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GTP/GDP binding stabilizes bacterial cell division protein FtsZ against degradation by FtsH protease *in vitro*

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Abstract

Factors contributing to the stability of bacterial cell division protein FtsZ remain unknown. In order to identify FtsZ-stabilizing factor(s), we exploited FtsH protease-based *in vitro* FtsZ degradation assay system. Whole cell lysate from an *ftsH*-null strain of *Escherichia coli* inhibited degradation of FtsZ by FtsH *in vitro*. However, activated charcoal-treated lysate did not inhibit degradation. The loss of ability of the activated charcoal-treated lysate to inhibit degradation of FtsZ was restored when it was replenished with GTP, but not when replenished with other NTPs or dNTPs. The lysate did not protect either FtsZ deletion mutants, which do not bind GTP, or FtsH substrates, σ^{32} and cI-108 proteins, against FtsH. GDP and GTPγS also stabilized FtsZ against FtsH. Neither GTP nor GDP inhibited proteolytic activity of FtsH *per se*. These observations demonstrate that binding of GTP/GDP ligands is responsible for the proteolytic stability of FtsZ against FtsH.

Keywords: Bacterial cell division protein; FtsZ protein; FtsZ polymers; FtsZ polymerisation; GTP; GDP; FtsH protease

Septal biogenesis in bacteria is initiated by FtsZ protein [1], which possesses GTP binding, GTPase [2,3], and GTP-dependent polymerisation activities [4,5]. It gets localized to the mid-cell site early in cell cycle and remains at the leading edge of the invaginating septum throughout the division process [1,6]. While the levels of FtsZ have been found to remain constant throughout the cell cycle in *Escherichia coli* and *Bacillus subtilis* [7,8], it was found reduced by 50% in late stationary phase in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* [9]. Similarly, in *Caulobacter crescentus*, the levels of FtsZ are proteolytically regulated where it is specifically degraded in the swarmer cells during the late stages of cell cycle [10]. However, the identity of protease(s), which degrade FtsZ in these bacterial systems, remains unknown. Although FtsZ has been found to be a stable protein *in vivo*, the intracellular factor(s) responsible for the stability of the protein remain unclear. In order to identify intracellular factor(s) that stabilize FtsZ, we initiated studies using *E. coli* FtsZ as the model system. Earlier we had demonstrated that recombinant FtsH protease of *E. coli* degrades recombinant FtsZ protein of *E. coli* in an ATP- and Zn^{2+}-dependent manner *in vitro* [11], as characteristic of degradation of substrates by FtsH [12–14]. Secondly, heat-denatured FtsZ was not degraded by FtsH [11], indicating structural specificity for the substrate degradation. These characteristics validated the use of FtsH-dependent assay system for the degradation of FtsZ *in vitro* as a model system, with *E. coli* (*ftsH* null strain) whole cell lysate as the source of the factor(s) that would stabilize FtsZ. We demonstrate that GTP/GDP binding stabilizes FtsZ against degradation by FtsH.

Materials and methods

Bacterial strains, media, and plasmid constructs. Escherichia coli AR5090 strain (Δ*ftsH3::kan*, *zad220::*Tn10 *sfhC21 degP5087*/*F'lacI*^{q}) and *E. coli* TYE024 (MC4100, *ompT::kan*/*F' lacI*^{q}) [both kind gifts from T. Ogura; 15,

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16] were grown in Luria–Bertani broth at 30 and 37 °C, respectively. *E. coli* C43 strain [kind gift from J.E. Walker; 17] was grown in Luria–Bertani broth at 37 °C. Ampicillin, kanamycin, tetracycline, and chloramphenicol were used at 100, 25, 10, and 10 μg/ml concentrations, respectively.

GTP binding. Two microgram of purified protein was incubated in 50 mM MES–NaOH (pH 6.5) buffer, containing 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.1 μM [α^{32}P]GTP (3000 Ci/mmol) for 10 min at 0 °C, and UV crosslinked (Hoefer) for 5 min at 0 °C [2,3]. Crosslinking was verified on 12% SDS–PAGE using Phosphorimager BioImage analyzer (FLA 2000, Fuji, Japan).

Depletion of nucleoside triphosphates from whole cell lysate. The exchangeable and free nucleotides were removed from whole cell lysate by repeated (thrice) mixing of 0.1 ml of activated charcoal suspension (15 mg/ml) per ml of extract for 5 min at 4 °C and centrifuging at 25,000*g* for 15 min [23]. Almost 99% of the extraneously added [α^{32}P]GTP was removed from the lysate upon treatment with activated charcoal.

FtsZ polymerisation assay. FtsZ polymerisation assay was carried out in 50 mM MES–NaOH (pH 6.5) buffer, containing 10 μM protein, 1 mM GTP, 50 mM KCl, and 5 mM $MgCl_2$, and the polymers were viewed as described [24].

90° light scattering assay. The 90° light scattering assay for verifying polymerisation of full-length FtsZ and FtsZΔN2 was carried out in 40 μl as described [23], using 5 μM protein, 25 μg whole cell lysate, and 1 mM GTP, but in 1× CGH buffer (1 mM citrate, 1 mM glycine, 1 mM Hepes–KOH, pH 6.5), containing 5 mM $MgCl_2$ and 50 mM KCl at 30 °C. For reasons not known to us, in MES–KOH buffer system [25], the denatured-refolded protein did not show appreciable level of polymerisation that could be measured using light scattering assay.

FtsH protease assay. The protease assay for FtsH was carried out in 40 μl as described [12], but with 20 mM ATP and 100 μg/ml bovine serum albumin, at 42 °C. AEBSF (1 mM) and Protease Inhibitor Cocktail (EDTA-free) (1 μl; Sigma) were included in the reaction mixture. GTP/GDP/GTPγS and *ortho*-phenanthroline (a Zn^{2+} chelator) were used at 1 and 10 mM concentrations, respectively. The reactions involving FtsZ protein/deletion mutants, cI-108 protein, and FtsZ polymers were carried out for 3 h, 30 min, and 60 min, respectively. For degradation of FtsZ substrates in the presence of GTP, the proteins were pre-incubated with GTP at 37 °C for 15 min and then incubated with FtsH for 60 min at 42 °C. The ATP regeneration system contained 10 units of creatine phosphokinase and 100 mM phosphocreatine [26] in the protease assay buffer.

Results and discussion

Whole cell lysate stabilizes FtsZ against degradation by FtsH protease in vitro

Incubation of denatured-refolded FtsZ with FtsH in the presence of 25 μg equivalent of whole cell lysate, which was prepared from *E. coli* AR5090 *ftsH*-null strain, inhibited degradation of FtsZ by FtsH protease *in vitro* (Fig. 1A). In the absence of the lysate, FtsZ was degraded by FtsH in an ATP-dependent manner. This finding indicated that the lysate is the source of FtsZ-stabilizing factor(s). However, in order to verify whether the lack of degradation was due to a possible depletion of ATP pool in the lysate, an ATP regeneration system was included in the protease assay. Degradation of FtsZ by FtsH when 0.5 mM ATP, which was insufficient for protease activity, was compensated with ATP from the regeneration system (Fig. 1B, lanes 5 and 6, respectively) showed the efficacy of the ATP regeneration system. However, the ATP regeneration system did not relieve the inhibition of proteolysis of FtsZ

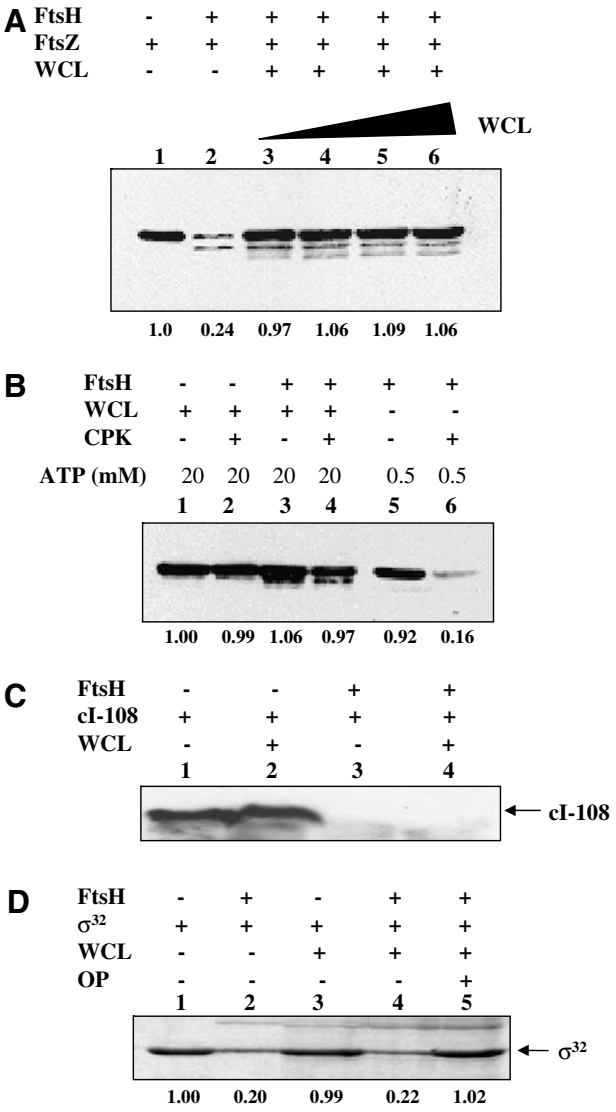

Fig. 1. Degradation of substrates by FtsH in the presence of WCL from AR5090 cells. Degradation of 6×His-FtsZ (1 μg) (A and B), 6×His-cI-108 (30 μM) (C), and σ^{32}-6×His (1 μg) (D). The amounts of 6×His-FtsZ, 6×His-cI-108, and σ^{32}-6×His, after FtsH assay, were detected with anti-FtsZ antibody, anti-polyhistidine antibody, and Coomassie blue staining, respectively, and are indicated below respective lane. (A) Lanes 3–6: increasing concentrations of WCL (25, 50, 100, and 200 μg, respectively). (B) In the presence or absence of ATP regeneration system and WCL. OP, *ortho*-phenanthroline; WCL, whole cell lysate.

by FtsH (Fig. 1B, lane 4). These observations confirmed that some cellular factor(s) present in the lysate might be conferring stability on FtsZ against FtsH.

FtsZ stabilization by whole cell lysate is not through FtsH inhibition

In order to verify the possibility whether the lack of degradation of FtsZ by FtsH in the presence of the lysate was due to the protease activity of FtsH itself being inhibited by the lysate, ability of the lysate to inhibit degradation of FtsH substrates σ^{32} and cI-108 proteins by FtsH was examined. The rationale for using σ^{32} protein was that its

degradation by purified FtsH preparations is not inhibited by cellular extracts [28], although FtsH is only one of the cellular proteases that degrade σ^{32} [29]. The reactions were carried out in the presence of protease inhibitor cocktail (EDTA-free), which inhibits other classes of proteases that are responsible for the degradation of σ^{32}. However, cI-108 is specifically degraded only by FtsH and not recognized by ClpAP or ClpXP proteases [22]. The lysate did not inhibit degradation of cI-108 and σ^{32} proteins by FtsH (Fig. 1C and D, respectively). Further, degradation of σ^{32} protein in the presence of the lysate by FtsH was completely inhibited by *ortho*-phenanthroline, demonstrating that the degradation was specifically mediated by the extraneously added recombinant FtsH protease and not by any other protease from the lysate (Fig. 1D, lane 5). Addition of lysate alone, without purified FtsH, served as the control for the degradation of σ^{32} by the lysate (Fig. 1D, lane 3). These findings showed that the lysate did not inhibit FtsH activity *per se* but rather it had specifically inhibited degradation of FtsZ by FtsH.

FtsH does not degrade polymeric FtsZ in vitro

Stability of FtsZ in the stalked cells of *C. crescentus*, but not in the swarmer cells wherein FtsZ undergoes degradation by an unknown protease, has been hypothesized to be probably due to the existence of an important fraction of the protein in the polymeric form [10]. Similarly, it is possible that the polymeric status of FtsZ, which can be induced by the high intracellular concentration of GTP [30], might have stabilized FtsZ against FtsH. In apparent concurrence with this premise, GTP-induced polymers of FtsZ and Ca^{2+}-induced and stabilized polymeric bundles of FtsZ polymers (Fig. 2A and B, respectively) were found to be resistant to proteolytic activity of FtsH *in vitro* (Fig. 2C, lanes 2 and 4, respectively). The unpolymerised FtsZ was degraded by FtsH (Fig. 2C, lane 6). Moreover, FtsZ deletion mutants FtsZΔN1 (30 kDa, lacks N-terminal 108 amino acids) and FtsZΔC1 (30 kDa, lacks C-terminal residues from 253 to 372), which do not bind GTP (Fig. 2D, lanes 2 and 4, respectively), were also degraded by FtsH even in the presence of GTP *in vitro* (Fig. 2E, lane 3 of upper and middle panels, respectively). The FtsZΔN2 mutant (lacks only the N-terminal 38 residues and contains the core GTP binding domain), which binds GTP (Fig. 2D, lane 3), was not degraded by FtsH in the presence of GTP (Fig. 2E, lane 3 of lower panel). These observations suggested that GTP-induced polymeric status of FtsZ might be the reason for the stability of the protein against proteolytic degradation.

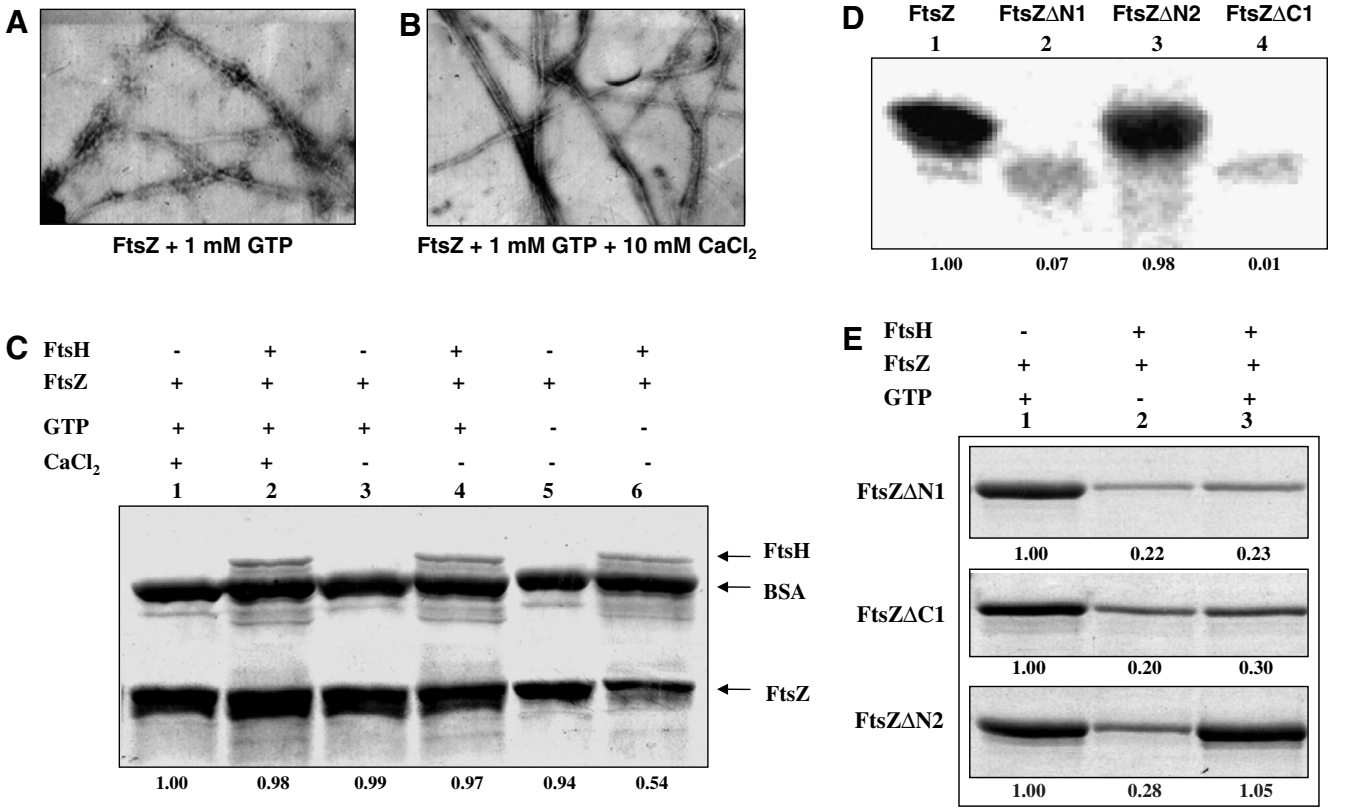

Fig. 2. Lack of degradation of FtsZ polymers by FtsH. (A) GTP-induced polymers of FtsZ at 40,000× magnification. (B) GTP-induced and Ca^{2+}-bundled FtsZ polymers at 27,000× magnification. (C) Coomassie blue stained profile for lack of degradation of: GTP-induced polymerised $CaCl_2$ stabilized FtsZ (lane 2) and GTP-induced polymerised FtsZ (lane 4). Degradation of unpolymerised FtsZ (lane 6). (D) Extent of GTP binding by full-length FtsZ (lane 1); FtsZΔN1 (lane 2); FtsZΔN2 (lane 3); FtsZΔC1 (lane 4), indicated below respective lane. (E) Degradation of deletion mutants of FtsZ in the presence of GTP. Levels of susbstrate (relative to the control sample), remaining after FtsH protease assay, are indicated below respective lane (C and E).

GTP in the lysate stabilizes FtsZ against FtsH

The necessary fallout from these experiments is that GTP in the lysate might have stabilized FtsZ against FtsH through the induction of polymeric status, and in which case, the lysate that is depleted of GTP should not stabilize FtsZ against FtsH. In concurrence with this premise, activated charcoal-treated lysate did not inhibit degradation of FtsZ by FtsH (Fig. 3, lane 4). Moreover, replenishment of activated charcoal-treated lysate with 1 mM GTP, but not with other nucleotides, restored the ability of the lysate to stabilize FtsZ against FtsH (Fig. 3, lanes 6 and 5, respectively). Addition of *ortho*-phenanthroline to the reactions containing activated charcoal-treated lysate inhibited degradation of FtsZ, showing that the degradation was mediated specifically by the Zn^{2+}-metalloprotease FtsH (Fig. 3, lane 7). These experiments demonstrated that the GTP, which was present in the lysate, was responsible for the inhibition of degradation of FtsZ by FtsH. This finding and the earlier observation wherein GTP-induced polymeric form of full-length FtsZ and FtsZ mutants that bind GTP alone were stable against FtsH, taken together, suggest the possibility that GTP present in the lysate might have conferred stability on the protein probably through the induction of polymerisation of FtsZ.

Verification of polymerisation of FtsZ and FtsZΔN2 in the presence of lysate

If intracellular GTP confers stability on FtsZ through induction of polymeric status, then the whole cell lysate that contains intracellular pool of GTP must be able to

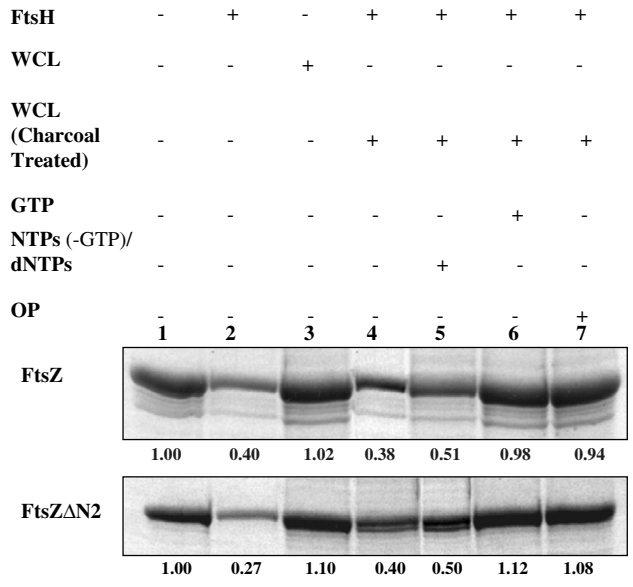

Fig. 3. Effect of GTP depletion/replenishment in WCL on FtsZ degradation by FtsH. The levels of respective FtsZ protein sample (relative to the respective control sample), remaining after FtsH protease assay in each sample, are indicated below respective lane. OP, *ortho*-phenanthroline; WCL, whole cell lysate.

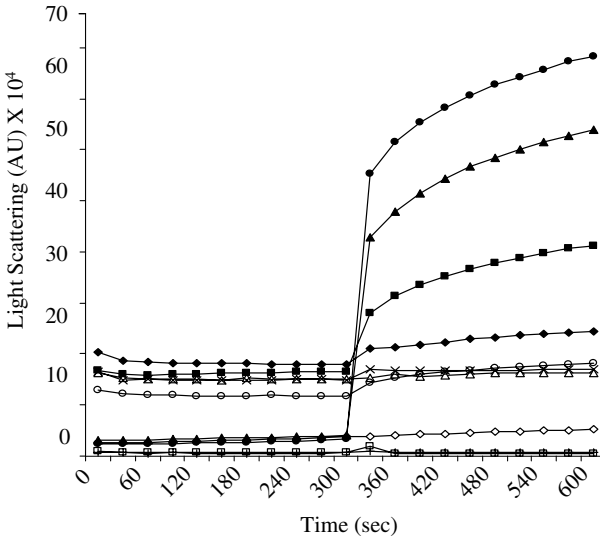

Fig. 4. 90° light scattering assay in the presence of GTP and WCL. (–◇–) FtsZ – GTP; (–▲–) FtsZ + GTP; (–△–) activated charcoal-treated lysate + FtsZ; (–♦–) lysate + FtsZ; (–■–) activated charcoal-treated lysate + FtsZ + GTP; (–□–) FtsZΔN1 + GTP; (–+–) FtsZΔC1 + GTP. (–●–) FtsZΔN2 + GTP; (–x–) activated charcoal-treated lysate + FtsZΔN2; (–○–) lysate + FtsZΔN2. WCL, whole cell lysate; AU, arbitrary units.

stimulate polymerisation of FtsZ *in vitro*. Surprisingly, 90° light scattering assays to monitor polymerisation of full-length FtsZ and FtsZΔN2 (GTP binding) mutant showed that the proteins did not undergo appreciable extent of polymerisation in the presence of the lysate, when compared to the polymerisation in the presence of externally added GTP (Fig. 4). Further, as expected, the activated charcoal-treated lysate did not support any polymerisation at all (Fig. 4). But the activated charcoal-treated lysate, when replenished with GTP but not with other nucleotides, did support polymerisation to a significant extent (Fig. 4). These experiments demonstrated that the lysate could not induce significant extent of polymerisation of full-length FtsZ and FtsZΔN2 mutant. Nevertheless, although the extent of polymerisation induced by the lysate was negligible, the finding that the lysate did confer protection on FtsZ and FtsZΔN2 mutant proteins against FtsH protease indicated that polymerisation of FtsZ and FtsZΔN2 proteins might not be the reason for their stability against FtsH. This finding is in contrast to the case of phage λ cII protein, wherein oligomerisation has been suggested as a possible cause for the stability of the protein against FtsH [31]. It raised the possibility that binding of the ligands (GTP or GDP) *per se*, rather than induction of polymeric status itself, might be sufficient enough to confer protection on the proteins against degradation by FtsH.

Ligand binding prevents FtsZ degradation by FtsH

The possibility that ligand binding, but not the polymeric status, might be sufficient to stabilize FtsZ against

FtsH implies that FtsZ would remain undegraded even if the protein had lost its polymeric status during FtsH assay conditions. When the FtsZ polymers, which we used for incubation with FtsH, were examined under FtsH assay conditions at 42 °C for 1 h, we found that FtsZ was completely depolymerised within 15 min of incubation itself (Fig. 5A and B). Thus, FtsZ was stabilized against FtsH in spite of the polymers of FtsZ being not stable under FtsH assay conditions (see Fig. 2). This finding strengthened the possibility that ligand binding might be sufficient to stabilize FtsZ against FtsH. In order to verify this possibility, we carried out *in vitro* protease assays in the presence of GTP as well as GTPγS (non-hydrolysable analogue of GTP) or GDP, both of which do not induce polymerisation of FtsZ [32]. However, it is known that GDP, but not GTPγS, can induce dimerisation and oligomerisation of FtsZ in the presence of Mg^{2+} [33]. Nevertheless, FtsH could not degrade FtsZ, which was preincubated with GTP, GTPγS, or GDP (Fig. 5C). Thus, binding of GTP, GTPγS, or GDP was sufficient to stabilize FtsZ against FtsH.

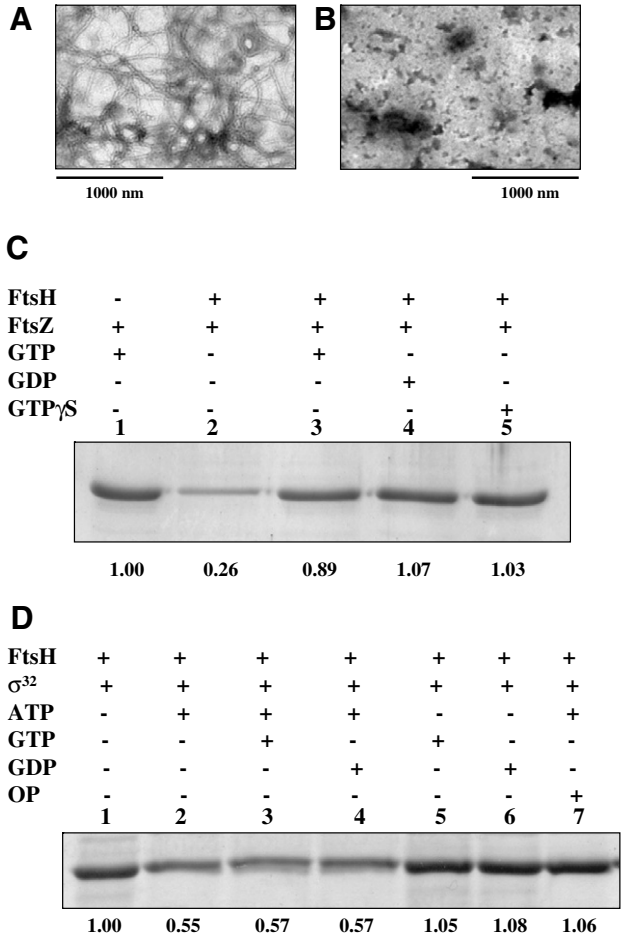

Fig. 5. Effect of GTP/GDP binding on the degradation of FtsZ by FtsH. (A) Electron micrograph of GTP-induced polymers of FtsZ before addition to FtsH protease assay. (B) Electron micrograph of polymerised FtsZ after 15 min of incubation under FtsH assay condition. (C) Coomassie blue stained profile of FtsZ exposed to FtsH in the presence of GTP/GDP/GTPγS. The levels of FtsZ (relative to the control sample), remaining after exposure to FtsH protease in each sample, are indicated below respective lane. (D) FtsH protease assay on σ^{32}-6×His protein in the presence of GTP/GDP. The amount of σ^{32}-6×His protein (relative to the control sample in lane 1) after the assay in each sample is indicated below respective lane. OP, *ortho*-phenanthroline.

GTP/GDP does not inhibit protease activity of FtsH

Although GTP/GDP ligand binding by FtsZ was sufficient to inhibit degradation of FtsZ by FtsH, it was possible that GTP/GDP itself might have inhibited proteolytic activity of FtsH. In order to verify this possibility, proteolytic activity of FtsH was tested on σ^{32}-6×His protein in the presence of GTP/GDP. It was found that even in the presence of GTP/GDP, FtsH degraded σ^{32}-6×His protein (Fig. 5D, lanes 3 and 4) in an ATP- and Zn^{2+}-dependent manner, which is characteristic of FtsH proteolytic activity [12]. Thus taken together, the data presented in this work demonstrate that ligand (GTP/GDP) binding by FtsZ is sufficient enough, and that polymerisation of FtsZ is not essential, for the stability of FtsZ against FtsH. This finding does not necessarily rule out the possibility of FtsZ polymers also being stable against FtsH. However, this point could not be verified as the FtsH assay conditions were fastidious and could not be carried out under FtsZ polymerisation conditions. Moreover, we have not investigated as to how the concentration of endogenous FtsZ in AR5090 cells might affect the polymerisation of purified 6×His-FtsZ since the amount of the lysate added was so low that the contribution of endogenous FtsZ in terms of FtsZ concentration would be negligible compared to the concentration of purified recombinant 6×His-FtsZ. Finally, although high intracellular concentration of GTP [30] would favour stabilization of FtsZ against proteolysis through ligand binding, it does not necessarily mean that FtsZ would always remain bound by GTP. It is quite possible that GTP binding and exchange (GTP for GDP) by FtsZ may be under regulation by hitherto unknown factors. Such regulation may be influencing turnover of the protein, in a concerted manner with the requirement of FtsZ protein for septal biogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.055.

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