MinC Protein Shortens FtsZ Protofilaments by Preferentially Interacting with GDP-bound Subunits*

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Background: The MinC protein of the site selection Min system targets FtsZ to prevent polar division ring formation. Results: At similar MinC concentrations, MinC protein shortens FtsZ protofilaments and interacts preferentially with GDP-bound FtsZ.

Conclusion: MinC disrupts FtsZ protofilaments by specifically targeting FtsZ-GDP subunits.

Significance: Biophysical analysis reveals how MinC disrupts FtsZ filaments in solution.

The interaction of MinC with FtsZ and its effects on FtsZ polymerization were studied under close to physiological conditions by a combination of biophysical methods. The Min system is a widely conserved mechanism in bacteria that ensures the correct placement of the division machinery at midcell. MinC is the component of this system that effectively interacts with FtsZ and inhibits the formation of the Z-ring. Here we report that MinC produces a concentration-dependent reduction in the size of GTP-induced FtsZ protofilaments (FtsZ-GTP) as demonstrated by analytical ultracentrifugation, dynamic light scattering, fluorescence correlation spectroscopy, and electron microscopy. Our experiments show that, despite being shorter, FtsZ protofilaments maintain their narrow distribution in size in the presence of MinC. The protein had the same effect regardless of its addition prior to or after FtsZ polymerization. Fluorescence anisotropy measurements indicated that MinC bound to FtsZ-GDP with a moderate affinity (apparent $K_D \sim 10 \mu M$ at 100 mM KCl and pH 7.5) very close to the MinC concentration corresponding to the midpoint of the inhibition of FtsZ assembly. Only marginal binding of MinC to FtsZ-GTP protofilaments was observed by analytical ultracentrifugation and fluorescence correlation spectroscopy. Remarkably, MinC effects on FtsZ-GTP protofilaments and binding affinity to FtsZ-GDP were strongly dependent on ionic strength, being severely reduced at 500 mM KCl compared with 100 mM KCl. Our results support a mechanism in which MinC interacts with FtsZ-GDP, resulting in smaller protofilaments of defined size and having the same effect on both preassembled and growing FtsZ protofilaments.

In Escherichia coli, the Min system prevents aberrant cytokinesis close to cell poles, ensuring a correct placement of the division machinery to produce two identical cells (1). Evidence indicates that this system blocks cell division by targeting FtsZ, a widely conserved tubulin homolog whose polymerization represents one of the first steps in the assembly of the cell division machinery (2, 3). The three components of the Min system, MinC, MinD, and MinE proteins, have been shown to form a pole-to-pole oscillator with a 1–2-min cycle in E. coli (4). The presence of MinD and MinE is sufficient for the emergence of this oscillatory behavior in vivo (4) or in vitro (5), whereas MinC is the actual inhibitor of FtsZ assembly (2, 6). It has been proposed that the oscillation of these proteins creates a gradient of MinC from the poles to midcell, the latter being then the only location where the formation of the division ring would be allowed (7). Remarkably, the Min system is widely conserved in prokaryotes with MinD and MinE homologs present in chloroplasts (3, 8).

Although not required for the oscillation, MinC is a key component of the Min system as it inhibits FtsZ polymerization and therefore connects the Min oscillation with the division machinery. FtsZ is a central component of this machinery whose reversible polymerization coupled to GTP hydrolysis triggers the formation of the Z-ring, a complex of FtsZ polymers and associated cell division proteins that coordinates membrane constriction with septum formation at midcell (9). FtsZ polymerization has been extensively studied in vitro, especially for the E. coli protein (9–13). Upon addition of GTP, FtsZ assemblies into head-to-tail polymers, usually called protofilaments, whose size and arrangement are highly variable (9, 10). These protofilaments are very dynamic, and they rapidly disassemble when the GTP-GDP ratio in the solution decreases due to hydrolysis. Strategies such as the addition of a GTP enzymatic regenerating system (14) have been developed to maintain...
the polymers at steady state for sufficient time to be used in biophysical methods (15, 16). Another approach to be used is to use slowly hydrolyzable analogs of GTP such as GMP-PCPP, a compound that permits polymerization of FtsZ for longer times (10). Using the GTP-regenerating system approach, we have recently shown that under close to physiological conditions, that is nearly neutral pH and 500 mM KCl, FtsZ assembles into a narrow size distribution of protofilaments comprising around 100 subunits in solutions containing 0.3–5 mM Mg²⁺ (16). The concerted formation of a narrow distribution of polymers was also observed under these conditions when polymerization was triggered by GMP-PCPP (16). Protofilaments are considered the basic structural unit of the Z-ring, but they are too short to encompass the whole bacterial circumference at midcell. Therefore, protofilaments must assemble into a larger structure to form the Z-ring observed in vivo (17, 18). The mechanism by which this is achieved is not known, although in vitro assembly of protofilaments into several higher order structures has been observed upon addition of crowding agents (12, 19) or calcium (20) or in certain buffer conditions (21).

Experiments with MinC tagged with MalE protein revealed that it inhibits FtsZ-GTP polymer sedimentation, although it does not affect FtsZ GTPase activity (2). Strikingly, GTPase activity was required for MinC inhibition as FtsZ polymerized with slowly or non-hydrolyzable GTP analogs like GMP-PCPP and GDP/AlF₄⁻ was unaffected by this inhibitor (6). The crystal structure of MinC from *Thermotoga maritima* reveals that it is formed by two structural domains connected by a short flexible linker (22). Studies with deletion mutants have revealed that both isolated domains are able to interact with FtsZ and inhibit polymer sedimentation, although the C-terminal domain (MinC") requires the presence of MinD (23). MinC" is responsible for MinC oligomerization (probably dimerization) and contains the binding site for MinD as indicated by structural and mutational studies (22, 24). Moreover, in vivo and in vitro assays with MinC and FtsZ mutants indicate that MinC" interacts with FtsZ through the last 15 C-terminal amino acids of FtsZ in the presence of MinD (25). In addition, a recent study described several mutations in *E. coli* FtsZ clustering to the H10 α-helix at the interface between two FtsZ molecules that render it resistant to MinC" (26). In this study, it was proposed that MinC severs FtsZ polymers by specifically breaking the interaction between FtsZ and GDP present in polymers and the adjacent FtsZ molecule through this α-helix. A more recent mutational study on *Bacillus subtilis* FtsZ found mutations that conferred resistance to MinC in a different, more lateral region of FtsZ than in *E. coli*, suggesting that the MinC inhibition mechanism may be different in this organism (27).

With the aim of obtaining further insight into the molecular mechanisms underlying the influence of MinC on *E. coli* FtsZ polymerization, we applied a biophysical approach entailing a combination of methods such as SV, DLS, and FCS that has proven very useful to thoroughly characterize FtsZ polymers (11, 16). Previous studies dealing with the effects of MinC on FtsZ polymerization were carried out principally under conditions favoring FtsZ bundling (6, 26). In contrast, our study was conducted at nearly neutral pH and moderate ionic strength and Mg²⁺ concentration, conditions under which single-stranded FtsZ protofilaments are observed. In this way, the effect of MinC on protofilament formation was specifically addressed in a quantitative manner and related with the interaction between the inhibitor and the GDP-bound form of FtsZ, providing crucial details on the molecular basis of MinC function.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—pWM2688 containing His₆-tagged MinC was obtained by amplifying the minC gene with primers 890 (GCGAGGCTCTAAACACCGCAATCGAGC) and 803 (CGGGATCCTTAATTTAACGGTTGAACGG) and cloning it as a SacI-BamHI fragment (sites underlined) into pWM2619 (23) to render pWM2622 and then into Ncol-BamHI-cleaved pET28a.

**Protein Expression, Purification, Labeling, and FtsZ Polymerization Conditions**—*E. coli* FtsZ was purified by the calcium-induced precipitation method (28). MinC was produced in an *E. coli* BL21(DE3) strain harboring pLys and pWM2688 plasmids. Expression was carried out at 30 °C for 3 h in LB medium in the presence of kanamycin and chloramphenicol after induction with 1 mM isopropyl-β-D-galactopyranoside at A₆₀₀ ~0.6. After cell pelleting, cells were resuspended in 25 mM Tris-HCl at pH 8, 25 mM NaCl, 10% glycerol and sonicated on ice. After centrifugation at 30,000 × g for 30 min at 4 °C, MinC was purified from the soluble fraction using a protocol adapted from Ref. 29 that includes two chromatographic steps, anion exchange chromatography and immobilized metal affinity chromatography. MinC was eluted from the affinity column using 25 mM Tris-HCl, pH 8, 25 mM NaCl, 500 mM imidazole, 10% glycerol. Fractions were centrifuged for 10 min at 4 °C, and the supernatants were desalted in a 5-ml HiTrap desalting column (GE Healthcare) against 50 mM Tris-HCl, pH 7.5, 500 mM KCl, 10% glycerol; frozen; and stored at −80 °C. Before each experiment, MinC fractions were thawed and dialyzed against the desired buffer.

FtsZ was covalently labeled in the amine groups with Alexa Fluor 488 carboxylic acid succinimidyl ester dye (Molecular Probes/Invitrogen) as described elsewhere (30). The degree of labeling, estimated from the molar absorption coefficients of the protein and the dye, was typically 30–50%. MinC was covalently labeled in the amine groups with Alexa Fluor 488 or Alexa Fluor 647 carboxylic acid succinimidyl ester dyes (Molecular Probes/Invitrogen). The reaction was allowed to proceed for 45 min at room temperature at pH 7.5 with a 2-fold molar excess of dye with respect to the protein. The degree of labeling, calculated using the molar absorption coefficients of the protein and the dyes, was 30–40%.

To analyze the effect of MinC on FtsZ protofilaments, samples were equilibrated in T500 buffer (50 mM Tris-HCl, pH 7.5, 500 mM KCl, 1 mM EDTA, 0.2 mM tris(2-carboxyethyl)phosphine, 5 mM MgCl₂) or T100 buffer (the same as T500 but with 100 mM KCl instead). 1 mM GTP or 0.4–1 mM GMP-PCPP was added to trigger polymerization. Unless otherwise stated, FtsZ-GTP polymers were kept in solution for at least 1 h by using an

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3 The abbreviations used are: GMP-PCPP, guanosine 5′-O-(6-carboxymethyl) triphosphate; DLS, dynamic light scattering; FCS, fluorescence correlation spectroscopy; SV, sedimentation velocity; RS, regenerating system; SE, sedimentation equilibrium.
enzymatic GTP-regenerating system (RS; 15 mM acetyl phosphate, 2 units/ml acetate kinase) (12).

Sedimentation Velocity—This technique was used to assess MinC homogeneity and oligomerization in the absence of FtsZ, to measure the effect of MinC on FtsZ polymer size, and to monitor the binding of MinC to the polymers. Samples were equilibrated in T500 or T100 buffer. In experiments where FtsZ polymers were present, MinC and FtsZ were mixed prior to starting polymerization, which was then promoted with the addition of GTP + RS or with GMPCPP.

SV experiments were carried out at 48,000 rpm in an XL-I analytical ultracentrifuge (Beckman-Coulter Inc.) equipped with UV-visible and interference detection systems. Sedimentation profiles were registered by interference or, when labeled MinC was present, by absorbance at the adequate wavelength. The sedimentation coefficient distributions were calculated by least square boundary modeling of sedimentation velocity data using the c(s) method as implemented in SEDFIT (31).

To be able to compare the effect of MinC on the sedimentation properties of FtsZ protofilaments in different conditions, s values of protofilaments were normalized as follows,

\[
s_{\text{norm}} = 1 - \frac{s_c - s_i}{s_o - s_u}
\] (Eq. 1)

where \(s_i\) and \(s_o\) represent the experimental \(s\) value of the polymer peak in the presence and absence of MinC, respectively, and \(s_u\) corresponds to the \(s\) value of fully unassembled FtsZ in the corresponding buffer conditions: 2.7 \(S\) for T500 buffer and 2.8 \(S\) for T100 buffer. An \(s_{\text{norm}}\) value of 1.0 corresponds to an \(s\) value equal to that of the protofilament peak in the absence of MinC, whereas an \(s_{\text{norm}}\) value of 0 represents an \(s\) value equal to that of the FtsZ monomer peak. MinC concentration values at which half of the maximum change in the \(s\) value is achieved (C_{1/2}) were calculated using the empirical Hill equation described below for FCS data (Equation 4).

Sedimentation Equilibrium—Sedimentation equilibrium (SE) was used to assess the MinC association state in the absence of FtsZ and to study the binding of labeled MinC to FtsZ-GDP. Buffer and sample conditions were similar to those used for sedimentation velocity. Short column (85 \(\mu\)l) SE was carried out at multiple speeds (from 9000 to 14,000 rpm), and the corresponding scans were recorded at 230 and 280 nm for unlabeled MinC, 450–500 nm for MinC labeled with Alexa Fluor 488 (MinC-Alexa 488), and 600–650 nm for MinC labeled with Alexa Fluor 647 (MinC-Alexa 647). After the equilibrium scans, a high speed centrifugation run (48,000 rpm) was done to estimate the corresponding base-line offsets. Whole-cell apparent weight average buoyant molecular weight for each sample (FtsZ, MinC, and mixtures) was determined by fitting a single species model to the experimental data using the Hetero-Analysis program (32). The corresponding protein molecular weights were calculated from the experimental buoyant mass using theoretical partial specific volumes and buffer densities calculated with SEDNTERP software (33).

Fluorescence Anisotropy—This technique was used to monitor the binding of FtsZ labeled with Alexa Fluor 488 (FtsZ-Alexa 488) to MinC. Measurements were performed on a PC1 photon counting steady-state spectrofluorometer (ISS, Inc.) regulated at 20 °C using 3 × 3-mm quartz cuvettes (Hellma Hispania or Starna Scientific). The anisotropy was repeatedly measured for each sample, and the average of at least 8–10 values, obtained after reaching equilibration, was calculated. Binding isotherms were built by titration of MinC into solutions containing a fixed 0.69 \(\mu\)M concentration of FtsZ-Alexa 488. Results presented correspond to the average of at least three independent measurements.

Analysis of the binding isotherm was conducted using BIOEQS software (34), which allows calculation of the free energy of formation of complexes from their free elements by using a numerical solver engine. Errors in the fitting parameters were calculated by rigorous confidence limit testing at the 67% confidence level using this software.

Fluorescence Correlation Spectroscopy—FCS was used to monitor titrations of FtsZ-GTP polymers with MinC to obtain a C_{1/2} value and to study the binding of fluorescently labeled MinC to FtsZ polymers.

FCS measurements were performed under two-photon excitation using a Microtime 200 system (PicoQuant) essentially as described elsewhere (30). Analysis of the autocorrelation profiles recovered from titration of MinC into solutions containing 120 nm FtsZ-Alexa 488 and 0.5 g/liter (12.5 \(\mu\)M) unlabeled FtsZ in the presence of GTP + RS was performed as shown previously for FtsZ-GTP polymers (30). Briefly, a three-component model was found to represent the experimental data. This model involved a fast component corresponding to the free dye whose diffusion time and contribution (<20%) were fixed in the analysis, and two other components assigned to assembled and unassembled FtsZ. The translational diffusion coefficient of the unassembled protein was constrained to that independently measured in the absence of GTP. The translational diffusion coefficient of the polymeric species and its fractional contribution to the autocorrelation curve were found to depend on the concentration of MinC in the solution. FCS-based titrations of FtsZ with MinC were performed either in T500 or T100 buffer at 21 °C. Some experiments were performed on FtsZ polymers elicited by 0.4 mM GMPCPP.

To evaluate the global effect of MinC on FtsZ polymerization, an average translational diffusion coefficient, \(D\), was calculated at each concentration of MinC as follows,

\[
<D> = f_uD_u + f_pD_p
\] (Eq. 2)

where \(D_u\) and \(D_p\) are the diffusion coefficients of unassembled and assembled FtsZ, respectively, and \(f_u\) and \(f_p\) are their molar fractions. Average \(D\) values were normalized as follows,

\[
<D>_{\text{norm}} = (\langle D \rangle - D_0)/(D_u - D_0)
\] (Eq. 3)

where \(D_0\) is the average translational diffusion coefficient measured in the absence of MinC. C_{1/2} values corresponding to the concentration of MinC at which half of the maximum effect on FtsZ polymerization was observed were obtained by fitting the dependence of normalized \(D\) on MinC concentration using the empirical Hill equation,
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\[
<\text{D}>_{\text{norm}} = \frac{\left( \frac{c}{C_{1/2}} \right)^{a}}{1 + \left( \frac{c}{C_{1/2}} \right)^{b}} \quad \text{(Eq. 4)}
\]

where \( c \) is MinC concentration and \( n \) is the cooperativity, here constrained to 1 (independent binding). User-defined scripts were written in MATLAB (version 7.10; MathWorks, Natick, MA) to analyze the experimental data. The interaction of MinC-Alexa 488 (0.45 \( \mu \)M) with 25 \( \mu \)M FtsZ-GTP in T100 or T500 buffer was measured and analyzed using one- or two-component models as described for ZipA reconstructed in nanodiscs (35).

**GTPase Activity**—The GTPase activity was determined by measuring released inorganic phosphate using the malachite green-molybdate reagent (36, 37).

**Dynamic Light Scattering**—We used DLS to measure the diffusion of FtsZ-GTP polymers in the presence of RS and variable concentrations of MinC following a procedure previously optimized for analysis of FtsZ filaments (11, 16). Samples were measured at 20 °C after filtration with a 0.1-\( \mu \)m Anotop syringe filter (Whatman) and centrifugation at 50,000 rpm in an MLA-130 rotor for 30 min. Experiments were performed, and data were analyzed as in Ref. 16. Briefly, an empirical exponential function that properly describes the diffusion behavior of the scattering species was used through which an apparent average diffusion coefficient of the species in solution was obtained.

**Electron Microscopy**—MinC and FtsZ were equilibrated separately in T100 buffer and mixed at the indicated concentrations. Polymerization was triggered upon addition of 1 mM GTP plus RS. After addition of GTP, samples were incubated for 5 min at room temperature and then adsorbed to carbon-coated glow-discharged grids. After 2 min, the sample was blotted and then stained for 1 min with 1% uranyl acetate. Images were recorded on a TemCam-F416 complementary metal oxide semiconductor camera (Tietz Video and Image Processing Systems) under low dose conditions at 50,000× nominal magnification on a JEOL-1200 electron microscope operated at 80 kV.

**RESULTS**

**MinC Reduces the Mass of the Narrow Size Distribution of FtsZ-GTP Protofilaments**—In a previous study, we extensively investigated the size and hydrodynamic properties of FtsZ-GTP polymers using physiologically meaningful polymerization conditions: pH 7.5, 500 mM KCl, 5 mM MgCl\(_2\) and a millimolar concentration of GTP constantly replenished by a GTP-regenerating system (11). Under these conditions, the FtsZ sedimentation profile includes a sharp peak at 14–15 S corresponding to FtsZ protofilaments (Fig. 2A). The extent of this reduction was dependent on the concentration of MinC present, although saturation was not reached at the highest concentration of MinC attainable (see below). Interestingly, the narrow size distribution characteristic of FtsZ protofilaments was still observed in the presence of MinC. Parallel experiments on FtsZ filaments obtained under slow hydrolysis conditions, that is in the presence of GMPCPP (FtsZ-GMPCPP), showed only a minor reduction in the s value of the polymer (\( s_{\text{norm}} = 0.92 \)) when using a 3:1 MinC:FtsZ ratio (Fig. 2B), whereas in the same conditions, \( s_{\text{norm}} \) was 0.69 for FtsZ-GTP filaments. In summary, SV results indicate that MinC provokes a change in the size and/or conformation of FtsZ-GTP protofilaments.
which remain narrowly distributed in size, whereas the filaments elicited by GMPCPP have a greatly reduced sensitivity to this protein.

To determine whether the effect of MinC on the s value of FtsZ polymers was related to a change in size, the diffusion coefficient of FtsZ-GTP + RS in the presence of 25 μM MinC (2:1 MinC:FtsZ molar ratio) was measured using DLS. In these conditions, MinC produced an increase in the diffusion coefficient of FtsZ-GTP compared with that observed in its absence (Table 1), whereas the sedimentation profile still showed a narrow peak of smaller s value than that obtained for FtsZ protofilaments in the absence of MinC. The number of subunits present in FtsZ polymers was calculated from their sedimentation and diffusion coefficients using the Svedberg equation as described previously (16). At a 2:1 MinC:FtsZ molar ratio, FtsZ polymers contained half as many subunits as in the absence of the inhibitor (Table 1), confirming that MinC reduces the size of FtsZ-GTP protofilaments.

The above described results correspond to samples in which MinC was added to FtsZ prior to triggering polymerization with GTP. To test whether MinC could also reduce the size of preformed FtsZ-GTP polymers, MinC was added to FtsZ previously incubated for at least 10 min with 1 mM GTP + RS. DLS profiles obtained adding MinC either before or after GTP were nearly identical, indicating that MinC can also disassemble preformed FtsZ protofilaments.

To further analyze the effect of MinC on FtsZ polymerization, we used FCS, a technique that reports on the diffusion of fluorescently labeled species that has been used to study FtsZ polymerization (16, 30). Autocorrelation profiles recovered for 12.5 μM FtsZ assembled by addition of GTP + RS in T500 buffer displayed a shift to shorter diffusion time scales when MinC was present (Fig. 3A). Analysis of these traces indicated an increase in the diffusion coefficient of the protofilaments (Table 2), compatible with a MinC concentration-dependent shortening of the fibers, in agreement with DLS analysis. At high MinC concentrations, a significant increase in the fraction of unpolymerized protein was also found. Autocorrelation traces obtained when MinC was added before triggering polymerization with GTP + RS or to preformed protofilaments overlapped (Fig. 3B). Remarkably, at all MinC concentrations assayed, the slowly diffusing FtsZ protofilaments were well represented by a single diffusion coefficient, indicating that they were narrowly distributed in size. To describe the global effect of MinC on FtsZ polymerization, an average diffusion coefficient ((D)) was calculated (see “Experimental Procedures”). (D) was found to increase with MinC concentration (Table 2), although saturation was not reached at the maximum concentration of MinC achievable in agreement with SV results (Fig. 4). Finally, FCS anal-

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**Quantitative Analysis of FtsZ Polymer Inhibition by MinC**

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**Biophysical analysis of FtsZ protofilaments in the presence of MinC**

| Table 1 | Biophysical analysis of FtsZ protofilaments in the presence of MinC |
|---------|---------------------------------------------------------------|
| [MinC]  | 500 mM KCl | 100 mM KCl |
| μM      | D*         | s<sub>20,w</sub> | FtsZ subunits<sup>a</sup> | D*         | s<sub>20,w</sub> | FtsZ subunits<sup>a</sup> |
| 0       | 4.0 ± 0.3  | ~16           | ~90            | 4.6 ± 0.5  | ~14           | ~70            |
| 12.5    | 5.9 ± 0.4  | ~13           | ~50            | 8.3 ± 0.6  | ~9            | ~25            |
| 25      | 12.7 ± 1.0 | ~14           | ~70            | 12.7 ± 1.0 | ~9            | ~25            |

<sup>a</sup> Diffusion coefficient of protofilaments obtained using 12.5 μM FtsZ upon addition of the indicated concentrations of MinC in T500 and T100 buffers.

<sup>b</sup> Sedimentation coefficient of protofilaments obtained using 12.5 μM FtsZ upon addition of the indicated concentrations of MinC in T500 and T100 buffers.

<sup>c</sup> The number of FtsZ subunits was calculated from s<sub>20,w</sub> and D using the Svedberg equation as explained previously (11, 16).
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FIGURE 3. Effect of MinC on the diffusion of FtsZ-GTP protofilaments as studied by FCS. A, normalized FCS autocorrelation profiles of FtsZ-GTP in T500 buffer in the absence (open circles) and presence of different MinC concentrations: 6.3 μM (gray diamonds), 12.5 μM (open triangles), and 37.5 μM (gray triangles). The profile of FtsZ-GDP (open squares) is displayed for comparison. B, normalized FCS autocorrelation profiles of FtsZ-GTP polymers formed in the presence of MinC (gray triangles) and FtsZ-GTP polymers where polymerization was triggered before MinC addition (open diamonds). MinC concentration was 37.5 μM. The profiles of FtsZ-GDP (open squares) and FtsZ-GTP (open circles) in the absence of inhibitor are displayed for comparison. C, normalized FCS autocorrelation profiles of FtsZ-GTP in T100 buffer in the absence (open circles) and presence of different MinC concentrations: 3.1 μM (gray diamonds), 6.3 μM (open triangles), and 18.1 μM (gray triangles). The profile of FtsZ-GDP (open squares) is displayed for comparison. In all panels, lines represent best fits of the model used to calculate the average diffusion coefficient, (D), for each curve (see “Experimental Procedures”). The concentration of FtsZ-Alexa 488 was always 120 nM, and unlabeled FtsZ was added to achieve the final 12.5 μM (0.5 g/liter) concentration. All measurements were conducted in the presence of RS.

Analysis revealed that the effect of MinC on the diffusivity of FtsZ-GMPCPP polymers was very small even when using a 3:1 MinC:FtsZ molar ratio ($D_p = 3.5 \pm 0.2 \mu m^2/s$ with MinC versus $3.1 \pm 0.4 \mu m^2/s$ without MinC) in agreement with the mild effects observed in SV experiments under the same conditions.

TABLE 2

| [MinC] | $D_p$ | $D_s$ | $D_p$ | $D_s$ |
|--------|-------|-------|-------|-------|
| μM     | μm²/s | μm²/s | μm²/s | μm²/s |
| 37.0   | 7.1 ± 0.2 | 31 ± 1 |
| 24.0   | 8.3 ± 2 | 24.7 ± 0.3 |
| 18.4   | 12.5 ± 3 | 33 ± 2 |
| 12.5   | 21.7 ± 2.0 ± 0.3 |
| 9.0    | 23 ± 2 | 23 ± 2 |
| 6.3    | 25 ± 1 | 25 ± 1 |
| 3.1    | 28.7 ± 0.7 | 28.7 ± 0.7 |
| 1.6    | 33 ± 2 | 33 ± 2 |
| 0.8    | 47 ± 0.2 | 47 ± 0.2 |

FIGURE 4. Dose-response curves for MinC-mediated depolymerization of FtsZ-GTP protofilaments. Variation of (D)$_{norm}$ (top panel; normalization of (D) data in Table 2 according to Equation 3) and of $s_{norm}$ (bottom panel; normalized profilament s values from Fig. 2A according to Equation 1) with MinC concentration in T500 (open circles) or T100 buffer (solid circles). The solid lines indicate the best fit of Equation 4 to the experimental data rendering $C_{1/2}$ values of $10 \pm 2 \mu M$ for diffusion data and $16 \pm 4 \mu M$ for depolymerization data. Dotted lines are only meant to guide the eye. Error bars represent S.D. of at least four independent experiments.

MinC Effects on FtsZ Assembly Are Highly Dependent on Ionic Strength—One of the factors that has been reported to influence the inhibitory function of proteins such as SlmA (39) and that also acts on FtsZ polymerization is K$^+$ concentration. Therefore, we tested whether shifting ionic strength from 500 mM KCl to 100 mM KCl without altering any of the other conditions had an impact on the effects of MinC on FtsZ protofilaments. First, we measured the s value and diffusion coefficient by DLS of FtsZ-GTP polymers at 100 mM KCl in the absence and presence of various concentrations of MinC (Fig. 2 and Table 1). We found that polymer size was also reduced in these conditions. Moreover, as observed at 500 mM KCl, at this lower salt concentration, the addition of MinC did not qualitatively alter the particular polymerization of FtsZ into preferred fibrils.
of defined size. Calculations using the Svedberg equation showed that, at a 1:1 molar ratio with FtsZ, MinC reduced the number of subunits per protofilament from ~70 in its absence to ~25. The reduction in size observed at this lower salt concentration was larger than that obtained in T500 buffer at a MinC concentration twice as high (see above and Table 1). Hence, lowering the salt concentration provoked a marked enhancement in the inhibitory effect of MinC on FtsZ protofilament assembly. Shortening of polymers under these conditions was further confirmed by transmission electron microscopy micrographs of FtsZ protofilaments in the presence of MinC (Fig. 5). Moreover, these micrographs also showed a reduction in the number of FtsZ polymers upon addition of MinC in qualitative agreement with the reduction in the fractional contribution of the polymers to the FCS autocorrelation curves in the presence of MinC.

To quantify the effect of MinC on FtsZ polymerization in T100 buffer, SV and FCS titrations of FtsZ-GTP with increasing MinC concentration were conducted. A progressive reduction in the average s value (Fig. 2) and an increase in the average diffusion coefficient (Fig. 3C and Table 2) of FtsZ at increasing concentrations of MinC were observed. It is worth noting that, as for T500 buffer, FCS autocorrelation profiles obtained in the presence of MinC were also compatible with a single or narrow distribution of FtsZ protofilaments. At 100 mM KCl and the highest MinC concentrations tested, the effect of MinC on FtsZ polymers was closer to saturation than at 500 mM KCl (Fig. 4). Analysis of the dependence of the normalized average translational diffusion coefficient on MinC concentration at 100 mM KCl rendered a C$_{1/2}$ of 10 ± 2 µM, corresponding to the concentration of MinC at which half of the maximum depolymerization effect was attained. A similar analysis for the normalized s value of protofilaments resulted in a C$_{1/2}$ of 16 ± 4 µM (Fig. 4).

Finally, we measured the GTPase activity of FtsZ and found that it remained unaffected by MinC under our experimental conditions (T100 and T500 buffers; data not shown) in good agreement with results obtained on FtsZ bundles by other groups (2). Overall, these results demonstrate that the effect of MinC on FtsZ polymers is of the same nature at 100 and 500 mM KCl, i.e. a reduction in the size of the protofilaments without altering the tendency of FtsZ to assemble into protofilaments of defined size and the GTPase activity. These effects are stronger at a low salt concentration, suggesting that FtsZ-MinC interaction is greatly affected by ionic strength.

MinC Binds to FtsZ-GDP with Similar Concentration Dependence as Its Effects on Protofilament Shortening—One of the potential mechanisms by which MinC could interfere with FtsZ assembly is by sequestering FtsZ monomers, making them unavailable for polymerization. Such a mechanism has been proposed for SulA (40, 41). For this mechanism to work, MinC should be able to bind to non-polymeric FtsZ, and in fact, it has been reported that MalE-MinC binds to immobilized biotinylated FtsZ with $K_D$ ranging from 0.9 to 6 µM when tested in a biosensor assay (2, 26). We investigated whether soluble His$_6$-MinC was able to bind FtsZ-GDP in T100 and T500 buffers using sedimentation equilibrium and fluorescence anisotropy.

First, we obtained binding isotherms using a fluorescence anisotropy assay in which the anisotropy of fluorescently labeled FtsZ-GDP was monitored upon addition of increasing concentrations of unlabeled MinC (Fig. 6A). No GTP was added to FtsZ, and hence, no protofilaments are formed under these conditions. We detected an increase in anisotropy in both low and high ionic strength conditions, suggesting that interaction between FtsZ-GDP and MinC takes place. The binding curve at 100 mM KCl was close to saturation at 30 µM MinC, whereas at 500 mM KCl, saturation was not reached even at 42 µM MinC, indicating a lower binding affinity. Analysis of the curve at 100 mM KCl using a simple 1:1 binding model compatible with the data in which the only assumption is that each MinC monomer (independently of its self-association state) interacts with an FtsZ monomer resulted in an apparent dissociation constant of 12 ± 2 µM ($\Delta G^0 = 6.6 \pm 0.1$ kcal/mol). This value is equivalent within error to the concentration of MinC rendering half of the maximal effect on FtsZ polymerization derived from the change in the normalized s or D values (Fig. 4).

The difference between the apparent dissociation constant determined here and those reported previously (1–6 µM) may be explained by the lower ionic strength (50 mM KCl) in those studies (2, 26). Rough analysis of the isotherms obtained at 500 mM KCl, assuming a total anisotropy change equal to that obtained at 100 mM KCl, rendered an apparent affinity of ~70
Interestingly, no change in the anisotropy of FtsZ-Alexa 488 was detected upon addition of over 40 μM MinC in the presence of GTP or GMPCPP (10). Note that the nanomolar concentration of FtsZ used in this assay is below the critical concentration of polymerization (30), and hence, FtsZ remained unassembled. Although we cannot rule out the formation of very flexible complexes between FtsZ and MinC that escape detection, this experiment suggests that MinC does not bind unassembled FtsZ-GTP or FtsZ-GMPCPP.

To further demonstrate the interaction of MinC with FtsZ-GDP, we performed sedimentation equilibrium experiments monitoring changes in the average molecular weight of fluorescently labeled MinC upon addition of unlabeled FtsZ in T100 buffer. MinC was found to oligomerize mostly into dimers by SV and SE (Fig. 1), although higher order oligomers whose proportion varied with protein concentration were also observed. Labeled MinC retained these oligomerization features. The average molecular mass of labeled MinC increased in the presence of FtsZ from 51,000 ± 1900 Da in the absence of FtsZ to 72,000 ± 700 Da in the presence of 12.5 μM FtsZ (Fig. 6B), indicating the formation of heterocomplexes, in good agreement with the anisotropy titrations. In summary, we have shown that MinC in solution interacts with FtsZ-GDP at neutral pH with a moderate affinity close to the concentration of MinC that produced half of the maximal effect on protofilament length and observed that this interaction is highly dependent on ionic strength.

Only a Minor Fraction of MinC Interacts with FtsZ Protofilaments—We have recently used SV and FCS to monitor the binding of fluorescently labeled ZipA nanodiscs to FtsZ polymers (35). Here, we used an analogous approach to address the interaction of MinC with FtsZ polymers.

Mixtures of MinC labeled with either Alexa Fluor 488 or Alexa Fluor 647 and FtsZ in the presence of GTP + R5 were
Quantitative Analysis of FtsZ Polymer Inhibition by MinC

In this work, we quantitatively analyzed the negative regulation of FtsZ assembly by MinC using powerful orthogonal biophysical techniques such as analytical ultracentrifugation, FCS, DLS, and fluorescence anisotropy in a combined manner. Our most salient result is the fact that MinC reduces the length of the fundamental unit of the Z-ring, the protofilament. Early studies suggest that MinC inhibits Z-ring assembly mainly by blocking the association of protofilaments into bundles without a major effect on the assembly of FtsZ into protofilaments (2, 6, 10). However, Shen and Lutkenhaus (26) have recently proposed a model for MinC inhibition in which MinC would bind FtsZ molecules within polymers through interaction between MinC6 and the C-terminal tail of FtsZ. When GTP is converted to GDP, helix H10 within FtsZ would become available to interact with MinC6, which would produce a disruption of the filament. The reduction in the length of FtsZ filaments by MinC was already suggested from transmission electron microscopy and atomic force microscopy images (15, 42). Moreover, under the conditions used in this work, FtsZ is known to polymerize into a narrow distribution of preferred fibrils (16, 42). Our results clearly support shortening of FtsZ protofilaments, still rendering polymers of defined size as a key feature of the mechanism by which MinC interferes with Z-ring assembly. Furthermore, we found that MinC is not only capable of shortening growing protofilaments but also protofilaments previously assembled in the presence of GTP.

Dajkovic et al. (6) proposed that MinC inhibits lateral interaction between filaments to prevent the formation of the Z-ring based on measurements of the elasticity of gels formed by FtsZ polymer networks. We could only detect the binding of a minor fraction of MinC to FtsZ polymers. This means that in our conditions MinC can only block lateral interactions for a few molecules in the polymer, and therefore, its effect on filament bundling might be minimal. As discussed above and unlike in our case, the conditions used in that work promote filament bundling. It is possible that, in conditions in which bundling is favored, MinC remains attached to FtsZ polymers and thus prevents lateral interactions. Moreover, the MinD-MinC complex might also have a different distribution between polymer-bound and monomer-bound species than isolated MinC. Finally, shortening of filaments may produce filament networks with a reduced elasticity modulus.

Our measurements indicate that MinC reduces the size of FtsZ protofilaments more efficiently upon lowering ionic strength, suggesting a strong electrostatic component in the interaction between the two proteins. Indeed, we observed that the interaction of MinC with FtsZ, particularly in the GDP form, is of higher affinity at 100 mM KCl compared with 500 mM KCl. In agreement with this result, both FtsZ regions proposed for the interaction with MinC, the H10 helix and the C-terminal tail, contain abundant charged residues. Moreover, several FtsZ mutants found to be insensitive to MinC presented mutations in some of these charged residues (D373E and K380M on the C-terminal tail and R271G and E276D on helix H10) (25, 26).

The similarity between the C50 value obtained for the polymer shortening effect and the apparent Kp for the interaction of MinC with FtsZ-GDP, together with the fact that both processes are affected by ionic strength in the same direction, strongly suggests that the effects of MinC on FtsZ polymerization are exerted through its interaction with FtsZ-GDP. Indeed, we investigated the binding of MinC to FtsZ containing several different nucleotides and found that MinC preferentially binds to FtsZ-GDP, whereas only a minor fraction of MinC attaches to polymers in good agreement with results from a FRET assay performed under buffer conditions favoring bundling (29). Moreover, our experiments are compatible with a lack of interaction between MinC and FtsZ-GTP or FtsZ-GMPCPP under non-polymerizing conditions (i.e. protein concentration below...
Quantitative Analysis of FtsZ Polymer Inhibition by MinC

The preferential interaction of MinC with FtsZ-GDP may point toward a sequestration mechanism similar to that described for SulA, one of the best characterized inhibitors of FtsZ assembly. This protein binds to FtsZ with higher affinity than MinC, decreasing the GTP hydrolysis rate and inhibiting FtsZ polymerization by sequestering monomers (41). However, this mechanism would be difficult to reconcile with the lack of effect of MinC on the GTPase activity of FtsZ under conditions promoting assembly into protofilaments as also observed for the bundles (2). Here we found that protofilaments triggered by GMPCPP had a highly reduced sensitivity to MinC in agreement with previous reports in which bundling conditions were used (6) probably because of the lower cycling of subunits compared with the FtsZ-GTP polymers (10). As already pointed out by other authors (10), it is difficult to understand why GTPase activity is required for MinC inhibition because this protein has no detectable effect on the ability of FtsZ to hydrolyze GTP. Alternatively, the insensitivity of the FtsZ-GMPCPP polymers to MinC could also be related to the preferential binding of MinC to FtsZ-GDP subunits.

From our results, it can be inferred that any model attempting to explain the particular MinC inhibition mechanism of FtsZ polymerization will have to take into account that FtsZ protofilaments remain narrowly distributed in size even in the presence of MinC, which would hardly support a mechanism involving random breakage of the polymers. We can speculate that MinC may target FtsZ-GDP subunits both unassembled and at the end of the protofilaments. Moreover, it is widely accepted that FtsZ polymers are dynamic assemblies with a high subunit turnover (10, 21) that may determine the size of the protofilaments. On the other hand, turnover is generally thought to be mainly controlled by GTPase activity. The results presented here regarding protofilament inhibition by MinC (summarized in Fig. 8) would suggest that, aside from the GTPase activity of FtsZ, the subunit exchange rate and hence the size of the filaments may be determined by additional factors as this inhibitory protein modifies the size of FtsZ protofilaments without altering the GTPase activity.

In conclusion, we have demonstrated that MinC acts both on formed protofilaments to shorten them and on unassembled FtsZ to limit the growth of the polymer when assembly is triggered by GTP. These effects are produced by specifically targeting FtsZ-GDP without influencing the GTPase activity and the tendency of FtsZ to form preferred fibrils of a particular size. These results provide further insights on the molecular basis of the inhibition of the Z-ring assembly by MinC, adding specific information about the effect of this protein on individual FtsZ protofilaments.

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FIGURE 8. Summary of MinC effects on FtsZ assembly. This scheme depicts the effects of MinC on the different steps of FtsZ protofilament assembly based on the results presented herein and in previous literature. In the absence of MinC (1), FtsZ-GTP (T) forms protofilaments with a defined size (15, 16), which is likely determined by the subunit turnover rate (10, 21). It is generally assumed that some GDP-bound subunits are present inside protofilaments, although the specific amount is still a matter of debate (9, 10, 21). In the presence of MinC (2), FtsZ protofilaments are shorter but remain narrowly distributed in size (Table 1 and Fig. 2A), which strongly suggests that MinC does not produce random fragmentation of filaments. Instead, MinC targets FtsZ-GDP (D) subunits (the unassembled subunits and probably subunits at filament ends) (Figs. 6 and 7), altering the dynamics of subunit exchange in a way that reduces the average size of filaments (3). This alteration may consist of a short lived attachment of MinC to filament ends, enhancing the disassembly of GDP-bound subunits. The MinCN binding site in FtsZ, the H10 helix, is probably inaccessible to MinC in subunits within protofilaments (26), and binding through MinCN, which targets the C-terminal tail, is much weaker (25). This would explain why only a minor fraction of MinC is attached to filaments (Fig. 7). The fact that MinC does not alter GTPase activity (this work and Ref. 2) strongly suggests that the GTP binding properties of FtsZ remain unaffected when bound to MinC.
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