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Mechanism of Regulation of Prokaryotic Tubulin-like GTPase FtsZ by Membrane Protein EzrA*

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At initiation of cell division, FtsZ, a tubulin-like GTPase, assembles into a so-called Z-ring structure at the site of division. The formation of Z ring is negatively regulated by EzrA, which ensures only one ring at the midcell per cell cycle. The mechanism leading to the negative regulation of Z-ring formation by EzrA has been analyzed. Our data reveal that the interaction between EzrA and FtsZ not only reduces the GTP-binding ability of FtsZ but also accelerates the rate of GTP hydrolysis, both of which are unfavorable for the polymerization of FtsZ. Moreover, the acceleration in rate of GTP hydrolysis by EzrA is attributed to stabilization of the transition state for GTP hydrolysis and reduction in the affinity of GDP for FtsZ. Clearly, EzrA is able to modify the GTP hydrolysis cycle of FtsZ. On the basis of these results, a model for how EzrA acts to negatively regulate Z-ring formation is proposed.

Cell division in bacteria is mediated by the Z ring, of which FtsZ is the key component (1). At initiation of cell division, FtsZ polymerizes at the midcell into a ring structure that is required for subsequent recruitment of other cell division proteins and assembly of the cell division machinery (2–6).

FtsZ is a prokaryotic tubulin homologue that possesses GTPase activity. Although its amino acid sequence includes only a short segment with a high degree of sequence homology to tubulins, there is a remarkable similarity between the higher order structures of FtsZ and tubulin (7–10). Polymerization of FtsZ along the inner circumference of the cytoplasmic membrane is GTP-dependent (4) and is regulated by the GTP hydrolysis cycle of FtsZ (11, 12). Mutational analysis of *Escherichia coli* FtsZ has identified three catalytic amino acid residues in the GTPase-activating T7 loop of FtsZ, which are located far from the active site and are important for the GTPase but not nucleotide-binding activity of FtsZ (13–15). On the basis of the structural data of FtsZ, it was assumed that the three catalytic amino acid residues in the T7 loop of one FtsZ subunit of the protofilament can be brought into the vicinity of the catalytic site of the second FtsZ by making head-to-tail contact (16). It is the FtsZ in head-to-tail contact that possesses the GTPase activity (14). However, the main form of nucleotide in FtsZ polymers was found to be GDP-P_i (17) or GTP (12), depending on the treatment of FtsZ polymers. In addition, excess GDP was found to promote disassembly of the preformed FtsZ polymers (18).

EzrA is a transmembrane protein involved in spatial regulation of Z-ring formation in *Bacillus subtilis* (19). It is uniformly distributed throughout the plasma membrane in both nondividing and dividing cells and concentrates at the cytokinetic ring in an FtsZ-dependent manner during cell division (19). It has been also reported that EzrA plays both positive and negative roles in cell division (19, 20). Evidence for the positive role of EzrA came from the observation that low level expression of EzrA increases both cell length and percentage of filamentous cells of *B. subtilis* during growth (20). The negative role of EzrA was noted by the fact that null mutation of EzrA lowers the critical concentration of FtsZ required for Z-ring assembly and leads to the formation of polar Z rings and septa (19). In addition, the null mutation of EzrA is able to suppress the instability of FtsZ polymer associated with *minCD* overexpression (21). However, how EzrA acts to fulfill both the positive and negative roles remains unclear.

Recently, a purified thioredoxin-EzrA fusion protein was found to interact with FtsZ and to block FtsZ assembly but not disassembly *in vitro*. However, it did not significantly alter the GTPase activity of FtsZ (22). This implies that EzrA binds neither to the nucleotide-binding site nor to the catalytic amino acids in the GTPase-activating T7 loop of FtsZ to drastically block the interaction between the FtsZ monomers and thus to eliminate the GTPase activity of FtsZ. Rather, EzrA may regulate FtsZ assembly through modulating the functional conformation of FtsZ. To verify the idea, we analyzed the effects of EzrA on the GTP hydrolysis cycle of FtsZ. On the basis of our data, a model for how EzrA acts to negatively regulate Z-ring formation during *B. subtilis* cell division is proposed.

EXPERIMENTAL PROCEDURES

Construction of Plasmids for Expression of EzrA and FtsZ—The DNA fragment encoding the cytoplasmic domain (amino acid residues 27–562) of EzrA was amplified from the chromosome DNA of *B. subtilis* by polymerase chain reaction and cloned into pET21d (Novagen). The resultant plasmid, pKM4, expressed a 59-kDa EzrA with a His_6 tag fused at its C terminus. The sequences of the forward and reverse primers used for *ezrA* synthesis and cloning were 5′-AGACTACCATGGCCGAAATCGACCGGCTG-3′ and 5′-CGTTACCTCGAGAGCGGATATGTCAGCTTTG-3′, respectively. The FtsZ having a C-terminal His_6 tag was expressed using the pET21d-derived plasmid, pKM2. The sequences of the forward and reverse primers used for the synthesis and cloning of *ftsZ* were

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5′-AGACTACCATGGCTTTGGAGTTCGAAACAAACA-TAG-3′ and 5′-CGTTACCTCGAGGCCGCGTTTATTAC-GGTTTC-3′, respectively.

Purification of EzrA and FtsZ—E. coli BL21/DE3 harboring pKM2 or pKM4 plasmid was inoculated into the 2xYT medium supplemented with 100 μg/ml ampicillin and grown at 37 °C. Protein expression was induced at A_{550} of 0.8 by 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. The cells were harvested 4 h later, pelleted at 8,000 rpm and 4 °C for 10 min, and resuspended with 20 ml of ice-cold buffer (25 mM Tris-HCl, pH 7.0, 100 mM KCl, 5% glycerol, and 1 mM phenylmethylsulfonyl fluoride).

Both His-tagged EzrA and FtsZ were purified using the same method. Briefly, the cells containing the target proteins were lysed with a French press at 16,000 p.s.i. The lysate was cleared by centrifugation at 12,000 rpm, 4 °C for 20 min. The target proteins in the supernatant were absorbed with TALON™ IMAC resin (Clontech) and washed extensively with the buffer (25 mM Tris-HCl, pH 8.0, 100 mM KCl, and 5% glycerol) supplemented with 10 mM imidazole. The target proteins were then eluted with the same buffer supplemented with 150 mM imidazole. Sample fractions containing the target proteins were pooled, applied onto Superdex 200HR column (Amersham Biosciences) preequilibrated with HEPES buffer (20 mM HEPES, pH 7.0, 100 mM KCl, and 5% glycerol). The purified EzrA and FtsZ were concentrated by Centricon 10 (Millipore), estimated to be at least higher than 95% in homogeneity, and stored in buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 50% glycerol at −20 °C. The proteins were dialyzed against a specific buffer before being used to assay the effect of EzrA on a specific property of FtsZ.

Cross-linking Assay—The protein mixture containing 5 μM each of FtsZ and EzrA in HEPES buffer (20 mM HEPES, pH 7.0, 100 mM KCl, and 5% glycerol) was cross-linked with a thiol-cleavable primary amine-reactive homobifunctional cross-linker, 3,3-dithiobis(sulfosuccinimidylpropionate) (Pierce) at room temperature for 30 min. The concentration of the cross-linker was 250 μM. The cross-linking reaction was quenched with 20 mM Tris-HCl (pH 7.4) by incubation at room temperature for 15 min. Finally, the sample was subjected to SDS-PAGE and immunoblot analysis with anti-FtsZ and anti-EzrA.

Effect of EzrA on Binding of BODIPY®FL-GTPγS[2] to FtsZ—The analysis was based on relief of the fluorescence quenching of BODIPY®FL-GTPγS, a GTP analogue, upon its binding to FtsZ. To measure the effect of EzrA on binding of the GTP analogue to FtsZ, 400 μl of 500 nM BODIPY®FL-GTPγS in buffer (25 mM Tris-HCl, pH 7.0, 100 mM KCl, and 10 mM $MgCl_2$) was loaded into a 1-ml cuvette. Basal fluorescence (λ_{ex} = 485 nm and λ_{em} = 530 nm) of the GTP analogue was taken at room temperature with an F4500 FL Spectrophotometer (Hitachi). Afterward, 250 nM FtsZ either alone or preincubated with an equal concentration of EzrA (or bovine serum albumin (BSA)) for 30 min was further added into the cuvette. Fluorescence of the GTP analogue for each treatment was again monitored.

The apparent K_D value for FtsZ and BODIPY®FL-GTPγS binding was determined by measuring the fluorescence of the GTP analogue (from 12.5 to 200 nM) after mixing with either 250 nM FtsZ or 250 nM FtsZ preincubated with an equal concentration of EzrA for 30 min. The data of relative fluorescence obtained at various concentrations of BODIPY®FL-GTPγS was fitted to the one-site-binding hyperbolic function of Grafit (version 5; Erithacus Software).

Effect of EzrA on Binding Affinity of GTP, GDP-AlF_4^- or GDP for FtsZ—The protein sample containing 500 nM FtsZ or 500 nM each of FtsZ and EzrA was mixed with 500 nM BODIPY®FL-GTPγS in a 1-ml cuvette. Fluorescence of the GTP analogue in each sample was followed immediately and referred to as 100% relative fluorescence, respectively. Then various concentrations of GTP, GDP-AlF_4^- or GDP were added into the samples to compete with BODIPY®FL-GTPγS for FtsZ binding. The fluorescence obtained for each treatment was then divided by the reference fluorescence to determine the percentage of relative fluorescence.

Effect of EzrA on the GTPase Activity of FtsZ—The GTPase activity of FtsZ was measured using the Enzchek free phosphate assay kit (Molecular Probes, Inc., Eugene, OR), which takes advantage of the spectrophotometric shift of maximal absorbance from 330 to 360 nm when the substrate, 2-amino-6-mercapto-7-methylpurine riboside, is converted into ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine through the purine nucleoside phosphorylase-dependent addition of phosphate to 2-amino-6-mercapto-7-methylpurine riboside. In order to accurately measure the GTPase activity of FtsZ, 1 mM GTP was preincubated with the kit reagent for 10 min at 22 °C to deplete free phosphate before mixing with the protein sample, which includes FtsZ, EzrA, or a mixture of FtsZ and EzrA in 50 mM HEPES, pH 7.5, 200 mM KCl, 175 mM NaCl, and 2.5 mM $MgCl_2$. The concentration of FtsZ or EzrA in each experiment was 5 μM. The absorbance at 360 nm of the sample was taken every 10 s at room temperature with a U2001 UV-visible spectrophotometer (Hitachi). The rate of GTP hydrolysis was calculated based on the regression line of phosphate standards.

To measure the effect of EzrA on the apparent k_{cat} and K_m of FtsZ, the GTPase activity of FtsZ or FtsZ preincubated with EzrA was measured at 25, 50, 100, 200, 300, 400, and 500 μM GTP. The initial rate of GTP hydrolysis for each reaction thus obtained was fitted to the enzyme kinetics program of Grafit (version 5; Erithacus Software) to determine the k_{cat} and K_m.

Effect of EzrA on FtsZ Polymerization Assayed by Sedimentation—Procedures for sedimentation analysis were similar to those described in a previous report (23). Before sedimentation analysis, the purified FtsZ was cleared by spinning at 250,000 × *g* and 4 °C for 15 min to remove any aggregate or preexisting polymer. Then FtsZ or FtsZ preincubated with an equal molar concentration of EzrA (or BSA) was diluted with polymerization buffer (50 mM MES, pH 6.5, 10 mM $MgCl_2$, and 30 mM KCl) at room temperature to a concentration of 5 μM for each protein. Afterward, 1 mM GTP or GTPγS was added to stimulate the polymerization of FtsZ. Bundle formation of FtsZ polymers was initiated by adding 0.1 mg/ml diethyl-aminoethyl-dextran hydrochloride (DEAE-dextran) to the assembly reaction. The

[2] The abbreviations used are: GTPγS, guanosine 5′-*O*-(thiotriphosphate); MES, morpholineethanesulfonic acid; BSA, bovine serum albumin.

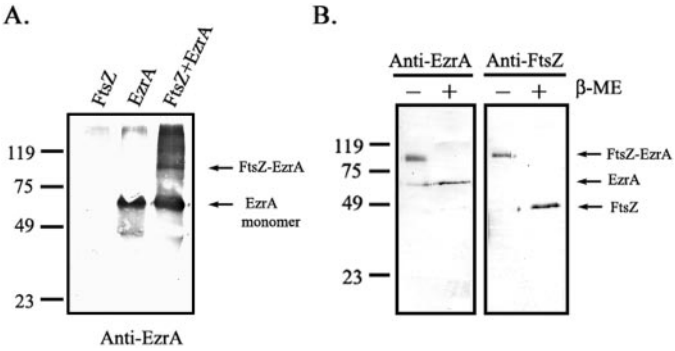

FIGURE 1. **Direct interaction between FtsZ and EzrA.** *A*, cross-linking of FtsZ and EzrA. FtsZ, EzrA, or FtsZ incubated with EzrA was cross-linked with 3,3-dithiobis(sulfosuccinimidylpropionate) (see "Experimental Procedures") and then subjected to Western blot analysis using anti-EzrA. *B*, identification of the cross-linked product of FtsZ and EzrA. The protein band with a gel mobility corresponding to a 100-kDa protein and supposed to be the cross-linked complex of FtsZ and EzrA (as shown in *A*) was excised from the SDS-polyacrylamide gel run in parallel with the Western blot. The recovered protein was subjected to another Western blot analysis using anti-FtsZ and anti-EzrA under both reducing and nonreducing conditions. β-ME, β-mercaptoethanol.

final volume of each assembly reaction was 100 μl. The bundles were spun down at 250,000 × *g* for 10 min at 20 °C and resuspended in 100 μl of polymerization buffer. Both the supernatant and suspended bundle polymers were subjected to 10% SDS-PAGE to determine the content of FtsZ.

RESULTS

The Interaction between EzrA and FtsZ—It has been reported that the thioredoxin-EzrA fusion protein is able to interact with FtsZ; however, the interaction did not significantly alter the GTPase activity of FtsZ (22). We speculated that the thioredoxin moiety of the fusion protein may have impaired the function of EzrA. To solve the potential problem, EzrA with a smaller His_6 tag fused at the C terminus was constructed and used to examine the effect of EzrA on FtsZ function. Before the examination, the capability of interaction between EzrA and FtsZ was first verified using a chemical cross-linking method. As shown in Fig. 1*A*, a protein with a gel mobility expected for the cross-linked FtsZ-EzrA (~100 kDa) was visualized when the mixture of FtsZ and EzrA was treated with the homobifunctional thio-cleavable cross-linker, 3,3-dithiobis(sulfosuccinimidylpropionate), and probed with anti-EzrA. To confirm that this protein was indeed FtsZ-EzrA, the specific protein was recovered from the SDS-polyacrylamide gel run in parallel with an immunoblot and then subjected to Western blot analysis using anti-FtsZ and anti-EzrA under either reducing or nonreducing conditions (Fig. 1*B*). Both free FtsZ and EzrA appeared when the recovered protein was treated with the reducing agent, β-mercaptoethanol; however, the fusion protein remained intact and reacted with both anti-FtsZ and anti-EzrA when β-mercaptoethanol was omitted (Fig. 1*B*). These results indicated that EzrA is able to interact directly with FtsZ.

EzrA Reduces the Binding of the GTP Analogue, BODIPY®FL-GTPγS, to FtsZ—The ability of EzrA to directly interact with FtsZ raised the possibility that EzrA may modify the GTP hydrolysis cycle of FtsZ through mutual interaction. Thus, the change in GTP-binding activity of FtsZ was analyzed. The fluorescent GTP analogue, BODIPY®FL-GTPγS, used to study the functional mechanism of G-protein, was adopted for this analysis due to its convenience and applicability toward real time and continuous measurement (24). Basically, the fluorescence of BODIPY (fluorophore) is significantly quenched in solution by intramolecular interaction between BODIPY and guanine base of the GTP analogue; however, the fluorescence increases appreciably upon binding of the analogue to the G-protein.

As expected, fluorescence quenching of BODIPY was detected for the free GTP analogue (Fig. 2*A*, *black line*). A similar phenomenon was observed when the analogue was incubated with EzrA, indicating that EzrA is unable to bind the analogue (Fig. 2*A*, *purple line*). However, upon binding to FtsZ, fluorescence quenching of the GTP analogue was relieved, and an approximately 95% increase in relative fluorescence was obtained (Fig. 2*A*, *blue line*). Instead, only 57% increase was obtained when FtsZ was preincubated with equal concentration of EzrA (Fig. 2*A*, *yellow line*). Taken together, these results suggested that EzrA is able to lower the affinity of the GTP analogue for FtsZ. To confirm this idea, fluorescence of the GTP analogue added to FtsZ preincubated with various concentrations of EzrA was measured. As shown in Fig. 2*B*, fluorescence of the GTP analogue decreased as the dosage of EzrA was increased. Minimal fluorescence was obtained as the molar ratio of EzrA to FtsZ reached 1:1 or 2:1 (Fig. 2, *B* and *C*), suggesting that EzrA and FtsZ are able to form a 1:1 complex. The ability of EzrA to specifically affect binding of the GTP analogue to FtsZ was further strengthened by the fact that BSA did not have any significant effect on relief of fluorescence quenching caused by FtsZ (Fig. 2*A*, *red line*). Since the effect of EzrA on relief of fluorescence quenching disappeared when FtsZ was incubated with the GTP analogue prior to mixing with EzrA (Fig. 2*A*, *green line*), we proposed that EzrA plays its role not by acting on FtsZ polymers.

Kinetic analysis of the binding affinity of BODIPY®FL-GTPγS for FtsZ was also performed (Fig. 2*D*). In the analysis, various concentrations of the GTP analogue were mixed with FtsZ or FtsZ preincubated with an equal concentration of EzrA, since FtsZ and EzrA may form a 1:1 complex. Fluorescence of the GTP analogue was then measured, and the data were fitted to the one-site-binding hyperbolic function of Grafit to determine the K_D value for FtsZ and BODIPY®FL-GTPγS binding. The value thus obtained was 27.30 ± 5.22 or 78.27 ± 28.98 nM, respectively, for FtsZ or FtsZ preincubated with EzrA, indicating that EzrA is able to reduce the binding of the GTP analogue to FtsZ.

EzrA Reduces the Binding Affinity of GTP for FtsZ—The reduction in binding of the GTP analogue to FtsZ by EzrA suggests that EzrA must be also able to modulate the affinity of GTP for FtsZ. To verify this, a competition assay was performed. FtsZ or FtsZ preincubated with EzrA was mixed, respectively, with BODIPY®FL-GTPγS before adding various concentrations of GTP. Competition between GTP and the GTP analogue for FtsZ was then followed (Fig. 3*A*). A decrease in fluorescence was observed for both FtsZ and FtsZ preincubated with EzrA, indicating that GTP is able to replace the

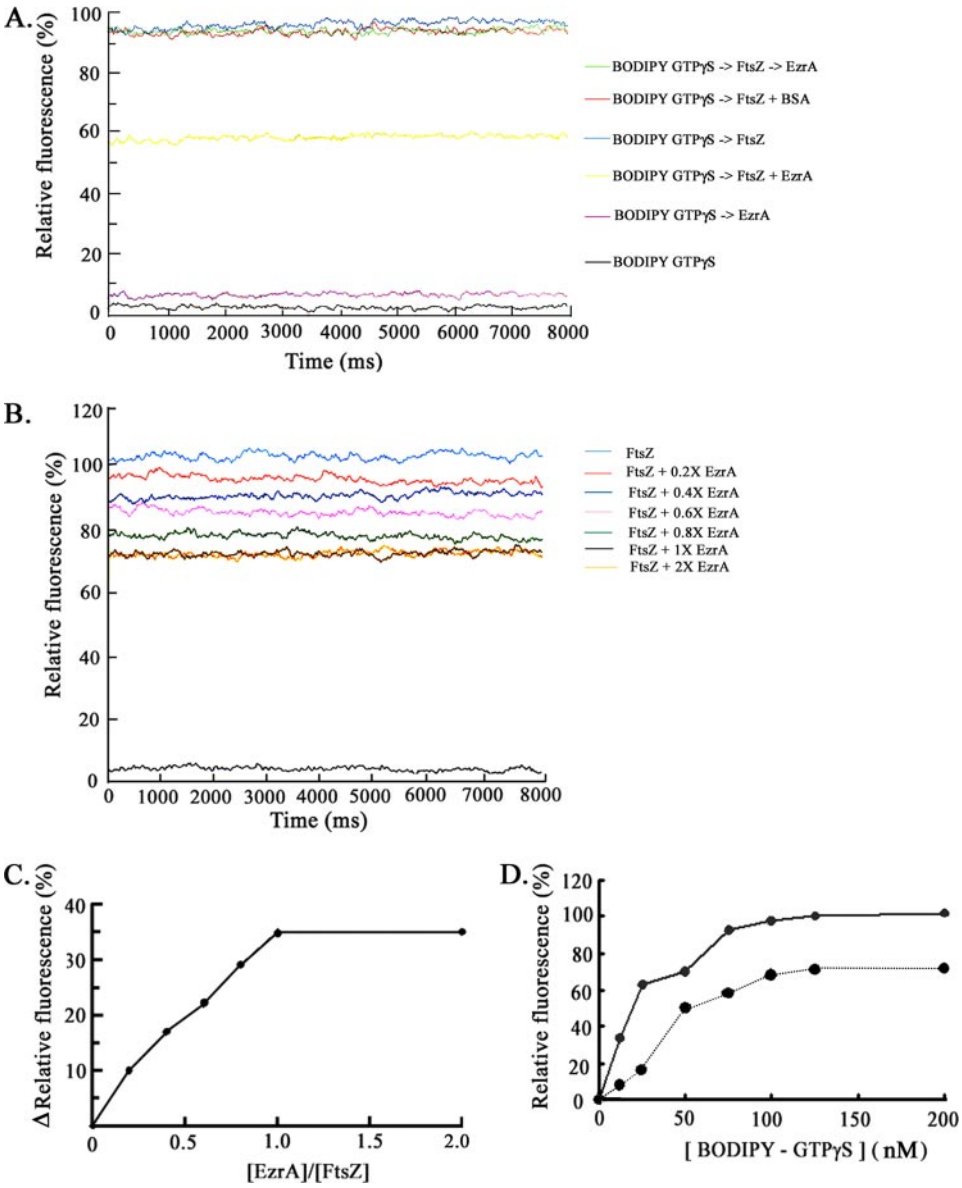

FIGURE 2. **Effect of EzrA on the binding of BODIPY®FL-GTPγS to FtsZ.** *A,* binding of BODIPY®FL-GTPγS to FtsZ. The method used for the analysis was described under "Experimental Procedures." *Black line*, fluorescence of the GTP analogue alone. *Blue*, *purple*, or *red line*, fluorescence of the GTP analogue mixed with FtsZ, EzrA, or FtsZ preincubated with BSA, respectively. *Yellow line*, fluorescence of the GTP analogue mixed with FtsZ preincubated with EzrA for 30 min. *Green line*, fluorescence of the GTP analogue preincubated with FtsZ for 1 min prior to the addition of EzrA. The relative fluorescence (%) = ((fluorescence of the GTP analogue for each treatment − fluorescence of the GTP analogue alone)/fluorescence of the GTP analogue alone) × 100%. The increase of fluorescence intensity to the same fluorescence level of GTP analogue alone is referred to as 100% of relative fluorescence. *B,* effect of EzrA dosage on binding of BODIPY®FL-GTPγS to FtsZ. Fluorescence of the GTP analogue mixed with FtsZ preincubated with various molar ratios of EzrA was measured. The method for calculation of the relative fluorescence was the same as that described in *A*. *C,* relation between the decrease of relative fluorescence and the ratio of [EzrA]/[FtsZ]. The data used for the plotting were calculated from *B*. *D,* kinetic analysis of the binding affinity of BODIPY®FL-GTPγS for FtsZ. *Solid line*, fluorescence of the GTP analogue incubated with 250 nM FtsZ alone. *Dotted line*, the fluorescence of the GTP analogue incubated with a mixture of 250 nM FtsZ and EzrA. Each of the experiments was carried out in triplicate at least three times with three independent preparations of EzrA and FtsZ, and similar results were obtained.

FtsZ-bound GTP analogue irrespective of whether or not FtsZ was preincubated with EzrA. However, the concentration of GTP (IC_{50}) required to remove 50% of the GTP analogue away from FtsZ preincubated with equal molarity of EzrA (3.22 ± 0.47 μM) was 1.6-fold higher than that from FtsZ alone (2.05 ± 0.06 μM) (Fig. 3*B*). This indicated that EzrA is able to reduce the GTP-binding activity of FtsZ.

We also analyzed the effects of different molar ratios of EzrA to FtsZ on IC_{50} of GTP. As shown in Fig. 3*C*, maximal IC_{50} of GTP was obtained as the molar ratio of EzrA and FtsZ reached 1:1, further indicating that a 1:1 complex of EzrA and FtsZ may be formed.

EzrA Enhances the Binding of the Transition State Analogue of GTP but Lowers the Binding of the Hydrolyzed Product to FtsZ—The capability of EzrA to modify the binding of GTP to FtsZ led us to propose that EzrA may also modify the binding of FtsZ to GDP-AlF_4^-, a transition state analogue of GTP (25–28) and also to the hydrolyzed product, GDP. The idea was put to test by adding various concentrations of GDP-AlF_4^- or GDP to FtsZ or FtsZ preincubated with equal molar concentration of EzrA in a fixed concentration of BODIPY®FL-GTPγS. With GDP-AlF_4^- as the competitor, fluorescence decrease of the GTP analogue was more pronounced for FtsZ preincubated with EzrA than for FtsZ alone (Fig. 4*A*). The concentration of GDP-AlF_4^- (IC_{50}) required to remove 50% of the GTP analogue away from FtsZ in the presence or absence of EzrA was 0.46 ± 0.07 or 1.27 ± 0.28 μM, respectively (Fig. 4*B*). The relatively lower IC_{50} of GDP-AlF_4^- for FtsZ preincubated with EzrA than for FtsZ alone implicated that EzrA is able to stabilize the transition state for GTP hydrolysis of FtsZ. Since the lowest IC_{50} was obtained when the molar ratio of EzrA to FtsZ reached 1:1 or higher than 1:1, it was further suggested that EzrA is able to complex with FtsZ in 1:1 stoichiometry (Fig. 4*E*).

To test whether EzrA can modulate the binding affinity of GDP for FtsZ, a competition assay was also performed. FtsZ or FtsZ preincubated with EzrA was mixed, respectively, with BODIPY®FL-GTPγS before adding various concentrations of GDP. A decrease in fluorescence was observed for both FtsZ and FtsZ preincubated with EzrA (Fig. 4*C*). The IC_{50} of GDP for FtsZ preincubated with EzrA (3.87 ± 0.10 μM) was 1.26-fold higher than from FtsZ (3.07 ± 0.22 μM) (Fig. 4*D*), indicating that EzrA is able to lower slightly the affinity of GDP for FtsZ. Similarly, the maximal IC_{50} of GDP was observed

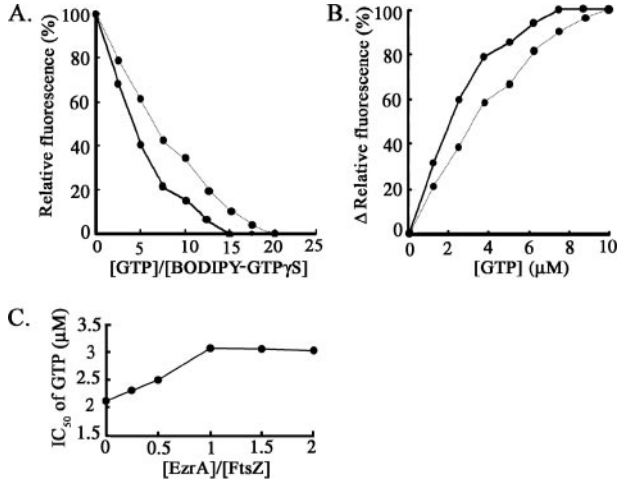

FIGURE 3. **Effect of EzrA on binding affinity of GTP for FtsZ.** *A,* competition between GTP and BODIPY®FL-GTPγS for FtsZ binding. The method used for the analysis was described under "Experimental Procedures." The relative fluorescence (%) = ((fluorescence of the GTP analogue in the presence of GTP − basal fluorescence of the GTP analogue alone)/(fluorescence of the GTP analogue in the absence of GTP − basal fluorescence of the GTP analogue alone)) × 100%. The fluorescence of BODIPY®FL-GTPγS incubated with FtsZ or with FtsZ preincubated with EzrA, both in the absence of GTP, was referred to as 100% relative fluorescence, respectively. *B,* reduction of the binding affinity of GTP for FtsZ by EzrA. The data used for plotting were calculated from *A*. In both *A* and *B*, the *solid line* indicates competition between GTP and GTP analogue for binding to FtsZ, whereas the *dotted line* indicates binding to FtsZ preincubated with EzrA. *C,* relation between the decrease of binding affinity of GTP for FtsZ and the ratio of [EzrA]/[FtsZ]. FtsZ (500 nM) preincubated with 125, 250, 500, or 750 nM or 1 μM EzrA for 30 min was assayed, respectively, for the relative fluorescence as described under "Experimental Procedures." The IC_{50} was calculated from the data of the competition assay as described in *B*. Each of the experiments was carried out in triplicate at least three times with three independent preparations of EzrA and FtsZ, and similar results were obtained. The S.E. values were calculated from the measurements of triplicate samples.

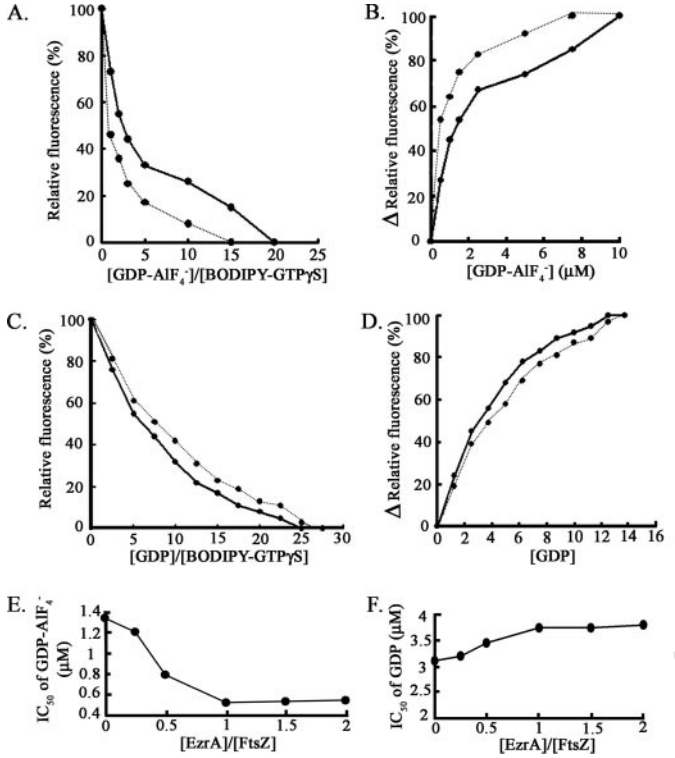

FIGURE 4. **Effect of EzrA on binding affinity of GDP-AlF_4^- or GDP for FtsZ.** *A* and *C,* replacement of BODIPY®FL-GTPγS by GDP-AlF_4^- (*A*) or by GDP (*C*). The method used for the analysis was described under "Experimental Procedures." The relative fluorescence (%) = ((fluorescence of the GTP analogue in the presence of GDP-AlF_4^- or GDP − basal fluorescence of the GTP analogue alone)/(fluorescence of the GTP analogue in the absence of GDP-AlF_4^- or GDP − basal fluorescence of the GTP analogue alone)) × 100%. The fluorescence of BODIPY®FL-GTPγS incubated with FtsZ or FtsZ preincubated with EzrA in the absence of GDP-AlF_4^- or GDP was referred to as 100% relative fluorescence, respectively. *B* and *D,* effect of EzrA on binding affinity of GDP-AlF_4^- or GDP for FtsZ. The data used for plotting were calculated from *A* or *C*, respectively. In *A–D*, the *solid line* indicates competition between GDP-AlF_4^- (or GDP) and GTP analogue for binding to FtsZ, whereas the *dotted line* indicates binding to FtsZ preincubated with EzrA. *E* and *F,* relation between the effect of binding affinity of GDP-AlF_4^- or GDP for FtsZ and the ratio of [EzrA]/[FtsZ]. FtsZ (500 nM) preincubated with 125, 250, 500, or 750 nM or 1 μM EzrA for 30 min was assayed, respectively, for the relative fluorescence as described under "Experimental Procedures." The IC_{50} was calculated from the data of competition assay as described in *B* and *D*. Each of the experiments was carried out in triplicate at least three times with three independent preparations of EzrA and FtsZ, and similar results were obtained. The S.E. values were calculated from the measurements of triplicate samples.

when the molar ratio of EzrA to FtsZ was 1:1 or higher, which is also an indication of the formation of a 1:1 EzrA and FtsZ complex.

EzrA Enhances the GTPase Activity of FtsZ—The increase in binding affinity of FtsZ for GDP-AlF_4^- by EzrA suggests that EzrA is able to increase the GTPase activity of FtsZ by lowering the activation energy for GTP hydrolysis. To corroborate this idea, we analyzed the effect of EzrA on the GTP hydrolysis rate of FtsZ using a spectrophotometric method that measures the phosphate released from GTP hydrolysis. Our data revealed that GTP was hydrolyzed by FtsZ at a rate of 0.66 ± 0.10 GTP/FtsZ min^{-1} in the absence of EzrA (Fig. 5, *solid triangles*). However, the rate was increased about 1.6-fold to 1.05 ± 0.15 GTP/FtsZ min^{-1} as an equal molar concentration of EzrA was present (Fig. 5, *solid* and *open circles*). Moreover, the acceleration of GTP hydrolysis was dependent on the molar ratio of EzrA to FtsZ; the highest acceleration was achieved as the molar ratio reached 1:1 or higher (Fig. 5*B*).

The apparent k_{cat} and K_m for GTP hydrolysis by FtsZ or FtsZ preincubated with equal molar concentration of EzrA were also measured. As shown in Table 1, the k_{cat} (0.75 min^{-1}) for GTP hydrolysis by *B. subtilis* FtsZ is comparable with that reported previously (22); however, it is lower than those reported for *E. coli* FtsZ (12, 29–32). Moreover, the K_m (39.45 μM) is also comparable with that reported for *E. coli* FtsZ (29). In the presence of EzrA, the k_{cat} for GTP hydrolysis by *B. subtilis* FtsZ increased about 1.67-fold (1.25 min^{-1}/0.75 min^{-1}), further confirming that EzrA is able to enhance the GTP hydrolysis rate of FtsZ. Moreover, consistent with the data from the competition assay (Fig. 3*B*), a 2.35-fold (92.92 μM/39.45 μM) increase in K_m for GTPase activity was observed as FtsZ was preincubated with EzrA (Table 1), again supporting that EzrA is able to lower the GTP-binding activity of FtsZ.

EzrA Is Able to Inhibit FtsZ Polymerization—To verify whether the reduction in GTP-binding activity of FtsZ by EzrA is unfavorable for FtsZ polymerization, GTPγS, a nonhydrolyzable GTP analogue, was adopted to simulate the condition of GTP binding. The effect of EzrA on FtsZ polymerization was then assayed using sedimentation analysis (23, 33, 34). This analysis relies on the formation of large bundles of FtsZ polymers in the presence of bundling agents, such as DEAE-dextran, and the efficient pelleting of high molecular weight bun-

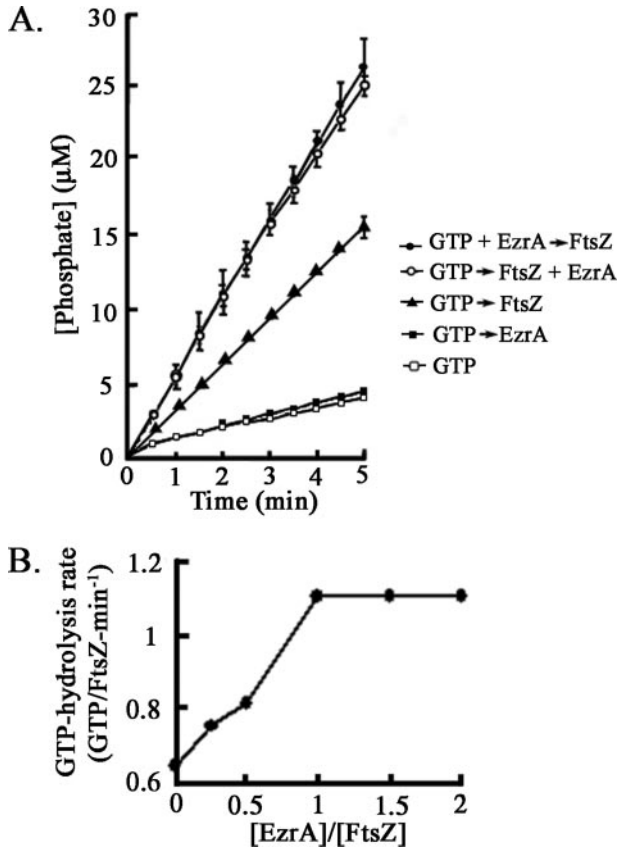

FIGURE 5. **Effect of EzrA on the GTPase activity of FtsZ.** *A*, the method used for the analysis was described under "Experimental Procedures." *Open square*, basal level of free phosphate in sample solution containing 1 mM GTP. *Closed square*, basal level of free phosphate in sample solution containing 5 μM EzrA and 1 mM GTP. *Closed triangle* or *open circle*, level of free phosphate in sample solution containing 5 μM FtsZ or 5 μM FtsZ preincubated with equal concentration of EzrA for 30 min before adding 1 mM GTP, respectively. *Closed circle*, level of free phosphate in sample solution containing 5 μM FtsZ added after the mixing of EzrA and GTP. *B*, relation between the increase of GTPase activity of FtsZ and the ratio of [EzrA]/[FtsZ]. FtsZ (5 μM) preincubated with 1.25, 2.5, 5, 7.5, or 10 μM EzrA for 30 min was assayed, respectively, for the GTPase activity as described under "Experimental Procedures." Each of the experiments was carried out in triplicate at least three times with three independent preparations of EzrA and FtsZ, and similar results were obtained. The S.E. values were calculated from the measurements of triplicate samples.

TABLE 1

The apparent k_{cat} and K_m of FtsZ or FtsZ preincubated with EzrA

The intracellular concentration of GTP is as high as 1–3 mM in exponentially growing cells (41), which is much higher than the apparent K_m determined in the present experiment.

	k_{cat}	K_m
	min^{-1}	μM
FtsZ	0.75 ± 0.02	39.45 ± 3.69
FtsZ + EzrA	1.25 ± 0.05	92.92 ± 13.14

dles during centrifugation. As shown in Fig. 6*B*, EzrA efficiently inhibited FtsZ sedimentation when equal concentration of EzrA and FtsZ was incubated prior to the addition of GTPγS and DEAE-dextran. Similar results were obtained when GTPγS was replaced with GTP (Fig. 6*A*). However, most of the FtsZ precipitated when EzrA was replaced with BSA. These results demonstrated that the decrease in GTP-binding activity of FtsZ caused by EzrA is enough to inhibit the formation of FtsZ polymers *in vitro*. Moreover, study of the effect of EzrA on FtsZ polymerization by 90° angle light scattering was performed in

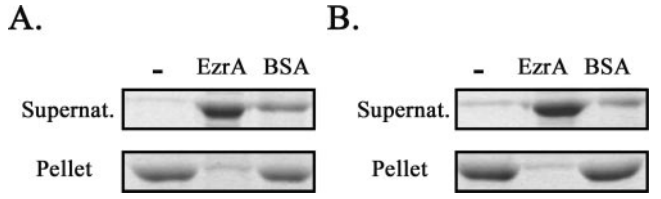

FIGURE 6. **Inhibition of FtsZ polymerization by EzrA *in vitro*.** *A* and *B*, effects of EzrA on sedimentation of FtsZ polymers in the presence of GTP (*A*) and GTPγS (*B*), respectively.

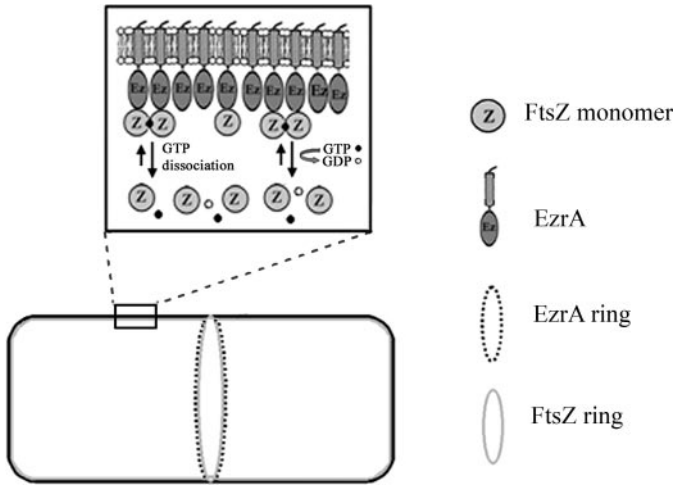

FIGURE 7. **Model for the negative regulation of Z-ring formation by EzrA in *B. subtilis*.** The plasma membrane-bound EzrA located at any cellular locus other than the midcell acts to inhibit FtsZ assembly and to prevent aberrant Z-ring formation. FtsZ assumes monomeric, dimeric, or multimeric conformation depending on the availability of cellular GTP. The inhibition or prevention of Z-ring formation is achieved through decreasing the binding affinity of GTP for FtsZ (with dimeric FtsZ shown) and increasing the rate of GTP hydrolysis of FtsZ.

the presence of GTP, since GTPγS is inappropriate for the assay (18). Similar to that reported previously (22), inhibition of FtsZ polymerization was also observed (data not shown).

DISCUSSION

We have investigated the detailed mechanism responsible for the negative regulation of FtsZ polymerization by EzrA. Our results reveal that EzrA is able to reduce the affinity of GTP for FtsZ and enhance the GTPase activity of FtsZ, both of which prevent the assembly of FtsZ (Figs. 3, 5, and 6). These data, coupled with the ability of EzrA to directly interact with FtsZ (Fig. 1) (22), demonstrate that EzrA participates in the FtsZ GTP hydrolysis cycle to regulate FtsZ polymer dynamic. They also support a model for the negative regulation of Z-ring formation by EzrA (Fig. 7). As a negative regulator, the plasma membrane-bound EzrA prevents inappropriate Z-ring formation at any cellular locus other than the midcell by direct interaction with FtsZ (Fig. 7). The interaction reduces the GTP-binding ability of FtsZ, which inhibits Z-ring formation. It also enhances the GTPase activity of FtsZ by stabilizing the transition state for GTP hydrolysis and reduces the GDP-binding activity of FtsZ (Fig. 4), both of which promote the disassembly of polymerized FtsZ.

The mechanism responsible for the enhancement of the GTPase activity of FtsZ by EzrA is similar to that reported for

RSG, a GTPase activator for heterotrimeric G protein (35–37). Both EzrA and RSG act to stabilize the transition state for GTP hydrolysis of the GTP-binding protein. The 1.5–1.7-fold increase in the GTPase activity of FtsZ by EzrA as observed in the experiment is subtle but significant and is different from that reported for the thioredoxin-EzrA fusion protein, which shows no appreciable effect on the GTPase activity of FtsZ (22). Apparently, the fused thioredoxin moiety has impaired the weak modulation activity of EzrA. Moreover, these results implicate that modulation of the polymer dynamics of FtsZ by EzrA is also subtle. In other words, prevention of Z-ring formation *in vivo* does not require a drastic change in the GTPase activity of FtsZ; a subtle change in GTPase activity may already have the ability to destabilize the Z-ring. A similar view for fine tuning of FtsZ polymer stability has been also reported by another research group (11).

On the basis of the effects of various molar ratios of EzrA to FtsZ on the IC_{50} of GTP, GDP-AlF_4^-, and GDP and on the GTP hydrolysis rate of FtsZ (Figs. 3–5), it was clear that EzrA and FtsZ may form a 1:1 complex. This raises a possibility that the two rings of FtsZ and EzrA as observed at the initiation of cell division (19) may associate in 1:1 stoichiometry at midcell.

Several inhibitors for FtsZ assembly have been identified in *E. coli* and *B. subtilis*. *E. coli* SulA is the one that is well characterized. This protein negatively regulates Z-ring formation by inhibiting the GTPase activity of FtsZ (23, 38). The inhibition is attributed to SulA binding to the GTPase-activating T7 loop (opposite to the nucleotide-binding site) of FtsZ (39), which in turn blocks the dimerization of FtsZ and therefore its GTPase activity (14, 40). However, the mechanism underlying the inhibition of Z-ring formation by EzrA is totally different from that found for SulA in terms of modulation of the GTPase activity of FtsZ (Fig. 5). The limited increase in the GTPase activity of FtsZ by EzrA suggests that EzrA does not contribute any catalytic amino acid residue to the active site of FtsZ to enhance the GTP-hydrolyzing activity of FtsZ. The seemingly contradictory effects of EzrA on FtsZ (*i.e.* the decrease in GTP-binding but increase in GTPase activity of FtsZ) raise the possibility that EzrA may act to fulfill its role simply by changing the conformation of FtsZ. This can be supported by the fact that EzrA is able to stabilize the transition state for GTP hydrolysis and to slightly reduce the GDP-binding activity of FtsZ (Fig. 4).

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