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Substitution of a conserved aspartate allows cation-induced polymerization of FtsZ

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Abstract The prokaryotic tubulin homologue FtsZ polymerizes in vitro in a nucleotide dependent fashion. Here we report that replacement of the strictly conserved Asp212 residue of *Escherichia coli* FtsZ by a Cys or Asn, but not by a Glu residue results in FtsZ that polymerizes with divalent cations in the absence of added GTP. FtsZ D212C and D212N mutants co-purify with GTP as bound nucleotide, providing an explanation for the unusual phenotype. We conclude that D212 plays a critical role in the coordination of a metal ion and the nucleotide at the interface of two FtsZ monomers.

Key words: FtsZ; Polymerization; Cell division; Tubulin; Cation

1. Introduction

The key prokaryotic cell division protein FtsZ forms a structural element known as the Z-ring at the site of cell division (for a recent review see [1]). FtsZ has been identified in all but four prokaryotic species studied to date [2] and is essential for division of chloroplasts and mitochondria in some eukaryotes [3,4]. In *Escherichia coli*, the Z-ring is critical for the localization of all other known protein components of the cell division machinery to the division site [1]. The Z-ring is likely to consist of polymers similar to the FtsZ polymers that can be formed in vitro in a GTP dependent manner [5–7].

FtsZ is the prokaryotic homologue of tubulin [8,9]. Both proteins share the unique GTP-binding motif known as G-box [10]. FtsZ displays a concentration dependent GTPase and polymerization activity suggesting that it acts through self-activation [11–13]. A comparison of tubulin and FtsZ structures arranged in their polymerized forms indicated an important role for the T7-loop of FtsZ in modulation of the GTPase activity. This loop may interact with the γ-phosphate of the GTP bound to the nucleotide-binding site on another monomer [14,15]. This hypothesis is augmented by various mutations in the T7-loop that abolish or inhibit FtsZ GTPase activity and polymerization [16–18] (D.-J.S., J.G.d.W., T.d.B. and A.J.M.D., in preparation).

The aspartate residue at position 212 of the T7-loop is conserved among all FtsZ amino acid sequences known to date. This residue is homologous to a glutamate in α-tubulin that modulates the hydrolysis of the GTP bound to β-tubulin, whereas a lysine at the similar position in β-tubulin prevents hydrolysis of the GTP bound to α-tubulin [9]. The D212G mutation in *E. coli* FtsZ abolishes GTPase but not GTP-binding activity and renders the protein resistant to the SulA, an inhibitor of FtsZ polymerization [16,18,19]. Here we report that FtsZ D212C and D212N mutants polymerize in the presence of divalent cations, without added GTP or GDP. The tightly bound nucleotide that co-purifies with these mutants is GTP, in contrast to wild-type (wt) FtsZ or the D212E mutant that contain bound GDP. Our results point at a critical role for D212 in nucleotide hydrolysis and coordination of the cation at the nucleotide-binding site.

2. Materials and methods

2.1. General procedures

Construction and purification of the D212 mutants are described elsewhere (D.-J.S. et al., in preparation). Protein concentrations were determined using a Bradford assay [20] with a correction factor of 0.82 for the FtsZ/BSA ratio [11].

2.2. GTP hydrolysis assay

GTPase activity was monitored by the release of inorganic phosphate [21]. FtsZ and mutant proteins (0.1 mg/ml) were incubated in polymerization buffer (50 mM Mes/NaOH, 50 mM KCl, pH 6.5) with 5 mM $MgCl_2$ at 30°C. The reaction was started by the addition of GTP to 1 mM, and hydrolysis was monitored over a period of 30 min.

2.3. FtsZ sedimentation assay

FtsZ and D212 mutants (0.2 mg/ml) were incubated for 5 min at 30°C in 50 μl polymerization buffer with divalent cations as indicated. Aliquots (45 μl) were centrifuged using an A-100 18° rotor in a Beckman airfuge at 28 psi for 10 min at room temperature. Pellet fractions were resuspended in 45 μl polymerization buffer, and analyzed by SDS–PAGE and Coomassie brilliant blue staining.

2.4. Electron microscopy

FtsZ and D212 mutants at 0.4 mg/ml were incubated at 30°C in polymerization buffer with divalent cations (5 mM). After 5 min, samples were prepared for electron microscopy as described [22] and viewed in a Philips 400T transmission electron microscope (a gift of the Shell Research and Technology Center, Amsterdam, The Netherlands).

2.5. Nucleotide content analysis and nucleotide removal

Nucleotides bound to FtsZ and D212 mutants were extracted as described [22], and quantified using a MonoQ HR5/5 anion-exchange

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Abbreviations: MTSEA, methanethiosulfonate-ethylammonium; MTSES, methanethiosulfonate-ethylsulfonate; NEM, *N*-ethylmaleimide; DTT, 1,4-dithio-DL-threitol

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column on an Äkta FPLC system using a linear gradient of 10 mM KH_2PO_4/K_2HPO_4, pH 8.0 to 50 mM KH_2PO_4/K_2HPO_4, 1 M NaCl, pH 7.4 (Amersham Pharmacia Biotech). The column was calibrated with GTP and GDP standards. Nucleotides were removed by dialysis against 50 mM KH_2PO_4/K_2HPO_4, 50 mM KCl, pH 6.5 [13].

2.6. Modification of FtsZ D212C

Sulfhydryl-specific reagents were used to modify FtsZ D212C. Protein was incubated with methanethiosulfonate-ethylammonium (MTSEA), methanethiosulfonate-ethylsulfonate (MTSES) (both from Anatrace) and *N*-ethylmaleimide (NEM) (Sigma) at 2.5 mM, 10 mM and 0.5 mM, respectively. After 10 min labeling at room temperature [23], protein was stored on ice until further use. To reverse the labeling, 20 mM 1,4-dithio-DL-threitol (DTT) was added and the incubation was continued for 5 min at room temperature.

3. Results

3.1. Characteristics of the D212 mutants

Site-directed FtsZ D212N, D212E and D212C mutants were constructed to analyze the role of the T7-loop in GTP hydrolysis and polymerization (D.J.S. et al., in preparation). All D212 mutants displayed a markedly reduced GTP hydrolysis activity (Fig. 1). FtsZ D212E polymerized with GTP in the presence of calcium, albeit at an increased critical protein concentration for polymerization. FtsZ D212N and D212C, however, behaved differently from wt FtsZ. First, a high basal level of light scattering precluded the spectroscopical analysis of the polymerization behavior of these mutants. Second, in the sedimentation assay, these mutants also appeared to polymerize with GDP (not shown). These unusual characteristics prompted us to characterize these D212 mutants in more detail.

3.2. Removal of the negative charge at position 212 allows cation-induced polymerization

Since FtsZ D212N and D212C sedimented irrespective of the nature of the added nucleotide, the polymerization solution was analyzed for another factor that induces sedimentation. Omission of $MgCl_2$ completely abolished sedimentation (Fig. 2). In the absence of added nucleotide, addition of 5 mM of $MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, and $NiCl_2$ led to the sedimentation of substantial amounts of FtsZ D212N and D212C (Fig. 2, Table 1), whereas wt FtsZ and FtsZ D212E did not sediment. Since $SrCl_2$ or $BaCl_2$ were unable to induce sedimentation (Fig. 2), the size exclusion limit for the (crystal) cation radius is ~1.1 Å (Table 1). With 5 mM $ZnCl_2$ all proteins sedimented as aggregates [24]. To establish whether the sedimentation was due to protein aggregation or true

Table 1
Sedimentation of FtsZ D212C and D212N in the presence of divalent cations

Divalent cation	Ionic radius[a] (Å)	Polymer sedimentation
Mg^{2+}	0.66	+
Ni^{2+}	0.69	+
Co^{2+}	0.72	+
Zn^{2+}	0.74	aggregation
Mn^{2+}	0.80	+
Ca^{2+}	0.99	+
Sr^{2+}	1.12	−
Ba^{2+}	1.34	−

Sedimentation assays were performed as described in the text.
[a]Taken from [33].

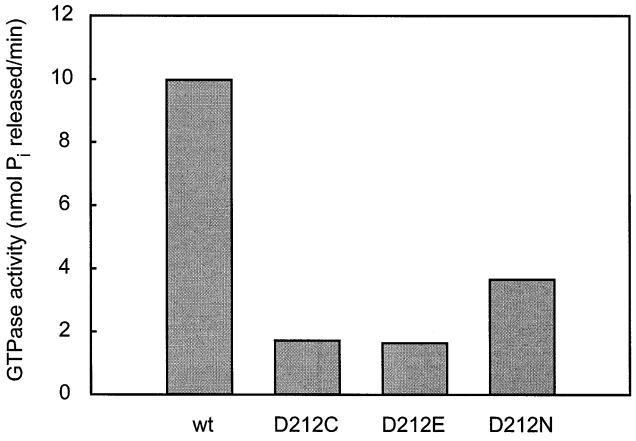

Fig. 1. GTPase activity of wt FtsZ and FtsZ D212C, D212E and D212N mutant proteins. GTP hydrolysis was measured as described in the text.

polymerization, samples were analyzed by electron microscopy. Addition of Mg^{2+} to FtsZ D212N and D212C leads to the formation of typical polymers (Fig. 3). These polymers were stable for at least 60 min, as tested by sedimentation (not shown). Cation-induced polymerization was reversible. Polymer pellets resuspended in the presence of EDTA sedimented only when cations were included in the buffer (not shown). We conclude that removal of the negative charge from position 212 enables polymerization of FtsZ with divalent cations.

3.3. Cation-induced polymerization requires GTP-bound FtsZ

Various studies have reported the retention of bound GDP by purified FtsZ with a stoichiometry of ~0.5–0.7 mol GDP per mol FtsZ [13,25]. Since the observed cation-induced sedimentation could be dependent on the retention of nucleotides, purified FtsZ and D212 mutants were analyzed for their nucleotide content. To our surprise, FtsZ D212C and D212N contained predominantly GTP, whereas wt FtsZ and FtsZ D212E contained solely GDP (Table 2). The bound nucleotides (~0.5 mol per mol of FtsZ) could be removed by extensive dialysis [13], but this procedure abolished the polymerization of the wt FtsZ (not shown) and could therefore not be used to determine if nucleotide-binding is critical for the cation-induced sedimentation. Next, we tested whether Mg^{2+}-in-

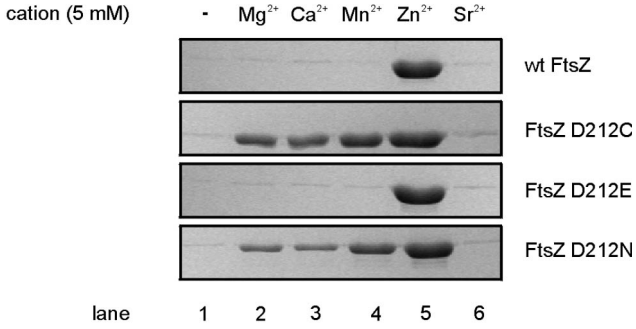

Fig. 2. Sedimentation of wt FtsZ and FtsZ D212C, D212E and D212N mutant proteins in the presence of various cations. Protein was incubated for 5 min at 30°C in polymerization buffer with no additions (lane 1) or 5 mM $MgCl_2$ (lane 2), $CaCl_2$ (lane 3), $MnCl_2$ (lane 4), $ZnCl_2$ (lane 5) or $SrCl_2$ (lane 6). Sedimentation and analysis of the protein pellets were performed as described in Section 2.

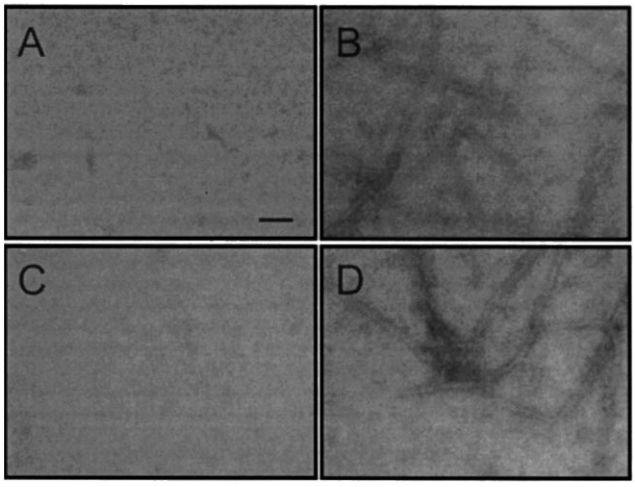

Fig. 3. Electron microscopy of wt FtsZ and FtsZ D212C, D212E and D212N mutant proteins in the presence of $MgCl_2$. Protein (0.4 mg/ml) was incubated in polymerization buffer with 5 mM $MgCl_2$ for 10 min at 30°C. Samples were analyzed by electron microscopy.

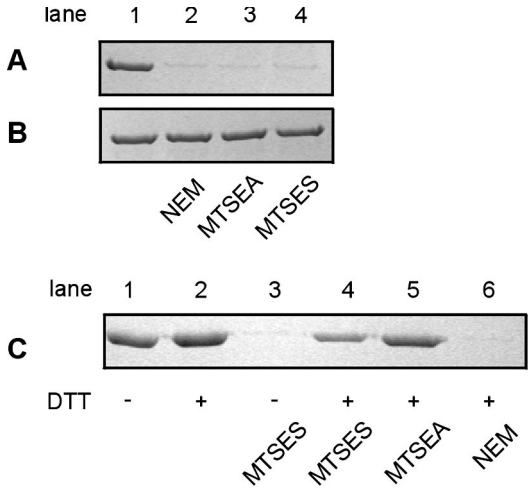

Fig. 4. Effect of sulfhydryl reagents on cation-induced sedimentation of FtsZ D212C. Sedimentation of FtsZ D212C (A) and FtsZ D212N (B) after incubation for 5 min at 30°C in polymerization buffer with 5 mM $MgCl_2$. The proteins were modified with nothing (lane 1), NEM (lane 2), MTSEA (lane 3) or MTSES (lane 4). (C) Reversal of cysteine modification with DTT. Sedimentation of FtsZ D212C after incubation for 5 min at 30°C in polymerization buffer with 5 mM $MgCl_2$ without (lanes 1 and 3) or with 20 mM DTT (lanes 2, 4, 5 and 6). FtsZ D212C was labeled with nothing (lanes 1 and 2), MTSES (lanes 3 and 4), MTSEA (lane 5) or NEM (lane 6).

duced polymerization leads to hydrolysis of the GTP bound to FtsZ D212C and D212N. After prolonged incubation with 5 mM $MgCl_2$, only a small fraction of the bound nucleotide was converted into GDP (Table 2). This observation is in agreement with the high stability of the Mg^{2+}-induced polymers. Taken together, these data show that FtsZ D212C and D212N retain significant amounts of GTP after protein purification, which in all likelihood explains the polymerization with cations.

3.4. Chemical modification of FtsZ D212C abolishes cation-induced polymerization

The role of the negative charge at position 212 was probed by modification of the FtsZ D212C mutant with the sulfhydryl reagents NEM, MTSEA and MTSES, which are neutral, positively and negatively charged, respectively. Modification of FtsZ D212C abolished cation-induced polymerization (Fig. 4A) while the reagents had no effect on FtsZ D212N (Fig. 4B). Addition of DTT to MTSEA and MTSES labeled FtsZ D212C re-established the cation-induced sedimentation, but it did not restore the activity of FtsZ D212C containing the covalently bound NEM (Fig. 4C). Addition of GTP after labeling did not restore polymerization (not shown). These data indicate that modification of FtsZ D212C with sulfhydryl reagents distorts either the interaction with cations or the interaction of FtsZ monomers in the polymer.

Table 2
Nucleotides associated with wt FtsZ and FtsZ D212 mutants

Protein		Bound nucleotide (mol per mol FtsZ)	
		GTP	GDP
FtsZ		0	0.74
FtsZ D212E		0	0.65
FtsZ D212C		0.68	0.14
+$MgCl_2$ [a]	15 min	0.49	0.13
	60 min	0.48	0.11
FtsZ D212N		0.49	0.08
+$MgCl_2$ [a]	15 min	0.35	0.10
	60 min	0.28	0.14

Nucleotides were extracted from the protein as described, and identified and quantified by anion-exchange chromatography.
[a]Proteins (1 mg/ml) were incubated in polymerization buffer with 5 mM $MgCl_2$ at 30°C for the indicated times.

4. Discussion

In this report we describe FtsZ mutants capable of polymerization without the addition of nucleotides. Removal of the negative charge on position 212 in FtsZ results in protein that purifies with GTP as its predominantly bound nucleotide. The addition of divalent cations allows polymerization of the mutants, suggesting that at the interface of two FtsZ monomers in a functional polymer both GTP and a coordinated metal are required for the formation of a stable structure. The aspartate residue described in this report is conserved throughout all FtsZ proteins known and its position corresponds to a residue that is critical for GTP hydrolysis in tubulins. D212 has been implicated in the coordination of Mg^{2+} at the nucleotide-binding site of FtsZ [26]. Exchange of this aspartate with a glutamate leads to a dramatic reduction of FtsZ GTP hydrolysis and polymerization activity, indicating that a conserved substitution does not fully restore FtsZ activity. While wt FtsZ and FtsZ D212E purify with GDP as associated nucleotide, the FtsZ D212C and D212N mutants contain GTP. This surprising observation is most easily explained by a critical role of the negative charge at position 212 in the coordination of Mg^{2+}. In the absence of added Mg^{2+}, wt FtsZ and FtsZ D212E retained the coordinated Mg^{2+} ion that mediates hydrolysis of the bound nucleotide. FtsZ D212C and D212N do not retain Mg^{2+} and therefore contain GTP. In the presence of added GTP and Mg^{2+}, both FtsZ D212C and D212N display GTP hydrolysis activity, although their activity is markedly reduced as compared to the wt.

The retention of GTP by the FtsZ D212C and D212N mutants permits polymerization in the presence of divalent

cations. The size exclusion limit for cation-induced polymerization indicates that the cations bind at a specific site. We propose that this cation-binding site comprises an occupied nucleotide-binding site on one FtsZ monomer and the T7-loop region on another FtsZ monomer. This requirement for an FtsZ dimer would explain the absence of a cation in the FtsZ crystal structure, which contains one FtsZ monomer per asymmetric unit [27]. Cation-induced polymerization of FtsZ D212C can be abolished by modification of the cysteine with neutral, positively and negatively charged sulfhydryl reagents. These reagents do not affect the nucleotide bound to FtsZ D212C and bulk GTP hydrolysis activity (D.J.S. et al., in preparation). Modification of D212C disrupts either cation-binding or the overall interaction of the FtsZ monomers at the nucleotide-binding interface. As previously reported for tubulin [28,29], nucleotide removal by extensive dialysis results in nucleotide-free, but non-functional FtsZ protein. The tubulin αβ-heterodimer is stabilized by Mg^{2+} and GTP bound at the β–α-interface, also known as the N-site [29]. It is likely that the FtsZ multimers found in vivo and in vitro [13,30–32] are also stabilized by the presence of nucleotide at the dimer interface. Summarizing, our results indicate that the highly conserved aspartate 212 plays a pivotal role in Mg^{2+} coordination and nucleotide hydrolysis. The D212 mutants provide a good starting point for the further characterization of the Mg^{2+}-binding properties of FtsZ.

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