in the absence of selective pressure was <1%, just like the level of the stability probe vector, whereas the level of segregational stability conferred by the wild-type *parFG* cassette was ~70%. These results indicate that residues G11 and K15 of ParF fulfil a crucial role in DNA segregation.

The polymerization kinetics and ATPase activities of ParFK15Q and ParFG11V are perturbed

The severe partition defect prompted an investigation of the biochemical properties of the mutated ParF proteins. The ATPase activity of ParFK15Q was reduced compared to that of wild-type ParF at low ATP concentrations (Figure 1E). However, at ATP concentrations of 250 μ M and 1 mM, the levels of hydrolysis by ParFK15Q were respectively ~70% and equivalent to that of ParF (Figure 1B and D). The nucleotide binding impairment predicted for this derivative correlates with the observed paucity of ATPase activity at low ATP concentrations (Figure 1E), that becomes alleviated at high ATP concentrations (Figure 1D). The $K_{0.5}$ for ATP of ParFK15Q (~200 μ M) is two-fold higher than that of wild-type ParF.

The ability of ParFK15Q to polymerize was first assessed by sedimentation assays. The mutant protein remained mostly in the supernatant, failing to respond to ATP or ATP γ S after 10 min incubation at 30°C (Figure 2B). In DLS trials, ParFK15Q remained stable in the absence of nucleotides, displaying a constant intensity of light scattering of ~70 kct/s for 25–30 min after which it began to polymerize with the count rate increasing up to ~600 kct/s after 40 min. The size of the particles increased in parallel up to a final value of ~130 nm. When ATP (500 μ M) was added, it did not trigger immediate polymerization of ParFK15Q. Instead ParFK15Q only began to polymerize after a lag period of ~25 min. After a further 15 min, the count rate reached a value of 1200 kct/s, two-fold higher than that attained in the absence of ATP (Figure 2E, bottom). The particle size increased up to ~200 nm (Figure 2E, top). As this protein displayed a prolonged lag before polymerization, its behaviour in the presence of ATP was followed by DLS for up to 90 min. After the initial lag, ParFK15Q steadily polymerized,

reaching a final count rate of ~4000 kct/s (data not shown). The pattern in the presence of ATP γ S (500 μ M) was very similar to that seen with ATP. These results show that ParFK15Q is less responsive to ATP and ATP γ S than wild-type ParF and that, even though the mutated protein retains the capacity to polymerize, polymerization is less extensive than that of ParF and the fibres formed are smaller. Interestingly, as for wild-type ParF, ADP totally suppressed ParFK15Q polymerization (Figure 2E).

To shed light on the ultrastructure of the polymers, the ParFK15Q polymerization process was investigated by EM. Sporadic, isolated fibres of ParFK15Q were visible 10 min after ATP addition, whereas numerous thin-bodied projections were observed after 60 min (data not shown). Although ParFK15Q retains the ability to polymerize, the polymerization is poor and probably incapable of supporting plasmid partition *in vivo*. Although it has yet to be rigorously shown, we are inclined to believe that ParFK15Q is impaired specifically in ATP binding on the basis of (i) the predicted interaction of this lysine with the β - and γ -phosphates of ATP (Hayashi *et al*, 2001); (ii) the reduced ATPase activity at low ATP concentrations, which attains wild-type levels at high ATP concentrations; and (iii) the poor responsiveness of this mutant to ATP and ATP γ S in polymerization.

ParFG11V failed to hydrolyse ATP efficiently and the residual activity was much lower (~10%) than that of ParF (Figure 1B–D). However, at low ATP concentrations (up to 500 nM), ParFG11V exhibited an ATP hydrolysis rate identical to that of wild-type ParF (Figure 1E). The $K_{0.5}$ of ParFG11V for ATP (~40 μ M) is slightly lower than that of wild-type ParF.

In DLS experiments, ParFG11V displayed a strong tendency to polymerize both in the absence and presence of triphosphate nucleotides (Figure 2F). In the absence of nucleotides, this mutant polymerized promptly without a lag, gradually reaching a plateau of ~3500 kct/s (compared to ~2000 kct/s for ParF and ~600 kct/s for ParFK15Q). ParFG11V still appeared responsive to ATP and ATP γ S, which elicited an immediate increase in light scattering intensity. Nevertheless, the final count rate in the presence of ATP or ATP γ S was ~4000 kct/s, similar to that attained by the protein without nucleotides (Figure 2F). However, the nucleotide sensitivity of ParFG11V is less than that of ParF, as illustrated by the response to ATP γ S, which was less dramatic for ParFG11V than that observed for the wild-type protein (~4500 and ~12 000 kct/s for ParFG11V and ParF, respectively) (Figure 2F and D). As observed for ParF and ParFK15Q mutant, ADP (500 μ M) also antagonized the polymerization of ParFG11V: both light scattering intensity (1400 kct/s) and particle size (~340 nm) were lower compared to those of ParFG11V alone (Figure 2F). At a higher concentration of ADP (5 mM), the inhibition of ParFG11V polymerization was more pronounced, as revealed by a lower count rate (~270 kct/s) and smaller fibre size (~140 nm) (data not shown).

The behaviour of the ParFG11V protein was also analysed by EM (Figure 5). Isolated, thick and long fibres were visible before ATP addition. This correlates with the pronounced tendency of this mutant to polymerize in the absence of ATP. At the first analysed time point after ATP addition, short projections were observed. These structures were morphologically comparable to those observed for wild-type ParF at the same juncture. The needles grew into longer and thicker filaments within 10 min. Some of the ParFG11V fibres were

Figure 5 ParFG11V exhibits a perturbed polymerization pattern. **(A)** EM time course of ParFG11V polymerization upon addition of ATP. Arrowheads indicate short growing projections. Bar = 500 nm. **(B)** Higher magnification image of another field of the ParFG11V grid revealing particularly elongated filaments. Bar = 1 µm. **(C)** Details of the intricate meshwork of highly interlaced filaments produced by ParFG11V after 20 min exposure to ATP. Bar = 500 nm.

particularly elongated, twice or three times the length of wild-type polymers (Figure 5B). The cross-sectional diameter of these polymers was also wider compared to wild-type fibres. Interestingly, polymerization of ParFG11V produced an intricate meshwork of overlapping, interconnected filaments within 20 min of exposure to ATP (Figure 5A, and details in Figure 5C). The EM data show that ParFG11V forms fibres and that the protein is prone to hyperfilamentation. However, the monomer–monomer and interprotofilament interactions within these fibres are likely to be intrinsically weaker or structurally different from those of wild-type ParF, as the fibres were never recovered in the pellet fraction in repeated sedimentation assays with and without nucleotides at 14 000 r.p.m. (Figure 2C) and only poorly (~25%) at 80 000 r.p.m. EM of these experiments also revealed that ParFG11V fibres remained in the supernatant fraction and exhibited a similar morphology as before centrifugation, suggesting that the ultrastructure of these fibres must be different from that of the wild-type protein.

ParG plays at least two distinct roles in ParF polymerization dynamics: enhancement of ATP hydrolysis and promotion of filament bundling

ParF and ParG interact *in vivo* and *in vitro* (Barillà and Hayes, 2003). The effect of ParG on the ATPase activity of ParF was examined at various ParG concentrations. ATP hydrolysis by ParF (0.5 µM) was strongly stimulated (~30-fold) by ParG, which alone exhibited no nucleotide hydrolysis (Figure 6A). At a higher ParF concentration (5 µM), a similar pattern of stimulation was observed (Figure 6B). Enhancement of nucleotide hydrolysis by a partner regulatory protein is a recurring theme among members of the ParA superfamily, including P1ParA (Davis *et al*, 1996; Fung *et al*, 2001), F SopA

(Libante *et al*, 2001), ParA of *Caulobacter crescentus* (Easter and Gober, 2002) and the MinD cell division protein of *E. coli* (Hu and Lutkenhaus, 2001; Suefuji *et al*, 2002), as well as more broadly in the case of Walker-type hydrolases like Haras-p21 (Vogel *et al*, 1988). The stimulation of ParF ATPase activity by ParG exhibited a sigmoidal behaviour at lower ParG concentrations (Figure 6A and B) analogous to the stimulation of *E. coli* MinD ATPase activity by MinE (Suefuji *et al*, 2002). The effect of ParG and DNA together on the ATPase activity of ParF was also examined. Both a cognate DNA fragment, containing the partition site, and an unrelated DNA fragment further augmented ParG stimulation of ATP hydrolysis by ParF by 40–50% (Figure 6C). When ParF was incubated with DNA alone, either cognate or unrelated, no enhancement of ATP hydrolysis was observed (data not shown).

The effect of ParG on ATP hydrolysis by the mutated ParF proteins was also investigated. ParG failed to enhance nucleotide hydrolysis by either ParFG11V or ParFK15Q (Figure 6A), suggesting that both mutations ablate the stimulatory response to ParG.

Sedimentation assays including both proteins were performed to assess whether ParG associates with ParF polymers (Figure 7A). In either the absence of nucleotides or in the presence of ATP or ATPγS (2 mM), a significant fraction of the input ParG cosedimented with ParF filaments. When ParG was tested alone, very little sedimentation was evident. Therefore, ParG is able to associate with polymeric forms of ParF. Intriguingly, the fact that ParF was recovered mostly in the pellet fraction in the presence of ParG even without added nucleotides suggests a function for the partner protein in promoting ParF bundling. In addition, ADP reduces the formation of ParF filaments (Figure 2A and D) and this effect was not counteracted by ParG (Figure 7A).

Figure 6 ParG stimulates ParF ATPase activity. (A) Levels of ATP hydrolysis driven by ParF, ParFG11V and ParFK15Q as a function of ParG concentration. ParF proteins were used at 0.5 μM. Diamonds, ParF; squares, ParFK15Q; triangles, ParFG11V. (B) Stimulation of ATP hydrolysis by ParF as a function of ParG concentration, using 5 μM ParF. The inset shows an expanded version of the early points of the curve. (C) Effect of DNA on the ATPase activity of ParF in the presence of ParG. The ParF protein was used at 0.5 μM, ATP at 50 nM and DNA at 500 ng per reaction. Filled circles, ParF + ParG; open circles, ParF + ParG + partition DNA; squares, ParF + ParG + non-partition DNA. The partition DNA was a PCR fragment containing the 259 bp upstream of *parF* start codon and the non-partition DNA was a similarly sized fragment comprising *ma-15 Saccharomyces cerevisiae* gene.

To address more directly whether ParG does indeed influence the polymerization behaviour of ParF, different ParF:ParG molar ratios were tested in sedimentation assays in the presence of ATP (100 μM) (Figure 7D). At low ParG:ParF ratio, ParG slightly enhanced the amount of ParF polymer mass (from 43 to 58%), suggesting that ParG promotes the association of ParF into polymers under these conditions. However, increasing the ParG molar ratio resulted

Figure 7 ParG has a stabilizing/remodelling role in filament bundling. (A) ParG cosediments with ParF in the pellet fraction. ParF (5.8 μM) was incubated with ParG (7.17 μM, dimer) either in the absence or presence of nucleotides and the reactions processed and analysed on a 15% SDS gel. (B) ParG partially rescues ParFG11V polymerization as detected by sedimentation assay. ParFG11V (7 μM) and ParG (7 μM) were coincubated and processed as in (A). (C) ParG rejuvenates ParFK15Q polymerization behaviour. ParFK15Q (3.62 μM) was mixed with ParG (3.62 μM, dimer) and the reactions processed as in (A). (D) ParG ratio-dependent modulation of ParF polymerization. ParF (10 μM) and ParG (varying molarity) were mixed in different ratios in the presence of ATP (100 μM) and the reactions processed and analysed as described in (A). In panels A-D, 100% of the pellet (P) and 33% of the supernatant (S) fractions were loaded on the gels. The amount of protein recovered in the pellets is shown at the bottom of each gel.

in a progressive decrease in the level of polymerized ParF until, at a ParG:ParF ratio of 2:1, both proteins were found predominantly in the supernatant (Figure 7D). Thus, depending on the relative concentrations of the proteins, ParG can either potentiate or attenuate ParF polymerization. EM analysis revealed that ParF polymers assembled in the presence of ParG were thicker (>70 nm) and longer (>1 μm) than ParF alone fibres, and apparently contained 2–3 times as

many protofilaments (Figure 8A). Furthermore, both ends were splayed in many fibres.

The influence that ParG exerts on ParF polymerization was emphasized by the observation that ParFK15Q, which alone exhibited no detectable polymerization in the presence of ATP or ATP γ S (Figure 2B), was diverted almost entirely (>95%) to the pellet fraction when coincubated with ParG. This effect was evident both in the absence of nucleotide and in the presence of ATP, ADP or ATP γ S (Figure 7C). In the presence of ParG, ParFK15Q polymerized into full-length thick filaments that were never observed for ParFK15Q alone (Figure 8B). In these fibres, one end appeared compact, whereas the other was frayed. In addition, the margins of the fibres were blurred and the protofilaments were densely packed. Although ATP hydrolysis by ParFK15Q is not stimulated by ParG, nevertheless the interaction with ParG apparently has a rejuvenating effect on the mutant protein modulating its behaviour so as to allow greatly improved polymerization. This suggests that the stimulatory activities of ParG in ATP hydrolysis and polymerization of ParF are separable.

ParFG11V polymerization was partially rescued by ParG, as only a portion of the mutant protein was recovered in the pellet fraction both in the absence and presence of nucleotides (Figure 7B). Clearly, an interaction occurs between the two proteins, as they proportionally cosediment, but the interaction is not entirely productive. Like ParFK15Q, the ParFG11V mutant alone exhibited negligible sedimentation in pelleting assays (Figure 2C and 7B).

Discussion

A growing number of dynamic polymerizing proteins that are ancestors of eukaryotic cytoskeletal proteins are being identified in prokaryotes (Lutkenhaus, 2002; Errington, 2003). These include actin-type and intermediate filament determinants of cell morphology (Jones *et al*, 2001; Ausmees *et al*, 2003), actin-like mediators of chromosome and plasmid segregation (Møller-Jensen *et al*, 2002; van den Ent *et al*, 2002; Kruse *et al*, 2003) and the tubulin homologue FtsZ required for cytokinesis (Romberg and Levin, 2003). These prokaryotic cytoskeletal factors associate with various auxiliary proteins. ParF, which epitomizes a discrete subgroup of the ParA superfamily that promotes plasmid and chromosome segregation (Hayes, 2000), can now be added to the expanding list of dynamic polymerizing proteins in prokaryotes. ParF shares sequence homology with two other prokaryotic cytoskeletal factors that have also been shown to involve filamentous proteins: the MinD factor of the cell division site-selection MinCDE system (Lutkenhaus, 2002; Suefuiji *et al*, 2002), and the ParA-type protein of plasmid pB171 that oscillates *in vivo* along a helical track (Ebersbach and Gerdes, 2004).

The ParF protein has a propensity to oligomerize without added nucleotides (Barillà and Hayes, 2003). These prefilamentous structures might act as nucleation sites for more extensive polymerization. Here, we have shown that ParF assembles into highly ordered filaments *in vitro* and that polymerization is strongly enhanced by ATP binding. The filaments can extend to lengths comparable to that of an *E. coli* cell and exhibit a multistranded ultrastructure of parallel protofilaments, each of which appears as a string of

bead-like particles (Figure 4B). The morphology of these filaments hints at a potential polarity: one end is compact with protofilaments closely packed together, whereas the opposite extremity has a distinctive frayed appearance with protofilaments more irregularly spaced and disarrayed. Intriguingly, the ends of shrinking microtubules are similarly splayed apart in eukaryotic cells, often adopting a 'ram's horns' appearance (Arnal *et al*, 2000). This characteristic morphology is associated with the switch between microtubule growth and shrinkage, and it is tempting to speculate that the frayed termini of ParF fibres analogously might be sites of dynamic protein interchange although this remains to be demonstrated.

Interestingly, ADP has an active role in antagonizing polymerization revealing that the ATP- and ADP-bound forms of ParF are proficient and suppressed, respectively, in filamentation. Thus, the stimulation of ParF polymerization by ATP and its inhibition by ADP might be fundamental regulatory mechanisms *in vivo*. The phenotypes conferred by mutations in the Walker A motif of ParF corroborated the importance of nucleotide binding and hydrolysis, and established a functional coupling between *sine vitio* polymerization and DNA segregation. Both ParFK15Q and ParFG11V mutants failed to support plasmid partition. The K15Q change in ParF resulted in altered ATPase kinetics compared to the wild-type protein, most likely due to reduced ATP binding. This hypothesis is based on (1) the slow response of ParFK15Q to ATP and ATP γ S in DLS experiments (Figure 2E); (2) a $K_{0.5}$ for ATP ($\sim 200 \mu\text{M}$) that is two-fold higher than that of wild-type protein; and (3) a pronounced defect in ATP hydrolysis at low ATP concentrations (Figure 1E) that becomes alleviated at higher ATP concentrations (Figure 1D), at which ParFK15Q attains the same level of ATPase activity as that of ParF. An impairment in ATP binding would also account for the hypopolymerization of this mutant.

The equivalent of G11 is also well conserved across Walker A boxes and is thought to have a key role in nucleotide hydrolysis: in Ha-ras-p21, the glycine-to-valine change gives rise to unfavourable interactions within the catalytic niche, resulting in a mutant characterized by altered GTP hydrolysis and insensitivity to activation by GAPs (Maegly *et al*, 1996). The G11V conversion in ParF strongly compromised ATPase activity, as well as triggering extensive nucleotide-independent polymerization. Furthermore, the ParFG11V protein exhibited hyperfilamentation as seen in EM experiments. The larger G11V fibres did not pellet either in the presence and absence of ATP/ATP γ S under conditions that harvested >95% of wild-type polymers. High-speed centrifugation also failed to pellet the G11V fibres. This mutant is less responsive than ParF to ATP and ATP γ S, as shown by DLS experiments. This feature might translate into filaments that are intrinsically less crosslinked, because the intermonomer and/or interprotofilament interactions lack the cementing action of ATP. In light of the above findings, the elusive link between ATP binding and plasmid partition by ParA-like proteins can now be mechanistically explained: correct polymerization of these proteins is crucial for proper DNA segregation.

ParG exerts a profound effect on ParF polymerization *in vitro*. ParG associates with ParF fibres, modulating the polymerization dynamics: ParG promotes filament bundling at low ParG:ParF ratios, but it antagonizes filamentation at

higher ratios. This dichotomy mirrors the ratio-dependent effects produced by MinE on MinD polymers (Suefuji *et al*, 2002). At low ratios, ParG apparently acts as a stabilizing factor. This role could be accomplished by lateral crosslinking of adjacent ParF protofilaments analogously to microtubule-associated proteins (MAPs) that promote microtubule stability often by crosslinking neighbouring tubulin monomers. One *modus operandi* of MAPs is through oligomerization (Bray, 2001). ParG oligomerizes: The recent solution of the protein structure has revealed that ParG is a symmetric dimer (Golovanov *et al*, 2003). Thus, the dimerization of ParG could mediate the pairing of ParF monomers that reside in adjacent protofilaments thereby promoting polymer bundling. Alternatively, ParG could stabilize the ParF monomer-monomer interaction along the length of individual protofilaments. High-magnification EM images show that ParF fibres assembled in the presence of ParG are thicker and longer than those observed in its absence (Figure 8A). The bundling activity of ParG is further illustrated by its effect on the mutant ParF proteins. ParFK15Q, which alone exhibits no detectable polymerization in sedimentation assays, was recovered in the pellet fraction when coincubated with ParG (Figure 7C) and fibres of normal length were observed by EM. The same stabilizing effect, although more moderate, was exerted on ParFG11V. Thus, ParG remodels the mutant polymers allowing detectable filament bundling. Interestingly, both ParFK15Q and ParFG11V mutants are insensitive to stimulation of ATP hydrolysis by ParG. This suggests that the stimulatory activities of ParG in ATP hydrolysis and polymerization are separable. The stimulation of ParF ATPase activity by ParG (Figure 6A and B) is likely to be an important regulatory factor for the ParF assembly-disassembly cycle *in vivo*. DNA also apparently has a role in modulating the ATPase activity of ParF.

It has been long speculated that ATP binding/hydrolysis by ParA proteins might provide a motive force for plasmid separation during the segregation process. The results presented here provide a potential molecular mechanism for ParA-mediated partitioning, not alone for immediate homologues of ParF (Hayes, 2000; Kalnin *et al*, 2000; Kwong *et al*, 2001; Ebersbach and Gerdes, 2004), but for the ParA family in general (Figure 9). Plasmid pairing is thought to be an initial event in the partition process (Edgar *et al*, 2001). After the initial pairing step, three different sequences of events could unfold. The simplest option involves bidirectional pushing of the paired plasmids in opposite directions. ATP-enhanced polymerization of ParF could provide the motive force for this process. A single, multistranded filament could elongate bidirectionally between the partition complexes promoting two-way plasmid movement (Figure 9) or, if the plasmids are tethered to host structures, discrete filaments could direct plasmid movement. A pushing force exerted by growing filaments could be generated by a thermal ratchet-type mechanism, in which small, random motions of the object to be moved create transient gaps between it and the end of the polymer (Peskin *et al*, 1993). Subunit molecules may then diffuse into the gap and add to the end of the polymer thereby increasing its length.

The second option for ParF-mediated segregation is bidirectional pulling. Host structures could anchor ParF filaments to the cell poles, which would allow depolymerization to pull plasmids on either side of the division plane in opposite

Figure 8 ParG remodels ParF fibres. EM reverse contrast images of ParF (A) or ParFK15Q (B) fibres assembled in the presence of ParG. Bar = 210 nm.

directions (Figure 9). Depolymerization of fibres can generate significant motive force as substantiated, for example, in the case of microtubule depolymerization, which has been shown to promote chromosome movement *in vitro* (Coue *et al*, 1991). A third possibility is that intact ParF filaments extending between cell poles might function as cables along which sister plasmids are moved apart in opposing poleward directions.

ParF polymerization *in vivo* might be coupled to the filamentation of another cytoskeletal element. It is intriguing that the ParF subgroup of proteins is closely related to the MinD cell division proteins (Hayes, 2000). Interestingly, the ATPase activities of both ParF and MinD are stimulated by dimeric partner proteins, ParG and MinE, respectively. The stimulation in both systems displays a sigmoidal pattern at low ParG/MinE concentrations (Figure 6A and B; Suefuji *et al*, 2002). The small proteins also modulate polymerization

Figure 9 Molecular models for ParF-mediated plasmid segregation. In both models, paired nucleoprotein partition complexes assemble at midcell. Complexes consist of the ParF (red rectangles) and dimeric ParG (green ovals) proteins arranged on the plasmid-located partition sites (yellow circles). A conformational change in ParF resulting from ATP binding induces polymerization (indicated by the square-to-diamond shape change). In bidirectional pulling, a pair of ParF filaments extends from midcell towards opposite cell poles where the filaments are anchored by a hypothetical host factor (blue half circles). Concerted depolymerization of the filaments from midcell draws plasmids away from the cell centres. In bidirectional pushing, the filaments extend from a tethered midcell position (orange oval) and plasmids are propelled towards the cell poles by the polymerization process.

of their cognate ParA-type proteins (Figure 7; Hu *et al*, 2002; Suefuji *et al*, 2002). Both MinD and ParF polymerize *in vitro* in response to ATP into ultrastructures with remarkably similar appearances with one end of the filaments displaying a characteristic brush-like morphology (Figure 4; Suefuji *et al*, 2002). These structures appear to be quite distinct from those of the FtsZ tubulin homologue required for cell division (Romberg and Levin, 2003) and of the actin-like protein involved in R1 plasmid partition (Møller-Jensen *et al*, 2002; van den Ent *et al*, 2002). MinD and ParF might also be very close structural homologues (unpublished data).

MinD and MinC oscillate between *E. coli* cell poles along a dynamic MinCDE spiral track (Raskin and de Boer, 1999; Fu *et al*, 2001; Hu and Lutkenhaus, 2001; Shih *et al*, 2003). Plasmid pB171 specifies a ParF-like protein (Hayes, 2000) that oscillates in a helical pattern between the nucleoid edges in *E. coli* (Ebersbach and Gerdes, 2004). A more distant ParA homologue, Soj, relocates between nucleoid patches in *B. subtilis* apparently in a MinD-regulated manner (Autret and Errington, 2003). By analogy, and considering its *in vitro* polymerization properties, the filamentous ParF protein is also a strong candidate for a cytoskeletal element that mediates DNA segregation, perhaps interacting with the spiral MinCDE protein scaffold during the process. This would allow for a coupling between plasmid segregation and cell division, thereby potentially providing an interconnected regulatory framework for these fundamental processes.

Materials and methods

Strains and plasmids

E. coli DH5 α was used for plasmid construction and BL21 (DE3) for protein overproduction. Strain BR825 was employed for partition

assays (Hayes, 2000). Plasmids used to overproduce ParF and ParG proteins have been described (Barillà and Hayes, 2003). Overexpression and partition constructs harbouring the point mutations in *parF* resulting in the G11V and K15Q changes were created by overlap extension mutagenesis.

Protein purification

His-tagged ParF and ParG proteins were purified as described (Barillà and Hayes, 2003). These proteins are as proficient as the wild-type proteins in supporting plasmid partition *in vivo* (Barillà and Hayes, 2003).

ATPase assay by TLC

ParF, at the concentrations indicated, was incubated with 1, 10 or 20 μ Ci of [α - 35 S]ATP (1250 Ci/mmol) in buffer (30 mM Tris·HCl pH 7.5, 100 mM KCl, 5 mM MgCl $_2$, 2 mM DTT) in a volume of 16 μ l at 30°C for 60 min. Cold ATP was added to attain the final concentration indicated. Aliquots (2.5 or 5.0 μ l) were spotted onto a PEI-cellulose plate that had been prerun in water, dried and subjected to TLC using 0.5 M KH $_2$ PO $_4$ (pH 3.5) as buffer. The plate was dried and exposed to Kodak BioMax MR film. Radiolabelled ATP and ADP spots were quantified by PhosphorImager (Molecular Dynamics) and normalized for the nonenzymatic hydrolysed ADP in the control lane. The data shown are typical results of experiments performed at least in triplicate.

Electron microscopy

Proteins were precentrifuged for 1 h at 14 000 r.p.m. at 4°C. Reactions containing ParF or mutant proteins (2.16 μ M) were set up in buffer F (30 mM Tris·HCl pH 8.0, 100 mM KCl, 2 mM DTT, 10% glycerol) to which 2 mM ATP and 5 mM MgCl $_2$ were added. The mixture was incubated at 30°C and 10 μ l aliquots were withdrawn at the time points indicated and applied to carbon-coated grids (400 mesh size) that had been glow discharged. After 30 s, the drops were blotted, the grids were stained with 2% uranyl acetate for 1 min and then blotted dry. Grids were examined with a Tecnai10 transmission electron microscope and photographed at 14–73 000 magnification. Micrographs were digitized with a UMAX PowerLook 3000 scanner.

Dynamic light scattering

ParF and mutant protein polymerization was measured by DLS in a Malvern Zetasizer Nano System (He-Ne laser, 633 nm). Proteins were centrifuged for 5 min at 14 000 r.p.m. and the supernatant was collected and used for experiments. A 47 µl Aliquot of protein (2.16 µM in 30 mM Tris·HCl pH 7.0, 100 mM KCl, 2 mM DTT, 10% glycerol) was added to a 50 µl quartz cuvette and incubated at 30°C in the Zetasizer chamber. Then ATP, ADP or ATPγS (500 µM) and MgCl₂ (5 mM) were added to a final volume of 50 µl. Values obtained every 20 s were plotted. The intensity or count rate measures the amount of scattered light expressed as photons detected per second. The intensity is proportional to the size and concentration of the scattering particles.

Polymerization assay

ParF or mutant proteins (6–8 µM) were incubated in buffer F in 60 µl in the absence or presence of nucleotides (2 mM) and MgCl₂ (5 mM) for 10 min at 30°C. In cosedimentation assays, ParG was included at the concentrations indicated. Reactions were centrifuged for 30 min at 4°C at 14 000 r.p.m. A 20 µl Aliquot of the supernatant was

collected for gel analysis, 10 µl was kept for Bradford quantitation and the remaining supernatant was carefully aspirated. The pellet was resuspended in 15 µl of water. Protein levels in the supernatant and the pellet were analysed by SDS-PAGE and Coomassie blue staining. In each gel, 100% of the pellet and 33% of the supernatant were loaded.

Partition assay

Partition assays were performed as described (Hayes, 2000). Plasmid pFH450 was used as empty vector and pFH547 was the construct containing the wild-type *parFG* cassette.

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