Microtubule-like Properties of the Bacterial Actin Homolog ParM-R1

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David Popp*,1,2, Akihiro Narita†, Lin Jie Lee§, Mårten Larsson‡, and Robert C. Robinson†

From the Institute of Molecular and Cell Biology, Proteos, 61 Biopolis Drive, 138673, Singapore, the Nagoya University Graduate School of Science, Structural Biology Research Center and Division of Biological Sciences, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan, the Department of Biochemistry, National University of Singapore, 8 Medical Drive, 117597, Singapore, and the School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551, Singapore

Background: ParM-R1 is a filament-forming actin homolog that segregates DNA in bacteria.

Results: ParM-R1 exhibits synchronous microtubule-like oscillations between assembly and disassembly and forms asters growing from a centrosome-like nucleus of interconnected nodes.

Conclusion: ParM-R1 exhibits microtubule-like properties.

Significance: These results imply that the selection pressure to reliably segregate DNA during cell division has led to common mechanisms within diverse segregation machineries.

Filamentous proteins with actin and tubulin folds are used interchangeably between prokaryotes and eukaryotes for vital cellular functions such as cytokinesis or DNA segregation. The eukaryotic contractile ring is formed from actin and actin-regulating proteins (1). Conversely, cytokinesis in bacteria depends upon the constriction of the FtsZ ring, a tubulin homolog (2). Chromosome segregation in eukaryotes relies on arrays of microtubules radiating from microtubule-organizing centers to position DNA (1). In contrast, some bacteria use polymerizing actin homologs to provide force for plasmid DNA segregation (3). The complex force-generating machineries in eukaryotes appear to be carried out by minimal systems in prokaryotes.

One important characteristic property of eukaryotic tubulin is the synchronous oscillations of polymerization and depolymerization (4–6). The energy for the microtubule oscillations is derived from GTP hydrolysis and oscillations stop on GTP depletion (5). Such self-organization of molecules in time is common in biology, generating clocks such as the circadian oscillating proteins KaiABC in cyanobacteria (7), whereas oscillations of bacterial Min proteins sense the geometry of the prokaryotic cell, regulate Z-ring formation, and subsequently cytokinesis, suggesting that spontaneous protein waves may be a general means of intracellular organization (8). Spontaneous oscillations in yeast glycolysis (9); spontaneous synchronous contractions in heart muscle (SPOC) (10); stretch activation-induced oscillations in insect flight muscle (11); and the Belousov-Zhabotinskii reaction in chemistry (12) are other examples of oscillators.

A second vital feature of microtubules is that they associate into cellular structures such as the dynamic asters found in mitotic and meiotic spindles (13). Astral microtubules can be reconstructed in vitro from a solution of motor proteins and microtubules (14). Self-organization in space is also a common phenomenon in biology that can be driven by molecular crowding, cation association, or associated proteins. For example, DNA (15) and the actin homolog FtsZ can both form toroids in vivo and in vitro (16, 17).

Bacterial plasmids encode partitioning (par) loci that ensure ordered plasmid segregation prior to cell division. The par locus of the R1 drug resistance plasmid encodes three components: a centromere-like site in the DNA (parC), a DNA binding protein (ParR), and ParM, an actin-like protein bearing the NTPase activity (18). In vivo, these components can form a linear assembly of multiple filaments (19), which position pairs of plasmids at opposite ends of the rod-shaped bacterium by a polymerization mechanism, ensuring equal distribution of the plasmids between the daughter cells (18).
In vitro, ParM-R1 has been shown to be not only distinct in structure from the F-actin, by forming a left-handed helix (20, 21), but also by its filament assembly dynamics. At low ParM-R1 concentrations (<10 μM), filament assembly consists of a classical time course, i.e. a short lag time precedes the development of polymers as monitored by light scattering up to a plateau, following a simple sigmoidal curve (21) as predicted for self-assembly of biological polymers (22). The critical concentration (C) for assembly is 2–3 μM (23). At low protein concentrations of monomer, filaments stochastically switch from elongation to shortening through dynamic instability (23, 24).

Hence, despite being an actin homolog, ParM-R1 is distinguished from actin by several important properties: (i) polymerization requires either GTP or ATP with a preference for GTP, whereas actin is an ATPase; (ii) an extremely rapid instead of slow spontaneous nucleation into filaments; and (iii) dynamic instability as a GTP-driven molecular switch resulting in a phase of steady elongation followed by a phase of shortening to complete disintegration of the polymer resulting in a phase of steady elongation followed by a phase of shortening to complete disintegration of the polymer instead of steady-state treadmilling (21, 23). These features of ParM-R1 have stronger similarities with microtubules than with actin.

Recently, the molecular mechanism of linear ParM-R1 bundle formation in the presence of crowding agents has been described (25). These in vitro experiments were performed at subphysiological salt concentrations (30–50 mM KCl) and in the presence of crowding agents (0.5–1.5% methylcellulose or 4–10% polyvinyl alcohol). ParM-R1 bundle formation consisted of two distinct phases. At the onset of polymerization, bundle thickness and shape were determined in the form of nuclei of short helically disordered filaments arranged in a liquid-like lattice. These nuclei then underwent an elongation phase whereby they rapidly increased in length (25).

Here, we show that ParM-R1 has additional supramolecular and dynamic properties that closely resemble features of microtubules. Under crowding conditions at physiological salt levels, ParM-R1 can form asters that are reminiscent of astral microtubules. We also observe that the behavior of ParM-R1 at concentrations >10 μM becomes synchronous, displaying oscillations in polymerization and depolymerization, redolent of microtubule dynamics.

EXPERIMENTAL PROCEDURES

Chemicals—Nucleotides and chemicals used were from Sigma.

Proteins—Two protocols were used to obtain native ParM-R1. Protein preparations behaved similarly (1). Native ParM-R1 was overexpressed in Escherichia coli BL21(DE3) cells (Invitrogen) and purified as described previously (2, 26). N-terminal His-tagged ParM-R1 and mutants cloned into the pS5 vector (27) were grown in E. coli BL21(DE3) cells to A600 ~1 and induced with isopropyl 1-thio-β-d-galactopyranoside (1 mM) at 25 °C overnight. ParM-R1 was purified on a HisTrap column (GE Healthcare), cleaved on-column with PreScission protease to remove the His tag, and purified by gel filtration (Superdex 75, GE Healthcare). The protein was flash frozen and stored at −80 °C in gel filtration buffer (30 mM KCl, 1 mM MgCl2, 0.5 mM DTT, 30 mM Hepes, pH 7.5). Protein concentrations were determined using a NanoDrop Spectrophotometer using theoretical extinction coefficients. Buffers used had the following compositions: High salt buffer (300 mM KCl, 1 mM MgCl2, 0.5 mM DTT, 30 mM Hepes, pH 7.5), Low salt buffer (50 mM KCl, 1 mM MgCl2, 0.5 mM DTT, 30 mM Hepes, pH 7.5). Oscillation studies were initiated by adding nucleotides to ParM-R1. Asters were assembled by adding nucleotides (1 mM final concentration) to a 5–15 μM ParM-R1 solution in high salt buffer including various amounts of crowding agents.

Light Scattering—Assembly and disassembly of ParM-R1 was followed by light scattering at 90 degrees using a Perkin Elmer LS 55 spectrometer or a BioLogic stopped-flow machine, monitored at 600 nm.

Electron Microscopy and Total Internal Fluorescence Reflection Microscopy—A drop of ParM-R1 solution was applied to carbon-coated copper grids stained with 1% uranyl acetate and visualized under a JEOL JEM-2010 HC electron microscope operated at 100 keV and a nominal magnification of 40,000. Films were digitized with a Photocan 2000 (Z/I Imaging, Zeiss) at 7-μm steps. Otherwise, images were taken on a Gatan Erlangshen ES500W CCD at a nominal magnification of 150,000 with a JEOL JEM 1010 electron microscope operated at 80 keV. Total internal fluorescence reflection microscopy was carried out using established protocols for ParM-R1 (21, 24).

Analysis of Electron Microscopy Data—Images of the ParM aster nuclei were extracted from the electron micrographs. To adopt our previous procedures used for cryo-electron micrographs without any change for negatively stained images, the contrast of the images was inverted. Fourier transforms, filtered images, determination of nodes, and connections between them, as well as cluster analyses were carried out using the EOS software package (28).

RESULTS

Aster Formation—We used electron microscopy (EM) to investigate ParM-R1 suprastructures formed under physiological salt concentrations in vitro. In the presence of crowding agents and salt concentrations >100 mM KCl, mostly asters were observed. Asters were almost exclusively observed when polyvinyl alcohol was used as the crowding agent, whereas with methylcellulose, a more mixed population of asters and bundles were formed (Fig. 1). 10–15 s after adding nucleotide, disc- or oval-shaped nuclei with diameters of ~100–300 nm were observed (Fig. 1A, supplemental Fig. S1). Structural details of the aster nucleus will be discussed in the next section.

From the elliptically shaped nucleus, filaments were seen to grow radially outward within 1 min (Fig. 1B). After several minutes, many filaments extended several hundred nm approximately isotropically from the nuclei, giving it the appearance of a fur ball (Fig. 1C). Filaments continued growing and gradually packed tightly (Fig. 1D) until after ~10–15 min, all structures had matured into asters (Fig. 1E). Aster formation was a slower process than bundle formation (24).

Growing of asters could also be followed by time-lapse total internal fluorescence reflection microscopy (Fig. 1F and supplemental Movie 1). Aster nuclei often associated into clus-
ters (Fig. 1A). The most regular asters grew with the non-hydrolysable nucleotides ATPγS and GMP-PNP (Figs. 1 and 2; supplemental Fig. S1).

Single mature asters grown with non-hydrolysable nucleotides had 13 or more arms (Figs. 1E and 2A). In the inner part of an arm (just outside the nucleus), filaments formed a net-like structure, whereas in the outer part of an arm, filaments were arranged approximately in parallel (Fig. 2A). Optical diffraction patterns of this region showed the typical set of layer lines also observed in the Fourier transforms of individual ParM-R1 filaments (Fig. 2B) (21).

Two salt bridges (Glu35–Lys258) and (Asp63–Arg262) were previously shown to be important for the formation of double stranded left-handed ParM-R1 filaments. Mutations of these amino acids resulted either in impaired or single-stranded ParM-R1 filaments (29). Here, ParM-R1 mutant D63K was capable of forming asters (Fig. 3A). However, ParM-R1 mutants R262D (Fig. 3B), E35K, K258D, as well as double mutants K258D/R262D and E35K/D63K (data not shown) assembled into linear bundles consisting of single-stranded filaments and were not able to support aster formation. This implies that the two salt bridges, besides being an integral part in helical filament formation, are also involved in the formation of the initial nuclei leading to asters.

Arms of asters formed in the presence of non-hydrolysable nucleotides were several μm long and appeared rather symmetric and regular (Figs. 1 and 2). GTP supported exclusively the growth of asters from nuclei, although their appearance was less regular than seen with non-hydrolysable nucleotides, and the arms appeared shorter (Fig. 3C). This may be due to dynamic instability of GTP-ParM-R1 filaments. In the presence of ATP, ParM-R1 grew asters and spindle-shaped bundles with very long arms (up to 10 μm; Fig. 3D). These ATP-induced asters did not show the initial nuclei as with GTP or non-hydrolysable nucleotides (Fig. 3D). Rather, they appear to involve overlapping bundles, which give rise to a superficial appearance of asters, indicating that the initial nucleation process and subsequent aster formation is nucleotide-dependent.

**FIGURE 1. Aster formation.** Shown are representative electron micrographs taken in the presence of 8% polyvinyl alcohol and physiological high salt buffer −15 s (A), −1 min (B), −5 min (C), −10 min (D), and −20 min (E) after adding GMP-PNP. Scale bars, A–D, 200 nm; E, 1 μm. The arrows point at filaments growing from the nucleus. F, asters visualized by total internal fluorescence reflection microscopy. Conditions were as follows: 8 μM ParM, 1% methylcellulose, physiological high salt buffer, ATPγS. The arrow points at free bundles that are also formed under methylcellulose conditions. Scale bar, 10 μm.

**FIGURE 2. Asters at higher magnification observed by EM.** A, close up of the inner part of an aster. Note the near parallel filaments in the aster arms. Scale bar, 200 nm. B, Fourier transform of the outer part of an arm marked within the black box in A. Layer lines observed are similar to those seen in single filaments (Ref. 21). As a reference, the arrow marks the fifth layer line at 6.4 nm.
Structure of the Aster Nucleus—The calculated Fourier transform (FT) of the ParM-R1 aster nuclei formed by non-hydrolysable nucleotides showed in general three discontinuous circles, with each consisting of 16 sharp spots (Fig. 4). The primary inner circle had a radial spacing of $\frac{1}{10}$ nm, the second circle was spaced at $\frac{1}{5.1}$ nm, and the weak third circle was located at $\frac{1}{3.3}$ nm spacing. The reflections were sharp, yet showed some spread in the radial direction, indicating some variability in position of the individual monomers contributing to the aster nucleus structure.

The second circle appeared unlikely to be the second order of the $\frac{1}{10}$ nm circle, as these 16 spots were spaced axially at approximately halfway between those of the inner circle (Fig. 4). The spots of the third circle were again approximately at the same position as those of the primary circle (Fig. 4). Despite the FT consisting of rather sharp spots indicative of a crystalline lattice, the whole filtered images were not easily interpretable, perhaps in part due to the fact that the aster nucleus structure may consist of multiple layers, which appear thicker in the middle of an aster nucleus than at the edges (Fig. 4).

We suspected that the 16-spot inner circle observed in the FT arises from ParM rings consisting of 16 individual molecules with a diameter of $\sim 10$ nm. Indeed, ring structures of $\sim 10$-nm diameter could be observed in the filtered image (Fig. 4). Yet, other square-like structures, many rings with a bright spot in the center, and rings with a substantially larger diameter also seemed apparent, suggesting multiple structures act as building blocks.

### FIGURE 3. Aster formation induced by various nucleotides and ParM-R1 mutants under crowding conditions (8% polyvinyl alcohol). A, ParM-R1 D63K mutant capable of forming asters. The nucleotide used was GMP-PNP. The inset shows the Fourier transform of the nucleus area, indicating the presence of 16-mer rings. Scale bar, 200 nm. B, ParM-R1 R262D only formed linear bundles. The inset shows the Fourier transform of such bundles. The arrow marks a meridional reflection at 4.8 nm, indicative of protofilaments. Scale bar, 200 nm. C, native ParM-R1 in the presence of GTP. The inset shows the Fourier transform of the nucleus area, indicating the presence of 16-mer rings. Scale bar, 200 nm. D, native ParM-R1 in the presence of ATP. The inset shows the Fourier transform of the nucleus area. Generally, only two reflections arising from packing of parallel filaments are observed. The arrow demarcates a single bundle. Scale bar, 500 nm.

### FIGURE 4. The aster nucleus observed by EM. A, a typical nucleus formed $\sim 15$ s after addition of GMP-PNP. B, the corresponding FT of A, which consists of three non-continuous circles of 16 discrete reflections. An arrow marks the first circle at $1/10$ nm spacing. C, the second circle is at $1/5.1$ nm, and the weak third circle is at $1/3.3$ nm spacing. C, the symmetrically averaged FT. D, the masked FT used to obtain the filtered image. E, the filtered image of the nucleus derived from the back-transformation of D. Image width of A and E, 255 nm.

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3 The abbreviation used is: FT, Fourier transform(s).
block of the nucleus, making a simple interpretation of the aster nucleus structure difficult (Fig. 4).

Therefore, we took a different approach in the analysis. First, we determined the “nodes” with the highest density in the filtered image (Fig. 5A). Next, the connections between nodes were identified (Fig. 5B). In this representation, the nucleus appears to show many square-like substructures (Fig. 5, B and C). Interestingly, the FT of this simplified structure of the aster nucleus already showed the main features of the original FT, in form of the inner 16 spot 1/10 nm⁻¹ circle (Fig. 5D).

To get a better idea of the building blocks leading to the aster nucleus structure, we performed “cluster analysis.” Individually, regions with a diameter of 42 nm were extracted around each node (420 in total) (Fig. 5A, supplemental Fig. S2). Clusters that were similar in appearance were averaged (12–33 individual images each, Fig. 6A). Many averaged clusters consist of a strong center node surrounded by eight strong nodes, indicating a pseudo 8-fold symmetry (Fig. 6A). Next, the FT of each cluster average was calculated, and the resulting 20 FT were averaged. This FT showed three circles. The inner circle at 1/10 nm⁻¹ appeared to consist of eight spots, the second circle at 1/5.1 nm⁻¹ consisted of 16 spots, which appeared shifted relative to the axial position of the spots in the first circle and a third circle at 1/3.3 nm⁻¹ included 16 spots, very similar to the FT of the original nucleus image (Figs. 6B and 4B).

A closer look at the inner circle revealed that it also consists of 16 spots and eight strong and eight weak spots (Fig. 6C). A log-plot of the inner circle from the FT of the original aster nucleus revealed that it also consisted of eight strong and eight weak spots (supplemental Fig. S3). This confirms the correctness of the cluster analysis, which gives a FT similar to the FT of the original nucleus.

To further elucidate the origin of weak and strong spots in the first circle of the calculated FT, each cluster average was filtered (Fig. 7) using a mask containing a strong central spot surrounded by eight strong spots (Fig. 7B). The filtered cluster averages emphasizing only the eight strong spots in the reciprocal space (Fig. 7C) did not appear very different from the low pass-filtered (using first 1/10 nm⁻¹ circle only) original cluster averages (Fig. 8A). Nevertheless, when filtered cluster averages with the eight spots were subtracted from the low pass-filtered original cluster averages (Fig. 8), the density appeared weak, and the corresponding FT (Fig. 8D) showed the weak eight spots originally observed in Fig. 6C.

Our interpretation is that the strong spots in the FT of the first circle arise from nodes, whereas the weak spots corresponded to the connection between nodes. This became more apparent when overlaying the subtracted cluster averages without the eight peaks with low pass-filtered original cluster averages in different colors (Fig. 9). These results indicate that the

FIGURE 5. **Node detection.** A, high density spots in the filtered image were detected (pink spots). We refer to these as nodes. B, connections between nodes were detected. A Gaussian filter was applied to C. D, the FT of C reproduces the first circle at 1/10 nm⁻¹ consisting of 16 spots. Image width of A–C, 467 nm.
structure of the aster nucleus consists of interacting nodes separated by \( \sim 10 \) nm, which are related by pseudo 8-fold symmetry. Such restricted orientation of connections between nodes appears to be a new phenomenon that has not been observed in other biological suprastructures. Interestingly, if the original aster nucleus image is filtered by artificially enhancing the spots of the second and third ring of the FT, which enhances the fine structure of the nucleus, we find evidence that the nodes and their interconnections appear to become elongated, suggesting that they may be constructed from short ParM protofilaments (Fig. 6D).

The nucleus itself seems to be composed of more than a single layer as the inner part of the nucleus appears thicker than at the edges (Fig. 4E). The shift of the spots in the second order circle at \( 1/5.1 \text{ nm}^{-1} \) relative to the first order circle may arise from a shift or beating between nodes in different layers.

In GTP-promoted aster nuclei, only the inner \( 1/10 \text{ nm}^{-1} \) circle could be observed in the FT. Although the reflections were less sharp and intense than with non-hydrolysable nucleotides, the circle consisted of 18 rather than 16 spots (Fig. 3C), indicative of a pseudo 9-fold symmetry. There appear to be two possibilities to explain the differences between these circles. The most likely is that the restriction of rings into a pseudo 8- or 9-fold symmetry is influenced by the nucleotide. This is plausible because ATP formed no aster nuclei (Fig. 3D). Supporting this explanation is the fact that small changes in surface charge arising from single mutations such as D63K also changed the symmetry from pseudo 8- to 9-fold in the presence of non-hydrolysable nucleotides (Fig. 3A). However, the possibility cannot be excluded that GTP hydrolysis may alter the symmetry. Visualizing such rearrangements will require high resolution time-resolved cryo-electron microscopy data.

**Oscillations**—To further investigate ParM-R1 dynamic instability, the concentration dependence of this process was explored. At \( 10 \mu M \) ParM-R1, in the presence of physiological GTP concentrations (1 mM), the bulk solution dynamics of growth and shrinkage started to become synchronized, leading...
to small oscillations in bulk solutions, which could be observed by light scattering (Fig. 10A). At higher ParM-R1 concentrations, addition of several millimolar GTP led to large periodic oscillations (Fig. 10). Repeated experiments were highly reproducible. The oscillation period remained constant for a given protein concentration regardless of the amount of GTP added (Fig. 10D); yet, the period increased approximately linearly with increasing protein concentration, from $\sim 530$ s at 18 $\mu$M ParM-R1 concentration to almost 900 s at 76 $\mu$M ParM-R1 concentration (Fig. 11A). More than 20 oscillations could be observed in the most favorable cases. The number of oscillations increased with increasing nucleotide concentration (Fig. 10D). Oscillation amplitudes were largest in the presence of GTP and considerably lower with ATP although the oscillation periods were similar (Fig. 10C). Over time, GTP filaments entirely disintegrated, whereas ATP filaments reached a steady state level (Fig. 10C). GTP dominated the behavior of filaments formed from an equal mixture of ATP and GTP, showing large oscillations and disassembly to completion (Fig. 10C). This confirms earlier results obtained at low ParM-R1 concentrations, which indicated ParM-R1 to be a GTPase rather than an ATPase (21).

Oscillations were observed in both non-physiological low salt (50 mM KCl) and physiological high salt conditions (300 mM KCl) (Fig. 10) (30). Addition of various amounts of non-hydrolysable nucleotide (GMP-PNP) to GTP slowed depolymerization but did not prevent oscillations (Fig. 11B). With non-hydrolysable nucleotides alone, a steady state level of ParM-R1 polymerization was rapidly reached, superimposed upon which were small oscillations with a larger oscillation period than observed with GTP (Fig. 11B). After the GTP-induced oscillations had died out, and filaments had entirely disintegrated as observed by light scattering, addition of fresh GTP restarted polymerization and oscillations (Fig. 10, B and D). The oscillation periods after restarting an oscillation remained unchanged. The total integrated intensity of oscillations peaks from the beginning to the end of a polymerization-depolymerization cycle was Gaussian-like in most experiments, except with non-hydrolysable nucleotides where the integrated intensity of oscillation peaks remained essentially constant (Fig. 11C). This behavior differs from microtubules, where the oscillations were damped in a linear fashion (31). Another difference concerned the influence of free GDP on oscillations. Whereas the presence of free GDP in microtubule oscillations provoked a significant increase in catastrophe (5, 31), this was not the case for ParM-R1, where addition of GDP or ADP had no major influence on
oscillations (data not shown). Build up of GDP, from a previous oscillation cycle, did not influence the restart of the next oscillation cycle induced by adding fresh GTP (Fig. 10).

Electron microscopy images were taken of negatively stained samples at different times of the oscillatory process (Fig. 12). Shortly after adding GTP, many ParM-R1 filaments were observed (Fig. 12B), resembling filaments grown in GMP-PNP (Fig. 12A). Near an oscillation minimum, numerous amorphous oligomers could be seen in addition to filaments (Fig. 12C). At this stage of cycling, many oligomers seemed to congregate at filament ends, which suggest that structures containing more than a single monomer dissociate from filament ends (Fig. 12D). At oscillation maxima, reduced amounts of oligomers, and many oligomer-free polymer ends were observed (Fig. 12E). Restarting oscillations, by adding fresh GTP after a previous oscillation cycle had died out, revealed almost entirely filaments by EM (Fig. 12F).

**DISCUSSION**

Mechanism of ParM-R1 Cycling—ParM-R1 polymerization can be described using the nucleated assembly model for actin that involves two steps, nucleation and elongation (22, 23). Nuclei formation is energetically less favorable than elongation, and nucleation becomes negligible once elongation takes over so that the number concentration of filaments remains roughly constant. Elongation proceeds until the free subunits are reduced below the critical concentration \( C_n \). ParM-R1 nucleotide hydrolysis is coupled to filament assembly (21, 23). Therefore, at least two types of subunits, ParM-GTP and ParM-GDP, should be considered in the dissociation reaction.

ParM-R1 depolymerization can be understood using the dynamic instability model of microtubules (32). The central portion of the ParM-R1 filaments, which consist mainly of GDP-bound protomers, is unstable; however, the decay of growing filaments is prevented by the presence of stabilizing GTP-ParM-R1 caps. Because ParM-R1 elongates by association of GTP-bound subunits, spontaneous hydrolysis of the incorporated GTP at a rate slower than the rate of elongation would automatically result in an extended GTP-cap (21, 23). Exhaustion of the GTP-ParM-R1 monomer pool below the \( C_n \) leads to the GTP-cap shrinking to zero and subsequent catastrophe.

Oscillations are only observed for microtubules under very specific conditions where two criteria are required to be fulfilled. First, the microtubule number concentration \( C_{\text{number}} \) exceeds a threshold value corresponding to a microtubule concentration of \( \sim 5 \text{ mg/ml} \) (31), which assuming 13-stranded microtubule filaments to have an average length of 2.4 \( \mu \text{m} \) (33), \( C_{\text{number}} \) can be calculated to be \( \sim 14 \text{ nM} \). Second, only when the regeneration of GTP-tubulin is slow, either due to a slower nucleotide exchange at high protein concentration (4) or that nucleotide exchange is normal (0.2 s\(^{-1}\), but regeneration is retarded by the slow dissolution of oligomers after microtubule breakdown (31). Microtubule oscillations in vitro require non-physiological levels of MgCl\(_2\) (10 mM) (4, 5). Sustained, stable oscillations were observed at microtubule concentrations of \( \sim 8 \text{ mg/ml} \).

In contrast, ParM-R1 oscillations were observed here in physiological salt and pH conditions (30), appearing at \( \geq 10 \mu \text{M} \) ParM-R1 concentrations. The composition of the ParM-R1
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oscillator is remarkably simple, requiring only two types of molecules, ParM-R1 and GTP (Fig. 11D). Plotting the oscillation period, which increases linearly with protein concentration, leads to an estimated period, which increases linearly with protein concentration, provided that it was high enough to induce oscillations. Red, GTP; yellow, ATP; green, GTP plus GMP-PNP; blue, GMP-PNP. B, oscillations in the presence of the non-hydrolysable nucleotide GMP-PNP. Blue, 54 μM ParM-R1, 4 mM GTP, and 1 mM GMP-PNP. Red, 60 μM ParM-R1 with 1 mM GMP-PNP. C, plot of the integrated intensity of individual oscillations over time, from the start to the end of a typical experiment. Note Gaussian-like appearance of most curves, except with GMP-PNP alone. 54 μM ParM-R1 with 1 mM GMP-PNP. D, model of the reaction cycle responsible for oscillations. ParM-R1 monomers (green conformation) are activated by addition of GTP (yellow conformation) (11) and assemble via a nucleation step (2) into filaments (3). GTP hydrolysis in the cap leads to disassembly of ParM-R1 filaments into oligomers (4, 5). These oligomers transiently lock the subunits in a non-polymerized state, which when dissociated into monomers (6), can be recharged with GTP (1), leading to a regrowth of existing ParM-R1 filaments and/or new nucleation.

Synchronous behavior can be found in a wide variety of biological and chemical systems. There seems to be consensus that although the subreactions of these systems are stochastic in detail, the systems as a whole react in a correlated and predictable way. The subreactions are coupled to one another through the output of one reaction serving as the input to the next reaction. If at least one of these steps is nonlinear, one can expect regular patterns or oscillations (12, 36). Non-linearity in ParM-R1 may arise from the cooperative interactions at filament ends and slow dissociation if GDP-bound aggregates to recycle GTP-bound monomers.

Aster Formation—In most cell types, microtubules form highly organized, polarized cell cycle-dependant arrays, which are built from microtubule-organizing centers. Two important microtubule-organizing center structures are the centrosome in animal cells and the spindle body in yeast (13). The base of the centrosome consists of a pair of centrioles, cylindrical structures composed of nine triplets microtubule “blades” organized around a central cartwheel (37) surrounded by pericentriolar material that nucleates a radial array of microtubules, similar in appearance to an aster. This is important in the formation of meiotic and mitotic spindles and subsequently chromosome segregation. Centrioles can also move to the cell surface and nucleate the formation of cilia and flagella (37). Many protein components of the pericentriolar material have been identified; however, only a subset of these are involved in the function of
the centrosome as a microtubule-organizing center, the major one being γ-tubulin (Fig. 13) (13). Spindles also form in the absence of centrosomes, as observed in meiotic cell division, and in in vitro experiments in Xenopus laevis egg extracts devoid of centrosomes (38). Purified motor protein kinesin and microtubules also assemble into asters (14). In the case of ParM-R1, asters were formed in vitro due to molecular crowding at physiological high salt concentrations. Initially, numerous pseudo-ring-like structures formed, which consisted of interconnected nodes, spaced 10-nm apart, and related by pseudo 8-fold symmetry. It is intriguing to consider these as analogs of centrioles. The pseudo-rings associated into a 100–300-nm-wide disk-like structure (Fig. 13). Although smaller in size, the ParM-R1 disk has a similar overall appearance in the electron microscope to isolated centrosomes (39).

In vivo, ParM-R1 asters have not been observed in bacterial cells; yet another DNA segregation system identified in E. coli, the F plasmid Sop system, forms asters both in vitro and in vivo as observed by low resolution light microscopy (40). The Sop system consists of two protein components SopA and SopB and a cis-acting DNA sequence sopC. Here, plasmid segregation is speculated to arise from SopA filaments growing radially outward from the SopB/sopC hub (40). SopA is a member of the ParA family of P-loop ATPases that is unrelated to actin and is not dynamically unstable (40). In eukaryotic cells, an aster-like microtubule mitotic spindle has the capacity not only to separate chromosomes but also to move itself to the center of the cell (41). The centering ability is accomplished through contacts of microtubules with the cell cortex. As forces applied to the spindle are propagated in a radial array along microtubules the spindle is moved to the center of the cell where all applied forces are equal. The discrete width of the 13-stranded microtubule will have a distinct advantage in the balancing forces, particularly in a multi-chromosome setting, in comparison with the distribution of widths displayed by bundle-forming proteins. The formation of asters by non-tubulin folds may also have been probed during evolution as a mechanism to center plasmids before replication (40).

ParM-R1 asters have not been observed in vivo; however, their formation in vitro invites speculation that this property may have been given up during evolution in favor of a centering/segregation mechanism driven by dynamic instability and periodic oscillations. This mechanism was recently proposed for the dynamically unstable DNA segregating protein and...
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actin homolog Alp7A from Bacillus subtilis (42). In conclusion, our results indicate that aster formation and synchronous oscillations of microtubules, which are exploited in eukaryotic chromosome segregation, can be reproduced in vitro by an actin-like polymer from a prokaryote.

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