Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 331 (2005) 1171–1177
BBRC
www.elsevier.com/locate/ybbrc

Mutations in the GTP-binding and synergy loop domains of *Mycobacterium tuberculosis ftsZ* compromise its function in vitro and in vivo

Malini Rajagopalan *, Mark A.L. Atkinson, Hava Lofton, Ashwini Chauhan, Murty V. Madiraju

Biomedical Research, The University of Texas Health Center at Tyler, 11937 U.S. Hwy @ 271, Tyler, TX 75708-3154, USA

Received 14 March 2005
Available online 19 April 2005

Abstract

The *Mycobacterium tuberculosis* FtsZ ($FtsZ_{TB}$), unlike other eubacterial FtsZ proteins, shows slow GTP-dependent polymerization and weak GTP hydrolysis activities [E.L. White, L.J. Ross, R.C. Reynolds, L.E. Seitz, G.D. Moore, D.W. Borhani, Slow polymerization of *Mycobacterium tuberculosis* FtsZ, J. Bacteriol. 182 (2000) 4028–4034]. In an attempt to understand the biological significance of these findings, we created mutations in the GTP-binding ($FtsZ_{G103S}$) and GTP hydrolysis ($FtsZ_{D210G}$) domains of FtsZ and characterized the activities of the mutant proteins in vitro and in vivo. We show that $FtsZ_{G103S}$ is defective for binding to GTP and polymerization activities, and exhibited reduced GTPase activity whereas $FtsZ_{D210G}$ protein is proficient in binding to GTP, showing reduced polymerization activity but did not show any measurable GTPase activity. Visualization of FtsZ-GFP structures in *ftsZ* merodiploid strains by fluorescent microscopy revealed that $FtsZ_{D210G}$ is proficient in associating with Z-ring structures whereas $FtsZ_{G103S}$ is not. Finally, we show that *Mycobacterium smegmatis ftsZ* mutant strains producing corresponding mutant FtsZ proteins are non-viable indicating that mutant FtsZ proteins cannot function as the sole source for FtsZ, a result distinctly different from that reported for *Escherichia coli*. Together, our results indicate that optimal GTPase and polymerization activities of FtsZ are required to sustain cell division in mycobacteria and that the same conserved mutations in different bacterial species have distinct phenotypes.

Keywords: *Mycobacterium tuberculosis*; *Mycobacterium smegmatis*; Cell division; FtsZ; Recombination; Fluorescent microscopy; GTPase activity; Polymerization activity; Gene replacement; Green fluorescent protein; Z-rings

Mycobacterium tuberculosis, the causative agent of tuberculosis, infects more than three million new people each year. *M. tuberculosis* is a slow grower with a doubling time of approximately 24 h. The genus *Mycobacterium* also includes other species with different doubling times: e.g., *Mycobacterium smegmatis* (2–3 h) and *M. avium-intracellulare* (10–12 h) [2]. The genetic and biochemical aspects of cell cycle, specifically the cell duplication process, in *M. tuberculosis* and other 'members of mycobacteria are largely unknown. A better understanding of the initiation and regulation of cell duplication process in *M. tuberculosis* would open the possibility of the development of novel drugs targeted to the cell duplication process.

FtsZ, the bacterial homologue of eukaryotic tubulins, is the initiator of the cell division process, and is a well-conserved protein [3]. The three-dimensional structure of *Methanococcus jannaschii* FtsZ protein shows considerable similarity to those of the tubulins, both α and β isoforms [4,5]. FtsZ protein binds GTP, shows GTPase activity associated with polymerization, and forms

* Corresponding author. Fax: +1 903 877 5969.
E-mail address: malini.rajagopalan@uthct.edu (M. Rajagopalan).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.239

protofilaments, similar to those of microtubules (reviewed recently [6,7]). FtsZ assembly dynamics are shown to be regulated by GTP hydrolysis [6,7]. In vivo, FtsZ protein forms a structural element called the Z-ring at putative mid-cell site and the dynamics of FtsZ assembly at the division site is thought to be critical for cell cycle progression [8,9]. FtsZ interacting proteins such as FtsA, ZipA, MinCD, and SulA are believed to influence the FtsZ-ring assembly and stability [3,10]. Two naturally occurring mutations in the *fts*Z gene of *Escherichia coli*, designated as *fts*Z84 and *fts*Z2, have been characterized [11,12]. Cells bearing these mutations are viable but show temperature sensitive phenotype. The *fts*Z84 mutant produces filamentous cells and does not form Z-rings at 42 °C, a non-permissive temperature for growth [13,14]. The *fts*Z2 mutant shows mini-cell phenotype at 30 °C but undergoes cell lysis at 42 °C [11,15]. In vitro both proteins show reduced GTPase activity [16–18]. Together, these results suggest that the mutant FtsZ proteins are functional in vivo although their GTPase activities are reduced significantly.

FtsZ protein of *M. tuberculosis* ($FtsZ_{TB}$) has been purified, characterized [1], and more recently, has been crystallized [19,20]. $FtsZ_{TB}$, unlike any of its eubacterial homologues, catalyzes slow GTP-dependent polymerization and hydrolysis activities. Furthermore, $FtsZ_{TB}$ polymers formed at steady state are found to be very stable and do not readily dissociate [1]. Presumably, interactions of $FtsZ_{TB}$ with other proteins are required for the disassembly of $FtsZ_{TB}$ polymers in vivo. The genome of *M. tuberculosis* lacks identifiable analogues of *E. coli* FtsA, ZipA, MinCD, and SulA, the putative FtsZ interacting proteins [21]. Given the slow GTP hydrolysis and polymerization kinetics of $FtsZ_{TB}$ combined with the absence of the identifiable analogues of FtsZ-interacting proteins in vivo, one would question whether the reported slow polymerization and hydrolysis activities of $FtsZ_{TB}$ are actual reflections of their activities in vivo and are critical for cytokinesis. To gain insights into these issues, we created two site specific mutations—one in the putative GTP-binding site by replacing glycine at position 103 with serine ($FtsZ_{G103S}$), and the other in the hydrolysis site by replacing aspartic acid at position 210 with glycine ($FtsZ_{D210G}$) and characterized the mutant proteins in vitro and in vivo. The following three questions were asked: (1) Do mutations in the GTP-binding and hydrolysis domains of $FtsZ_{TB}$ interfere with GTP binding, hydrolysis, and GTP-dependent polymerization activities in vitro? (2) Are mutant FtsZ proteins proficient in forming Z-ring structures? (3) Are cells producing mutant FtsZ proteins viable and competent for cytokinesis? We show that purified $FtsZ_{G103S}$ and $FtsZ_{D210G}$ proteins are defective for GTP hydrolysis and polymerization activities and in stark contrast to the situation seen in *E. coli*, cells producing the mutant *fts*Z proteins are non-viable, indicating that the observed slow GTP-dependent polymerization and hydrolysis activities are essential for mycobacterial cell division in vivo.

Materials and methods

Bacterial strains, transformation, and cell culture conditions. The sources of *E. coli* strains (Top10 and BL21 (DE3)/pLysS), *M. smegmatis*, and *M. tuberculosis* were as described [22,23]. *E. coli* cultures were propagated in Luria–Bertani (LB) broth in the presence of appropriate antibiotics at 37 °C and the transformants were selected on LB agar plates supplemented with ampicillin (100 μg/ml), kanamycin (kan) (50 μg/ml) or chloramphenicol (10 μg/ml), and ampicillin (100 μg/ml). Mycobacterial cultures were grown in Middlebrook 7H9 media supplemented with 0.05% Tween 80 and oleic acid–albumin–dextrose (OAD) [22,23].

Site-specific mutagenesis and construction of ftsZ mutant alleles. Mutagenesis at codons 103 and 210 of the *fts*Z gene of *M. tuberculosis* and *M. smegmatis* was carried out using the enzymatic inverse PCR method using Ex-*Taq* DNA polymerase and the respective genomic DNA as templates as described [24]. To replace GGC coding for glycine at codon 103 with AGC coding for serine, two PCR products were generated using the primer pairs Tb-A, Tb-C, Tb-D, Tb-B (see Table 1 for sequences of primers). The PCR products were gel purified, mixed in equal proportion, and the mixture was used as template along with primers Tb-A and Tb-B that bind to the 5′ and 3′ ends of *fts*Z to generate the full length $ftsZ_{tb}$ gene. Similarly, oligonucleotide primer pairs Tb-A, Tb-C1, and Tb-D1, Tb-B were used to replace GAT coding for aspartic acid at codon 210 with GGC coding for glycine. The *fts*Z coding regions were confirmed by sequencing, cloned in pET15b, and transformed into *E. coli* strain BL21-pLysS for overproduction of the mutant proteins.

For mutagenesis with *M. smegmatis fts*Z, oligonucleotide primers SmegA and SmegB (see Table 1) were used to amplify the DNA fragment bearing *fts*Z promoter and the coding sequence. Mutagenic primer pairs SmegC–SmegD and SmegC1–SmegD1 were used to create G103S and D210G mutations in the *M. smegmatis fts*Z coding region. The PCR products were cloned into pMV306, an integration proficient vector, and transformed into *M. smegmatis* [25].

Overexpression and purification of recombinant $FtsZ_{TB}$ protein. Recombinant *E. coli* strains overproducing FtsZ (pSAR1), $FtsZ_{G103S}$ (pRR3) or $FtsZ_{D210G}$ (pRR7) were grown to an OD_{600} of 0.6 and *fts*Z expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Wild-type and mutant FtsZ proteins were purified under soluble conditions on Ni–NTA affinity columns (Qiagen) essentially following the manufacturer's recommendations. Peak fractions containing FtsZ were pooled and dialyzed for 4 h in storage buffer (25 mM Hepes buffer, pH 7.2, 0.1 mM EDTA, 10% glycerol, and 1 mM DTT). The purity of $FtsZ_{TB}$ protein preparation was judged to be approximately 92% based on Coomassie blue and SYPRO Red (Molecular Probes) stained SDS–polyacrylamide gels. The $FtsZ_{TB}$ has only one tyrosine residue and no tryptophan residues, and therefore the absorbance at 280 nm could not be used to accurately determine the protein concentration. Furthermore, the $FtsZ_{TB}$ protein concentration determined by Bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard gave variable inter-assay results. Consequently, for accurate $FtsZ_{TB}$ protein concentration determinations, acid hydrolysis of with 6 N HCl followed by the determination of free amino acids by a quantitative ninhydrin reaction was carried out for each $FtsZ_{TB}$ preparation [26].

GTPase activity of $FtsZ_{TB}$. The GTP hydrolysis activity of $FtsZ_{TB}$ was assayed by measuring the release of inorganic phosphate from [γ-^{32}P]GTP. The $FtsZ_{TB}$ protein was incubated at various concentrations in buffer B (50 mM Mes, pH 6.5, 5 mM $MgCl_2$, and 100 mM