Ca²⁺-mediated GTP-dependent dynamic assembly of bacterial cell division protein FtsZ into asters and polymer networks *in vitro*

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FtsZ, a tubulin-like GTPase that forms a dynamic ring marking the division plane of prokaryotic cells, is essential for cytokinesis. It is not known what triggers FtsZ ring assembly. In this work, we use a FtsZ–green fluorescent protein (Gfp) chimera to assay FtsZ assembly over time by using fluorescence microscopy. We show that FtsZ polymers can assemble dynamically in solution in a GTP-dependent manner. Initially, FtsZ nucleation centers grow into aster-like structures that dramatically resemble microtubule organizing centers. As assembly proceeds further, protofilament bundles emanating from different asters interconnect, mimicking the closure of the FtsZ ring *in vivo*. Surprisingly, millimolar levels of Ca²⁺ promote FtsZ dynamic assembly. FtsZ can undergo repeated GTP-dependent assembly and disassembly in solution by sequential addition and removal of Ca²⁺. In addition, GTP binding and hydrolysis by FtsZ are regulated by Ca²⁺ concentration. Although the concentration of Ca²⁺ required for FtsZ assembly *in vitro* is high, its clear and specific effect on FtsZ dynamics suggests the possibility that Ca²⁺ may have a role in regulating FtsZ ring assembly in the cell.

Keywords: bacterial cell division/cell cycle/FtsZ/green fluorescent protein

Introduction

During cell division of *Escherichia coli* and other prokaryotes, FtsZ forms a ring at the cell midpoint before cytokinesis begins (Bi and Lutkenhaus, 1991; Lutkenhaus, 1993; Rothfield and Justice, 1997). After segregation of the daughter chromosomes, the ring constricts along with the invagination of the cell membrane and wall in the process of cell septation. After the cell divides, the ring disappears. Without FtsZ, *E.coli* cells continue to grow without dividing, forming long multinucleate filaments. The factors governing the timing and placement of assembly and disassembly of the FtsZ ring *in vivo* are not well understood.

FtsZ has been found in all eubacteria and archaea examined so far, and is probably essential for cell division in all prokaryotes (Baumann and Jackson, 1996; Margolin *et al.*, 1996; Wang and Lutkenhaus, 1996). Many other proteins are essential for the cell division process in *E.coli*, such as ZipA, a recently characterized putative membrane anchor for FtsZ that co-localizes to the FtsZ ring (Hale

and de Boer, 1997), and FtsA, which also co-localizes to the FtsZ ring, but seems to act at a later step (Addinall and Lutkenhaus, 1996a; Addinall *et al.*, 1996; Ma *et al.*, 1996). However, except for FtsZ, no other homologs of essential cell division proteins have been found in the wall-less *Mycoplasma* species or in the several species of archaea that have been examined. So it is likely that FtsZ alone is responsible for marking the division site and possibly for the force generation to constrict the cell at its midpoint.

Because FtsZ has some sequence similarity to tubulin, and it can bind and hydrolyze GTP and assemble into polymers, it was hypothesized that it is a prokaryotic homolog of tubulin (de Boer *et al.*, 1992; Raychaudhuri and Park, 1992; Mukherjee *et al.*, 1993; Mukherjee and Lutkenhaus, 1994; Erickson, 1995). This hypothesis has been supported by recent electron microscope studies which demonstrated that FtsZ assembles into sheets of tubulin-like protofilaments and minirings, with the dimensions of the minirings being closest to those made by γ tubulin (Erickson and Stoffler, 1996; Erickson *et al.*, 1996). This resemblance to γ tubulin is further supported by phylogenetic data (Margolin *et al.*, 1996) as well as evidence that FtsZ first nucleates at a point on the cell membrane before polymerizing (Addinall and Lutkenhaus, 1996b; Ma *et al.*, 1996). However, *in vitro* polymerization of FtsZ into filaments was shown to be dependent on special buffers such as DEAE–dextran or lipid monolayers, and the former could be assembled with either GDP or GTP (Mukherjee and Lutkenhaus, 1994; Erickson *et al.*, 1996). Therefore, the possible importance of the GTPase activity for assembly of the FtsZ ring remained unclear. Moreover, up until now, tubulin-like dynamic assembly of FtsZ had not been demonstrated. Therefore, it was likely that the optimal conditions for FtsZ assembly *in vitro* had not yet been discovered.

To measure and optimize FtsZ dynamic assembly, we have used an *E.coli* FtsZ–green fluorescent protein (Gfp) fusion protein. When expressed at relatively low levels in *E.coli* cells, FtsZ–Gfp acts as a fluorescent reporter of FtsZ ring formation before visible septation has begun and during the septation process (Ma *et al.*, 1996). In this paper, we use purified FtsZ–Gfp fusion protein in conjunction with purified wild-type protein to demonstrate dynamic assembly of FtsZ *in vitro*, as monitored by fluorescence microscopy. We show that FtsZ initially forms aster-like nucleation sites, and that these sites are the source of growing protofilament bundles that eventually connect to other bundles from different nucleation sites. We show that this dynamic assembly can occur in solution in simple buffers and is strictly GTP-dependent. We discovered unexpectedly that this assembly can occur with millimolar concentrations of Ca²⁺. We provide evidence that Ca²⁺ may mediate assembly of FtsZ by altering its GTP-binding and hydrolysis properties.

Fig. 1. Purification of FtsZ and FtsZ-Gfp. SDS-PAGE analysis is shown, after staining with Coomassie blue. Lane 1, protein molecular mass markers; lane 2, purified FtsZ; lane 3, whole-cell lysate from strain WM617 after IPTG induction; lane 4, 25% $(\text{NH}_4)_2\text{SO}_4$ cut; lane 5, 25–35% $(\text{NH}_4)_2\text{SO}_4$ cut; lane 6, FtsZ-Gfp eluted from the first DEAE-sephacel column; lane 7, FtsZ-Gfp eluted from the second DEAE-sephacel column.

Results

Ca²⁺-mediated polymerization of purified FtsZ-Gfp and FtsZ

Our strategy was to use an *E. coli* FtsZ-Gfp chimera in order to assay FtsZ polymerization under a variety of conditions by using fluorescence microscopy. Since this fusion protein can localize to the FtsZ ring *in vivo* and can participate in dynamic FtsZ ring assembly in dividing cells without perturbing septation (Q. Sun and W. Margolin, submitted), we reasoned that it was likely to co-assemble with wild-type FtsZ *in vitro* and faithfully reflect its properties. In addition, FtsZ-Gfp was able to complement an *ftsZ84* mutant when expressed at low levels (data not shown). FtsZ and FtsZ-Gfp were both purified to ~95% purity from overproducing *E. coli* cells (Figure 1). Whereas FtsZ-Gfp was soluble, FtsZ was purified as a refolded protein from inclusion bodies or as a soluble protein (see Materials and methods). The properties of soluble and refolded FtsZ were determined to be similar in the experiments described; the data shown below are with the refolded preparation, except where noted.

To find optimal conditions for the assembly of purified FtsZ-Gfp, we performed polymerization reactions in various buffers in the presence of GTP, then measured the extent of polymerization by applying the mixture to a glass slide and observing the fluorescent filaments. We found that in Tris buffer, Ca^{2+} could stimulate efficient polymer formation by FtsZ-Gfp in solution. The critical concentration of FtsZ-Gfp for Ca^{2+} -mediated polymerization was 2.5–3 μM , 3- to 5-fold lower than previous FtsZ concentrations used for polymerization into protofilaments (Mukherjee and Lutkenhaus, 1994; Erickson *et al.*, 1996). FtsZ-Gfp concentrations of <2.5 μM could not polymerize detectably under these conditions. However, if wild-type FtsZ was added to the FtsZ-Gfp solution, fluorescent polymers formed at an FtsZ-Gfp concentration <2 μM for the refolded protein and <1 μM for the soluble protein, supporting our idea that FtsZ can co-polymerize with FtsZ-Gfp *in vitro*. The concentration of 1–2 μM FtsZ-Gfp gave an easily detectable fluorescence signal; higher

concentrations tended to be too bright and gave a higher background fluorescence. Therefore, most of the assembly experiments described below were carried out with a mixture of FtsZ and FtsZ-Gfp to maintain the protein concentration over the FtsZ critical concentration and at the same time keep the fluorescence intensity optimal for detection.

Interestingly, polymers that were formed at FtsZ concentrations >2.5 μM depolymerized very rapidly when the protein was diluted to a level below the critical concentration. This suggested that there is an equilibrium between FtsZ polymerization and depolymerization under these conditions, similar to the classic dynamic instability of tubulin. The cycling experiment (see below) also supports this idea.

FtsZ assembles into asters and protofilament bundles

Figure 2A–D shows representative polymers of FtsZ formed in a solution containing 10 mM Ca^{2+} . Polymers assembled under these conditions reproducibly formed a large web-like network, reminiscent of actin networks in higher eukaryotic cells. High-resolution transmission electron microscope (TEM) analysis of the polymers suggested that the single polymers we observed in the fluorescence microscope were actually bundles of protofilaments, with each protofilament having a diameter of ~5–6 nm (Figure 2E). This size is similar to those reported previously for FtsZ (Erickson *et al.*, 1996). The number of protofilaments per bundle varied because of the connections, but was usually in the range of 10–20. Using the calculations of Erickson *et al.*, a bundle of this thickness consisting of all 10 000 FtsZ molecules estimated to be in the *E. coli* cell would encircle the cell once, exactly what the FtsZ ring appears to do. The TEM analysis also suggests that the interconnection between protofilament bundles to make a network occurs by lateral alignment of the protofilaments (Figure 2E). The occasional bifurcation of the polymers observed with fluorescence microscopy supports this idea (Figure 2B).

In addition to the protofilament bundle networks, we often observed structures that bore a striking resemblance to microtubule asters of animal cells (Figure 2C and D). These asters were sometimes separate from and sometimes a part of the protofilament network (Figure 2A). Often, polymers emanating from one aster could be seen connecting with polymers from an adjacent aster (Figure 2A and C). The asters often contained a dense fluorescent spot in the center that could represent a nucleation site for FtsZ assembly.

The requirements for Ca²⁺ and GTP are specific

Because the polymers described above assembled in the presence of GTP and Ca^{2+} , we tested whether GTP and Ca^{2+} were specifically required and if they were sufficient for FtsZ assembly. The Ca^{2+} -mediated FtsZ polymerization under our conditions did not require DEAE-dextran or cationic lipid monolayers, conditions previously necessary to observe FtsZ protofilaments. However, GTP was absolutely required. Neither ATP nor the non-hydrolyzable GTP analogue GTP γ s supported Ca^{2+} -dependent FtsZ assembly. Although GDP has been reported to support FtsZ polymerization mediated by DEAE-dextran, it could

Fig. 2. Polymerization of FtsZ and FtsZ-Gfp *in vitro*. Micrographs of assembled FtsZ are shown. For (A–F), proteins were incubated in 50 mM Tris (pH 7.5) containing 1 mM MgCl_2 , 1 mM GTP and 10 mM CaCl_2 at 37°C; for (G), incubations were in the same buffer lacking CaCl_2 but containing 2.5 mM ZnCl_2 . (A–C, E and G) Reactions included 2 μM of FtsZ-Gfp and 10 μM of FtsZ purified from WM684; (D) reaction included 2.5 μM FtsZ-Gfp and 5 μM FtsZ purified from WM688; (F) reaction included 5 μM of partially purified C-terminally truncated FtsZ-Gfp from WM630. For (A–D, F and G), the samples were loaded directly onto a glass slide and visualized by fluorescence microscopy; for (E), the samples were loaded onto EM grids and stained with 1% uranyl acetate. Bar = 100 nm for (E); 13 μm for all other panels. The arrow in (B) highlights a visible bifurcation of the bundle.

not support the formation of asters or networks in solution containing Ca^{2+} under our experimental conditions. FtsZ was occasionally observed to form some sheets and single long filaments in the presence of GDP, but these events were very rare.

Since charge shielding is thought to stimulate tubulin polymerization (Wolff *et al.*, 1996), the effects of different cations on FtsZ assembly were tested. When Ca^{2+} was replaced with $(\text{NH}_4)^+$, K^+ , Na^+ , Mg^{2+} , Cu^{2+} , Co^{2+} , Mn^{2+} , Fe^{2+} , Ni^{2+} or Zn^{2+} at low concentration (<1 mM), FtsZ assembly into protofilament bundles was not observed by our assay. When the concentrations of these divalent ions were increased to 2.5–10 mM, some of them, such as Cu^{2+} , Co^{2+} , Mn^{2+} , Fe^{2+} , Ni^{2+} and Zn^{2+} , induced

FtsZ aggregation (Figure 2G). However, the aggregation occurred even in the absence of GTP and Mg^{2+} . One reason for aggregation at high concentrations of certain ions is probably non-specific binding of these ions to the negatively charged residues in FtsZ, resulting in charge neutralization of the protein. The buffer composition was not important for FtsZ polymerization. Although most experiments were performed in Tris buffer at pH 7.5, FtsZ could also polymerize in PIPES and MES buffers in the presence of Ca^{2+} at pH 6.0 for MES or PIPES and at pH 8.0 for Tris (data not shown). These results strongly support the idea that Ca^{2+} specifically and reproducibly stimulates GTP-dependent polymerization of FtsZ into protofilament bundles.

The Ca^{2+} concentration was also critical for polymerization. When Ca^{2+} was <5 mM, FtsZ did not form any detectable polymers or aggregates, suggesting that no multimerization had occurred. When the FtsZ concentration was between 2.5 and 15 μM , levels similar to those in the cell, the optimal Ca^{2+} concentration range for the formation of visible polymers was 7–25 mM. Although this level seems high, it is equivalent to the total bound Ca^{2+} concentration in dividing *E. coli* cells as measured by electron optical microanalysis (Chang *et al.*, 1986). When the Ca^{2+} concentration was >25 mM, some FtsZ tended to aggregate, but protofilament bundles were the dominant species. Aggregation, which did not require GTP, became dominant at Ca^{2+} concentrations >40 mM. Therefore, FtsZ assembly into protofilament bundles occurred in a narrow concentration range of Ca^{2+} . To ensure that the requirement of millimolar Ca^{2+} concentration was not due to the presence of EDTA, which was used for purification and storage of the protein, the protein preparation was dialyzed extensively against 50 mM Tris-HCl (pH 7.5) to remove EDTA prior to use in all tests.

The C terminus of FtsZ does not mediate the Ca^{2+} effect

Interestingly, Ca^{2+} in the micromolar to millimolar range has been shown to have the opposite effect on tubulin as it does on FtsZ: tubulin polymerization is inhibited and tubulin depolymerization is stimulated (Berkowitz and Wolff, 1981). A C-terminal truncation of tubulin is Ca^{2+} resistant, implicating the C terminus of tubulin in Ca^{2+} binding (Solomon, 1977; Serrano *et al.*, 1986). FtsZ has an overall structure similar to tubulin, with a highly conserved N terminus and a highly divergent, polar C terminus. The FtsZ C terminus is not essential for polymerization, because a Gfp-tagged C-terminal truncation of FtsZ can polymerize *in vivo* (Ma *et al.*, 1996). We found that partially purified truncated fusion protein can assemble into bundles *in vitro* that are similar to those assembled from the full-length fusion protein (Figure 2F). Moreover, the polymerization was dependent on GTP and Ca^{2+} . This suggested that the C terminus of FtsZ is non-essential for GTP- and Ca^{2+} -dependent assembly into protofilament bundles. The differences in the Ca^{2+} effect on FtsZ and tubulin, and the protein domains mediating the effect, suggest that FtsZ may interact with Ca^{2+} by a different mechanism.

Effects of Ca^{2+} on FtsZ GTP binding and hydrolysis

Since GTP binding and hydrolysis appear to be required for the Ca^{2+} -dependent assembly of FtsZ, we determined

Fig. 3. Effects of Ca^{2+} on GTP binding and GTPase activity of FtsZ and FtsZ-Gfp. (A) Effect of Ca^{2+} concentration on the GTP binding of FtsZ-Gfp (circles) and FtsZ (squares). (B) Effect of Ca^{2+} concentration on GTPase activity of FtsZ-Gfp (circles) and FtsZ (squares) in the presence of 50 mM KCl. (C) Effect of Ca^{2+} concentration on GTPase activity of FtsZ-Gfp (circles) and FtsZ (squares) without added KCl. Data points represent the average from four independent experiments. In (B) and (C), 1 min^{-1} is equivalent to 1 mol GTP hydrolyzed/mol FtsZ/min.

whether Ca^{2+} could modulate these activities. Figure 3A shows the effect of Ca^{2+} on GTP binding of FtsZ and FtsZ-Gfp. Increasing the Ca^{2+} concentration from 0.1 to 40 mM resulted in a reproducible increase in GTP binding by FtsZ and FtsZ-Gfp. The stimulation of GTP binding approaches a maximum of 4- to 6-fold at ~10–20 mM Ca^{2+} . On the other hand, Ca^{2+} affected GTPase activity of FtsZ in a more complicated pattern. In the presence of KCl, which promotes GTPase activity (Mukherjee *et al.*, 1993), both proteins had similar GTPase activities in the absence of added Ca^{2+} , with turnover numbers of ~1.8 per min. This activity is comparable to the results reported previously (de Boer *et al.*, 1992; Raychaudhuri and Park, 1992) which were 1.2–2 per min and 1 per min, respectively, when the assays were performed at a protein concentration similar to ours. As shown in Figure 3B, GTPase activity of FtsZ was stimulated at low Ca^{2+} concentrations, but inhibited at higher Ca^{2+} concentrations. Maximal stimulation of 30–70% for FtsZ and ~110% for FtsZ-Gfp occurred at 2.5 mM Ca^{2+} , just below the concentration required to initiate detectable FtsZ poly-

Fig. 4. Depolymerization of FtsZ polymers by EGTA. A mixture of FtsZ-Gfp (2 μM) and FtsZ (10 μM) in 50 mM Tris-HCl (pH 7.5) containing 10 mM CaCl_2 , 1 mM MgCl_2 and 1 mM GTP was incubated at 37°C for 10 min. Then 5 μl of sample were mixed with 5 μl of 50 mM Tris-HCl (pH 7.5) containing 10 mM EGTA and 1 mM MgCl_2 , and 4 μl of the mixed sample were loaded onto a glass slide and immediately monitored by fluorescence microscopy. Numbers represent minutes after acquiring the first image.

merization. When KCl was omitted from the reaction, GTPase activities of FtsZ and FtsZ-Gfp in the absence of Ca^{2+} were reduced to 0.2–0.5 per min, but, interestingly, the stimulation of GTP hydrolysis at 2.5 mM Ca^{2+} was 4- to 5-fold (Figure 3C). We conclude that GTP hydrolysis still occurs under polymerization conditions, and Ca^{2+} can strongly stimulate GTPase activity in the absence of KCl. Moreover, addition of 50 mM KCl to assembly reactions had no detectable effect on the ability of FtsZ to assemble dynamically or on the morphology of the fluorescent polymers (data not shown), suggesting that the absolute level of GTPase activity was not critical for polymer growth.

Cycling of FtsZ assembly and disassembly

We next tested whether Ca^{2+} was also needed to stabilize the FtsZ polymers once they were assembled. FtsZ was incubated in buffer containing 10 mM Ca^{2+} for 10 min, and the presence of polymers in this mixture was verified by fluorescence microscopy. After EGTA was added to the solution to make a final concentration of 5 mM Ca^{2+} and 3.5 mM EGTA, the polymers completely disassembled in 1 h at 4°C . However, when the EGTA concentration was <2 mM under the same conditions, the polymers were very stable. These data suggest that stabilization of existing polymers requires Ca^{2+} , but at a concentration lower than that needed for nucleation in solution. Figure 4 shows an example of a time course of EGTA-mediated disassembly of pre-existing FtsZ protofilament bundles. EGTA was added to the polymer solution (both final Ca^{2+} and EGTA concentration were 5 mM) and the sample was immediately loaded onto a glass slide and monitored. Most of the bundles depolymerized within 2 min, and after 8 min only a barely detectable amount of fluorescent polymer remained (Figure 4D). EGTA concentrations below 1 mM had no effect on FtsZ assembly into protofilament bundles when the Ca^{2+} concentration was >7 mM, suggesting that EGTA *per se* had no effect. To confirm that the disappearance of fluorescent polymers was not due to photobleaching of Gfp, a control experiment was carried out by exposing a sample lacking EGTA to the same amount of blue light excitation as used for the

Fig. 5. Cycling of FtsZ-Gfp assembly and disassembly in solution by using Ca^{2+} . SDS-PAGE analysis of the results of the cycling experiment is shown, after staining with Coomassie blue. Lane 1, whole-cell lysate of strain WM 617; lane 2, supernatant from first cycle of polymerization; lane 3, recovered protein from polymers from the first cycle; lane 4, supernatant from the second cycle of polymerization; lane 5, recovered protein from polymers of the second cycle; lane 6, molecular mass markers.

experiment. Photobleaching was not significant when the sample was exposed to 10–20 exposures of blue light of 1–2 s each at 2 min intervals. These experiments suggest that the EGTA-mediated depolymerization of FtsZ was due to depletion of Ca^{2+} .

If the assembly and disassembly of FtsZ were truly dependent on GTP and Ca^{2+} concentration, then it should be possible to enrich for FtsZ by repeated cycles of assembly and disassembly with Ca^{2+} and EGTA. We used a whole-cell lysate of the WM617 strain overproducing FtsZ-Gfp for polymerization by addition of Ca^{2+} to 10 mM and GTP to 1 mM. The polymers were collected by centrifugation and depolymerized by addition of EGTA at a concentration the same as that of Ca^{2+} . This solution was recentrifuged to remove aggregates that presumably could not disassemble, and was subjected to the second cycle of polymerization after dialysis against buffer lacking EGTA. Figure 5 shows SDS-PAGE analysis of FtsZ-Gfp at different stages of cycling. Comparison of lanes 2 and 3 indicates that ~70% of FtsZ-Gfp in the whole-cell lysate was able to assemble into polymers. The unassembled FtsZ-Gfp in the supernatant was able to polymerize again after removal of the existing polymers by centrifugation (data not shown), suggesting that there was an equilibrium between polymerization and depolymerization. Most of the other proteins remained in the supernatant. The pelleted FtsZ-Gfp could be depolymerized by addition of EGTA, and the depolymerized FtsZ-Gfp was able to reassemble after EGTA was removed by dialysis (lanes 4 and 5). As with the first cycle, only around 70% of the FtsZ-Gfp was recovered from polymers in the second cycle. This result is different from the previous report that described the precipitation of essentially 100% of FtsZ by GTP (Bramhill and Thompson, 1994), which was later found to be caused by a decrease to pH 5.5–6 because the GTP was unbuffered (Erickson *et al.*, 1996). Under our conditions, addition of GTP (pH 7.5) and Mg^{2+} failed to precipitate FtsZ, which confirmed the previous result and suggested that our Ca^{2+} -mediated polymerization of FtsZ was not due to low pH. Furthermore, the pH of the sample

Fig. 6. Time lapse of nucleation, growth and interconnections of FtsZ polymers. The time course of FtsZ assembly was monitored on a microscope slide as described in Materials and methods. Two representative time courses are shown. Micrographs of the extent of polymerization at each time interval are shown in minutes, starting with the acquisition of the first image. Time zero is within 1 min after the mixture was put onto the microscope slide. (A) A dense field of nucleation sites. Long arrows point to several sites per field where polymers, some initially growing in different directions, connect to each other. Short arrows show areas where connections did not occur between adjacent growing (single arrow) and apparently non-growing (double arrow) protofilaments. Three different nucleation centers are denoted by A, B and C. (B) A sparser field of asters, with arrow highlighting a long-range interconnection.

solution before and after polymerization was verified, and found to be maintained at 7.5.

Real-time assembly of FtsZ from asters to network

The Gfp tag is a powerful tool because it makes possible the observation of the process of FtsZ polymerization in real time. To accomplish this, we took advantage of a serendipitous observation. Despite the fact that 5 mM Ca^{2+} in solution was insufficient to support FtsZ assembly, transferring a pre-warmed solution of FtsZ in 5 mM Ca^{2+} to a glass slide allowed polymers to grow at a slow rate. This phenomenon may be due to concentration of the solution by evaporation as well as leaching of Ca^{2+} from the glass. These conditions allowed us to follow FtsZ assembly dynamics *in vitro*, from nucleation to connections to formation of a polymer network. Two representative time courses of FtsZ aster growth are shown in Figure 6.

The dramatic interconnections between polymers from different asters, shown earlier in steady state in Figure 2, could now be verified to be due to dynamic polymer growth and joining. Some of the most obvious interconnections are indicated by long arrows (Figure 6), whereas apparent crossing of the polymers without making connections is indicated by short arrows.

Several conclusions can be drawn from these time-lapse observations. The first is that many connections were made by polymers which nucleated a significant distance ($>10\ \mu\text{m}$) away from each other. Secondly, many connections occur between two polymers which were previously extending their ends in different directions. Because the growing polymers were in solution on the glass and were able to vibrate (data not shown), it is likely that fluid flow allowed adjacent polymers to make contact transiently, inducing a connection. This attraction was more likely to be via lateral than head-to-head or head-to-tail interactions, based on the merging protofilaments shown in Figure 2B and data not shown. The third conclusion is that protofilaments are either able to associate with each other within a bundle, since this is how the bundle forms, or with protofilaments from another bundle. The thickening of bundles growing outward from nucleation centers (compare times 0 and 4) implies that protofilaments are all in a parallel arrangement within a given bundle. However, because most merged bundles were from individual bundles growing toward each other (black arrows), then the merged bundles logically should consist of protofilaments oriented in both directions. These observations suggest either that FtsZ protofilaments do not have microtubule-like polarity or, if they do, that bundles can contain protofilaments with opposite polarity. The fourth conclusion is that many outward-growing and some convergent polymers apparently failed to join with others growing in the vicinity (short or double arrows). The few cases where convergent polymers did not join (double arrows) could be explained by the slowdown in polymer growth as the experiment proceeded.

Our data are the first to show that FtsZ can assemble dynamically *in vitro* by nucleating, polymerizing outward as a protofilament bundle, and joining other protofilament bundles. The connections made between different FtsZ protofilament bundles are reminiscent of microtubule overlap during mitosis, and also may be a model for the final assembly steps of the FtsZ ring. In addition, the sequence of events from nucleation to aster to formation of a polymer network has remarkable similarity to the dynamics of microtubules in animal cells.

Discussion

By using a fluorescent assay for FtsZ assembly in solution, we have provided the first evidence that Ca^{2+} may have an important role in the dynamic assembly of FtsZ. Since the FtsZ ring has already been proposed to consist of a protofilament bundle or sheet based on EM analysis of purified FtsZ assembled on cationic lipid monolayers (Erickson *et al.*, 1996), it is likely that the dynamic structures in solution that we have characterized are physiologically relevant. Moreover, our *in vitro* data showing interconnections between growing polymers provide evidence that FtsZ assembly in the cell begins as a

nucleation center or centers and that the FtsZ ring forms once protofilament bundles growing outward from these centers connect.

Ca^{2+} has been implicated as a possible trigger for bacterial cell division, despite the lack of direct evidence (Norris *et al.*, 1988, 1996; Norris, 1989). In fact, the role of Ca^{2+} in bacteria is enigmatic, except possibly for its function in chemotaxis (Ordal, 1977; Tisa and Adler, 1995). Indirect evidence for a role for Ca^{2+} in cell division comes from an electron optical microanalytic study, which determined that the total Ca^{2+} concentration in *E. coli* is ~ 30 -fold higher in dividing cells (20 mM) than in non-dividing cells (0.65 mM) (Chang *et al.*, 1986). These high total concentrations of Ca^{2+} fit well with the concentrations in solution that support and fail to support *in vitro* FtsZ assembly, respectively. In addition, since FtsZ often assembles at the daughter cell midpoints as the mother cell is dividing (Q. Sun and W. Margolin, submitted), the higher Ca^{2+} level found in dividing cells may occur at the appropriate time for assembly of new FtsZ rings. However, the free Ca^{2+} concentration in *E. coli* is only around 0.1–1 μM (Gangola and Rosen, 1987), so it is unlikely that FtsZ would ever be exposed to free Ca^{2+} levels in the millimolar range *in vivo*. This suggests that if Ca^{2+} is involved in potentiating FtsZ assembly *in vivo*, other factors are also probably required. If an additional soluble factor exists, it was not active in the soluble fraction of *E. coli* whole-cell extracts, since the polymerization of FtsZ–Gfp in such extracts also required similar millimolar Ca^{2+} concentrations (data not shown). Since the Ca^{2+} concentration in the membrane is significantly higher than in the cytoplasm (Chang *et al.*, 1986), perhaps Ca^{2+} binding proteins or phospholipids may serve to present Ca^{2+} to FtsZ and initiate FtsZ ring formation, which might reduce the Ca^{2+} concentration requirement relative to what is required *in vitro*. The stimulation of FtsZ assembly by cationic lipid suggests that this is possible (Erickson *et al.*, 1996), but also suggests that the stimulus in this mixture may have been Ca^{2+} itself. It should be noted that millimolar levels of Ca^{2+} are also required for *in vitro* phosphorylation of EnvZ, a membrane-bound sensor kinase (Rampersaud *et al.*, 1991) and for *in vitro* activity of a membrane-associated endonuclease from *Mycoplasma* (Bendjennat *et al.*, 1997). Clearly, future work to image Ca^{2+} in bacterial cells and to isolate additional factors that promote FtsZ assembly will be required to establish a physiological role of Ca^{2+} in cell division.

FtsZ and tubulin share several important biochemical properties, such as GTP binding and hydrolysis, ability to form polymers, and similar predicted secondary structures (de Pereda *et al.*, 1996). In addition, we have found that as for tubulin, a critical concentration of FtsZ is required for assembly into protofilament bundles. This concentration, 2.5 μM , is similar to that estimated previously for pH-dependent FtsZ polymerization (Bramhill and Thompson, 1994) and is much lower than that for tubulin. This property may be due to the requirement *in vivo* for FtsZ to nucleate its own assembly, whereas microtubule assembly depends on microtubule organizing centers (Archer and Solomon, 1994). Another interesting difference between the two proteins is the stimulation of FtsZ assembly by Ca^{2+} , in striking contrast to the strong inhibition of tubulin assembly by Ca^{2+} . This suggests that

although FtsZ may bind to Ca^{2+} directly, as does tubulin, the conformational changes and potential modification of the proteins may be very different. The binding site on FtsZ for Ca^{2+} appears to be in a different domain than on tubulin, since the C terminus of tubulin binds Ca^{2+} whereas the assembly of a C-terminally deleted FtsZ is still dependent on Ca^{2+} . The cold resistance of FtsZ assembly under our conditions is another feature of FtsZ that is remarkably different from that of normal microtubules. It is interesting to note that when cold-stable microtubules are isolated, they are also resistant to millimolar levels of Ca^{2+} (Job *et al.*, 1981). Perhaps the Ca^{2+} interaction with FtsZ is similar to that with cold-stable microtubules.

The ability of FtsZ to assemble into asters and protofilament bundles may be evolutionarily significant, because γ tubulin also nucleates asters and forms short polymers on its own (Shu and Joshi, 1995). Our data support the model, developed from phylogenetic analysis (Baumann and Jackson, 1996; Margolin *et al.*, 1996), and from structural analysis of FtsZ and γ tubulin minirings (Erickson and Stoffler, 1996), that γ tubulin evolved from FtsZ. It is also conceivable that FtsZ polymer nucleation occurs by a ring mechanism similar to models proposed for γ tubulin (Moritz *et al.*, 1995; Zheng *et al.*, 1995; Erickson and Stoffler, 1996). Putative nucleation sites for FtsZ with polymers emanating from them have been observed in *E. coli* cells with FtsZ-Gfp or immunostained for FtsZ (Addinall and Lutkenhaus, 1996b; Ma *et al.*, 1996), and may be the *in vivo* counterparts to the asters we saw on the glass slides. The mechanism of nucleation is unclear, although our results suggest that Ca^{2+} may be involved and that FtsZ itself may be sufficient for the process. Since FtsZ asters have not been observed in bacteria, it is likely that nucleation and polymerization of the FtsZ ring occur by a mechanism that is constrained by the size and shape of the bacterial cell as well as the specific localization of nucleation factors. However, because bacterial FtsZ can form structures *in vitro* such as asters that are hallmarks of the eukaryotic tubulin cytoskeleton, it is tempting to speculate that the ancestral prokaryotic FtsZ already had the potential, apparently untapped, to form centrosome-like structures and networks before eukaryotes arose. Interestingly, recent evidence supports a role of γ tubulin in animal and plant cell cytokinesis (Liu *et al.*, 1993; Shu *et al.*, 1995), suggesting that perhaps FtsZ and γ tubulin have retained some similar cellular roles over the course of evolution.

The dramatic interconnections made between growing protofilament bundles *in vitro* over time suggest that the same process may occur *in vivo* to close the FtsZ ring. Our data suggest that FtsZ protofilament bundles can merge laterally, either to thicken the existing bundle or to connect with another bundle growing from a separate nucleation site. Such a configuration is reminiscent of microtubule overlap in the mitotic spindle, except for the crucial difference that microtubules never attach to each other, but instead are cross-linked by MAPs (Vallee and Bloom, 1983). Our *in vitro* data support the model proposed by Addinall and Lutkenhaus (1996b) in which FtsZ nucleates at a single midcell site and polymerizes bidirectionally around the cell, with the polymers connecting on the opposite side. Other potential models

invoking additional nucleation sites would also be consistent with our data. More detailed fine structural studies of FtsZ polymers, which should be facilitated by our assembly conditions, should be able to address the mechanisms of FtsZ protofilament polarity, growth and interconnections.

The roles of GTP binding and hydrolysis in the FtsZ ring cycle remain unclear, although the results presented here provide some new clues. On the one hand, our data suggest that GTPase activity may be essential for the correct assembly of FtsZ, because neither GDP nor GTP γ S could support assembly into protofilament bundle networks under our conditions. On the other hand, although it is intriguing that GTPase activity was specifically stimulated only in a narrow range of Ca^{2+} concentrations (1–5 mM), these concentrations were mostly just below the level required for us to detect FtsZ assembly. This suggests that Ca^{2+} does not mediate assembly solely by stimulating GTPase activity. It is not clear at present why GTPase stimulation and detectable FtsZ assembly occur at different Ca^{2+} concentrations. Interestingly, when KCl was omitted from the reaction, the stimulation of GTPase activity at 1–5 mM Ca^{2+} was stronger and the inhibition at higher concentrations of Ca^{2+} was weaker, perhaps because the absence of added KCl lowered the basal activity level. In contrast to GTP hydrolysis, GTP binding was clearly stimulated in the same range of Ca^{2+} required for GTP-dependent assembly. Therefore, part of the promotion of FtsZ assembly by Ca^{2+} may be due to its effect on GTP binding. These various findings can be investigated further by measuring the assembly, GTP-binding and GTP-hydrolysis properties of mutant FtsZ proteins with our assay, as well as refining the assay conditions. These and other studies to determine how the GTP cycle affects FtsZ conformation should help us to understand the fundamental roles of GTP, Ca^{2+} and other factors in the proper timing and placement of FtsZ ring assembly.

Materials and methods

Strains and plasmids

Escherichia coli strain JM105 was used as the host for protein induction. Strain WM617 contained plasmid pZG, which has Gfp fused to the C terminus of *E. coli* ftsZ in pBC SK+ under lac promoter control (Ma *et al.*, 1996). Strains WM684 and WM688 contain plasmids in which ftsZ was cloned between the SacI and ApaI sites of pBluescript SK+ (WM684) and pBC SK+ (WM688) such that ftsZ expression is under the control of the lac promoter. Plasmids from WM617, 684 and 688 all allowed complementation of an ftsZ84 temperature-sensitive mutant for colony formation, provided that the expression from the vector lac promoter was kept very low, indicating that FtsZ and FtsZ-Gfp were functional. WM630 harbors plasmid pZGA, described previously (Ma *et al.*, 1996).

Protein purification

FtsZ was overproduced from strain WM684 in 1 l of LB + 50 $\mu\text{g/ml}$ ampicillin, grown at 37°C and induced overnight with 1 mM IPTG. Cells were collected, washed and resuspended in 20 ml of 50 mM potassium phosphate buffer (pH 8.0), containing 0.3 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% glycerol. The cells were then broken by three passages through a French Pressure cell at 1000 p.s.i. and centrifuged at 25 000 g for 35 min. The inclusion bodies were washed and resuspended in 50 ml of buffer A (50 mM Tris-HCl buffer pH 7.5, 5 mM MgCl_2 , 1 mM EDTA, 10% glycerol) and solubilized by adding urea to 6 M and incubation on ice for 30 min. The urea concentration was lowered by gradual addition of 350 ml of buffer A. The solution was clarified by centrifugation and loaded onto

a DEAE-sephacel column (1.5×40 cm) pre-equilibrated with buffer A. The column was washed extensively with the same buffer and eluted with a gradient (300 ml) of 0–1 M KCl. The major elution peaks were pooled together and adjusted to 35% saturation with $(\text{NH}_4)_2\text{SO}_4$, and centrifuged at 25 000 g for 35 min. The resulting pellet was resuspended in and dialyzed against buffer A. FtsZ was also purified as a soluble protein from a cell lysate supernatant of WM688 as described previously (Raychaudhuri and Park, 1992), except that an additional DEAE-sephacel column (1.5×40 cm) was used to purify the protein further with a 300 ml gradient of 0.25–0.6 M KCl.

FtsZ-Gfp was overproduced from strain WM617 by IPTG induction as described above for FtsZ. Soluble FtsZ-Gfp was purified from the cell lysate supernatant by addition of $(\text{NH}_4)_2\text{SO}_4$ to 25% saturation and centrifuged at 25 000 g for 35 min. The resulting pellet was resuspended in 20 ml buffer B (20 mM potassium phosphate buffer pH 7.0 containing 0.1 M NaCl, 1 mM EDTA and 10% glycerol) and dialyzed against the same buffer. The non-dialyzable fraction was loaded onto a DEAE-sephacel column pre-equilibrated with buffer B. The column was washed with 6 vols of buffer and eluted with a gradient (300 ml) of 0.1–1 M NaCl. The green peak was FtsZ-Gfp fusion protein, which was >90% pure as judged by SDS-PAGE. The minor impurities were removed by loading the protein onto a second DEAE-sephacel column and eluted with a gradient of 0.25–0.6 M NaCl. The protein was then extensively dialyzed against buffer A. The truncated FtsZA-Gfp fusion protein was partially purified from WM630 cells as described for FtsZ-Gfp, except that the second column purification step was omitted. For all purification experiments, protein concentration was determined with the bicinchoninic acid method (Smith *et al.*, 1985) using bovine serum albumin as a standard.

GTP binding and GTPase assays

GTP binding and GTPase assays were performed as described previously (Raychaudhuri and Park, 1992) with minor modifications. Briefly, for UV light cross-linking of GTP, 2 µM of protein was incubated with 0.3 µM [α - ^{32}P]GTP (3000 Ci/mmol, Amersham) in 10 µl of 50 mM Tris-HCl (pH 7.5) containing 1 mM MgCl_2 and various concentrations of CaCl_2 at 4°C for 30 min, 2 cm from a UV lamp. A total of 10 µl of 2× SDS-PAGE loading buffer were added, and boiled at 100°C for 3 min; 18 µl of samples were subjected to SDS-PAGE. For GTPase assays, 5 µM of protein was incubated in 50 mM Tris-HCl (pH 7.5) containing 1 mM [γ - ^{32}P]GTP (0.5 Ci/mmol, Amersham), 5 mM MgCl_2 and various concentrations of CaCl_2 at 37°C. To some reactions, 50 mM KCl was added. At different time intervals, 5 µl aliquots were added to an equal volume of 1% SDS and 20 mM EDTA, and heated at 75°C for 2 min. The nucleotides and phosphate were resolved by chromatography on polyethyleneimine (PEI) cellulose in 0.75 M KH_2PO_4 (pH 3.4). Both the protein bands of SDS-PAGE gels from UV cross-linking of GTP and the GTP or Pi spots on PEI cellulose were visualized and quantitated with a Packard Instant Imager.

Assembly of FtsZ and FtsZ-Gfp

Polymerization reactions were performed with a mixture of 2.5–15 µM total FtsZ and FtsZ-Gfp, with FtsZ-Gfp generally at 1–2 µM. The mixture was incubated in 50 mM Tris, 1 mM MgCl_2 and 10 mM CaCl_2 at 37°C for 30 min. The mixture was then added directly to a glass slide, covered with a cover glass, and polymers were visualized using an Olympus BX60 epifluorescence microscope equipped with a 100 W HBO lamp, standard fluorescein isothiocyanate filter set, and a 100× fluorite oil immersion objective (NA = 1.3). Images were captured with an Optronics DEI-750 video camera and a Scion LG3 framegrabber, and manipulated in Adobe PHOTOSHOP 3.0. A Tektronix Phaser 440 was used for printing the images. For TEM analysis, the same mixture was applied to a formvar-coated grid, stained by 1% uranyl acetate, and visualized with a Jeol 1200 EX transmission electron microscope.

Dynamic assembly assays

To track FtsZ assembly in real time with the fluorescence microscope, 2 µM FtsZ-Gfp and 10 µM FtsZ were incubated in 50 mM Tris-HCl (pH 7.5) containing 5 mM CaCl_2 , 1 mM MgCl_2 and 1 mM GTP at 37°C for 5–10 min. Then 4 µl of the pre-warmed solution were transferred to a glass slide and covered with a cover glass. Polymers usually started to form in 1–2 min, and were photographed at time intervals with the imaging system described above. Photobleaching was minimized by keeping exposure to blue excitation light as short as possible over the time course. For the assembly-disassembly cycling experiment, a whole-cell lysate of FtsZ-Gfp-overproducing strain WM617 was prepared as described in the purification procedure, except that the buffer was

changed to 50 mM Tris-HCl (pH 7.5) plus 10% glycerol and 1 mM PMSF. Cell debris was removed by centrifugation at 35 000 g for 40 min. Then, 10 mM CaCl_2 , 1 mM MgCl_2 and 1 mM GTP were added to the lysate, which was incubated at 37°C for 30 min followed by centrifugation at 35 000 g for 40 min. The pellet was resuspended in 50 mM Tris-HCl (pH 7.5) containing 10 mM EGTA and 10% glycerol, and incubated on ice for 30 min. EGTA was then removed by dialysis against 50 mM Tris-HCl (pH 7.5) plus 10% glycerol. Insoluble aggregates were removed by centrifugation at 35 000 g for 40 min. The second cycle of polymerization and depolymerization was initiated by the addition of CaCl_2 , MgCl_2 and GTP to the supernatant as described above.

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