Human S100B protein interacts with the *Escherichia coli* division protein FtsZ in a calcium-sensitive manner

Peter L. Ferguson and Gary S. Shaw*

*Department of Biochemistry, The University of Western Ontario, London, Ontario, Canada, N6A 5C1

*Author to whom correspondence should be addressed

Email: gshaw1@uwo.ca
Fax: 519-661-3175

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Abbreviations: DIC, differential interference contrast; EGFP, enhanced green fluorescent protein; S100B-EGFP, S100B protein with a C-terminal EGFP fusion; IPTG, isopropyl-β-D-thiogalactoside; TCA, trichloroacetic acid
Summary

S100B is a small, dimeric EF-hand calcium-binding protein abundant in vertebrates. Upon calcium binding, S100B undergoes a conformational change allowing it to interact with a variety of target proteins including the cytoskeletal proteins tubulin and glial fibrillary acidic protein. In both cases, S100B promotes the \textit{in vitro} disassembly of these proteins in a calcium-sensitive manner. Despite this, there is little \textit{in vivo} evidence for the interaction of proteins such as tubulin with S100B. In order to probe these interactions, we studied the expression of human S100B in \textit{Escherichia coli} and its interaction with the prokaryotic ancestor of tubulin, FtsZ, the major protein involved in bacterial division. Expression of S100B protein in \textit{Escherichia coli} results in little change in FtsZ protein levels, causes a filamenting bacterial phenotype characteristic of FtsZ inhibition and leads to missed rounds of cell division. Further, S100B localizes to similar positions as FtsZ in bacterial filaments: the small foci at the poles, the mid-cell positions and between the nucleoids at regular intervals. Calcium-dependent physical interaction between S100B and FtsZ was demonstrated \textit{in vitro} by affinity chromatography and this interaction was severely inhibited by the competitor peptide TRTK-12. Together these results indicate that S100B interacts with the tubulin homologue FtsZ \textit{in vivo}, modulating its activity in bacterial cell division. This approach will present an important step for the study of S100-protein interactions \textit{in vivo}. 
Introduction

S100B is one of more than 20 known members of the small (9-12 kDa), acidic, S100 family of EF-hand calcium-binding proteins, which are expressed in vertebrates, generally in a tissue-specific manner (reviewed by (1)). Most of these proteins form homo- and heterodimers that respond to signal-induced increases in intracellular calcium levels. Upon binding calcium, these proteins undergo conformational changes that allow them to bind to target proteins and modulate their activity. S100B is abundantly expressed in glial cells, where its best-characterized roles involve modulating protein-protein interactions of all three classes of cytoskeletal structures. The assembly-disassembly of microtubules can be controlled by S100B through its interactions with tubulin (2-4) and the microtubule-associated protein, tau (5). In addition, S100B can regulate the dynamics of intermediate filaments through its interactions with the glial fibrillary acidic protein (GFAP), vimentin (3,6) and annexin VI (7,8). The S100B-mediated regulation of microfilaments has been suggested based on calcium dependent in vitro interactions with the actin capping protein, CapZα (9), and by disruption of the interactions of F-actin with caldesmon (10) and calponin (11).

S100B has been implicated in the calcium-dependent binding of at least twenty target proteins in mammals (1). However, since many of these target proteins have been identified only by in vitro methods, they require identification in vivo and a rationale for physiological relevance. In many cases, the S100B recognition site on putative targets has been characterized by the peptide chemical pattern +OXO*XOO (+ = basic, O = hydrophobic, * = hydrophilic, X = variable) which is found in the strong competitor peptide TRTK-12 (TRTKIDWNKILS) (12,13).

During our study of the mechanism(s) by which S100B recognizes its target proteins, we observed that human S100B expressed in bacteria causes a filamentation phenotype, likely the result of a specific interaction with one or more bacterial proteins. Our initial candidate was the protein FtsZ, the evolutionary precursor to tubulin. FtsZ is required for the division of bacteria, archaea, chloroplasts, and the mitochondria of some algae (14-23). When FtsZ is nonfunctional or under-expressed, bacteria form long filaments lacking signs of invagination at the division sites (24-26). Multinuclear filaments can also be formed when FtsZ is over-expressed (27), or misdirected to
secondary division sites by defects in the proteins of the minB locus (28). However, this phenotype is characterized by the additional presence of anucleate minicells, which are not observed when FtsZ is dysfunctional or under-expressed. Evidence that FtsZ is the evolutionary precursor to tubulin (reviewed by (29)), includes their similar GTPase sequence motifs and enzymatic activities (30-32) and three-dimensional structures (32-34). As well, bacterial FtsZ in vitro can form structures resembling protofilaments, rings, and tubes, similar to those formed by tubulin (35-38). One structure is a bundle of 6-7 FtsZ protofilaments that forms the septal ring as the first step in bacterial septation (39,40). This FtsZ ring provides the scaffold upon which at least nine proteins (FtsA, ZipA, FtsK, FtsQ, FtsL, YgbQ, FtsW, FtsI, and FtsN) sequentially assemble to form the division apparatus (41-44). FtsZ then provides either the mechanical force that causes constriction and eventual pinching off of the daughter cells or FtsZ forms the rigid framework against which the force will be applied (37). The proteins FtsA and ZipA, which bind to FtsZ independently of each other, are not required for assembly of the FtsZ ring, but stabilize the bundle and recruit other proteins to the division site (44,45). In a further analogy to tubulin function, ZipA is the bacterial homologue of the microtubule associated protein, tau, (45-47), whereas FtsA (along with the cell-shape regulator protein MreB) is a homologue of actin (22,48,49). Thus, the similarities between the bacterial cytoskeletal proteins to those of eukaryotes have become even more compelling since the original discovery of FtsZ.

The identification of in vivo protein interactions with the calcium-binding protein S100B, and indeed many of the other S100 proteins has proven difficult. Since the interactions are transient, and modulated by calcium, traditional methods such as yeast two-hybrid experiments have failed to identify interacting target proteins (50). The single exception is S100A10, which does not bind calcium and undergoes a calcium-insensitive interaction with the phospholipid-binding protein annexin AII (51). Further, the high sequence similarity of the S100s limits studies using monoclonal antibodies. In addition, several of the S100 proteins including S100B, S100A6 and S100A11 have the ability to form heterodimeric S100 species in vivo complicating protein interaction analyses since interactions can in principle occur with either homo- or heterodimeric
S100 species. Since the S100 proteins are restricted to vertebrates, one method to address protein interactions and biological function of these proteins is through the use of a lower non-vertebrate species. In this regard, E. coli is particularly attractive because the bacterium contains homologues of many of the proposed S100B interacting proteins, especially those involved in cytoskeletal assembly, but lacks all S100 protein homologues. Thus, protein interactions and their affect on function in E. coli can be studied for S100B without interference from other S100 proteins. In this report we present in vivo and in vitro evidence demonstrating the physical interaction between the calcium-binding protein S100B and the cytoskeletal tubulin homologue FtsZ found in E.coli. This interaction interferes with bacterial division causing a filamentous phenotype as a result of missed rounds of cell division.

**Experimental Procedures**

*Plasmids and bacteria* - S100B, S100B-EGFP and FtsZ proteins were expressed in E. coli strain N99, a galK derivative of K-12 (52). S100B bearing a C-terminal fusion of EGFP (Clontech) was made by inserting the BamH I - Not I segment of EGFP-N1 (Clontech) site into a version of expression plasmid pSS2 (53) modified to have a Bgl II restriction site replace the S100B stop codon. The S100B-EGFP protein has a hexapeptide linker (DPPVAT) between the S100B and EGFP moieties. A soluble EGFP-protein (sol-EGFP), was constructed by digesting the pS100B-EGFP plasmid with Neo I, and re-ligating the plasmid. Most of the S100B protein is removed except for the first 6 residues of helix I (SELEKA) fused in frame to the last 13 residues of helix IV (MVTTACHEFEHE), followed by the DPPVAT linker. The E. coli FtsZ gene was amplified by PCR using strain N99 DNA as template. PCR primers based on the FtsZ sequence in strain K-12 (54) were designed to introduce BamH I (forward: 5'-GGTGGTTGGATCCGGAGAAATATGTTGGGAACTATTGCCCATGGAACCC-3') and Hind III (reverse: 5'-AAATTCCAGTCAAAGCTTAATCAGCTTGCTTACGCAGG-3') restriction sites for cloning into pcDNA3 (Invitrogen) for sequencing and to obtain an EcoR I restriction site upstream of the BamH I site for construction of the FtsZ expression plasmid pFtsZ-Nex by
replacement of the S100B gene in pSS2 with the FtsZ gene. All constructs were confirmed by DNA sequencing. All proteins were confirmed by ESI-MS mass spectrometry.

Protein purification - S100B was purified as described previously (53,55). S100B-EGFP required a modification in the purification protocol in which a 30% cut of ammonium sulfate was selected for further purification. FtsZ was purified essentially as described by Mukherjee and Lutkenhaus (56). Briefly, E. coli strain N99 cells bearing pFtsZ-Nex was grown in 1L LB medium with 40 µg/ml carbenicillin to an OD$_{600}$ reading of 0.5, then induced with 1 mM IPTG for 5 hours. Bacteria were pelleted and stored at -80°C. Bacteria were lysed by French press and the FtsZ protein was suspended in TKC buffer (50 mM Tris-HCl, pH 7.9, 50 mM KCl, 1 mM EDTA, 10% glycerol) during purification and storage. FtsZ protein purity was 80-90% based on SDS-PAGE.

Inclusion body assay - The presence of inclusions was tested by a modification of a well-established method (57). N99 cells bearing S100B, S100B-EGFP, or FtsZ expression plasmids were grown in 20 ml of LB medium with 40 µg/ml carbenicillin to an OD$_{600}$ reading of 0.6. Cultures were induced with 1 mM IPTG for 4 h, then pelleted, resuspended in 10 ml of 50 mM Tris-HCl, pH 8.0, 5 mM MgCl$_2$ and stored at -80°C. Bacteria were thawed in the presence of 20 mg PMSF and ruptured by French Press. The lysates were split into 5 ml fractions. One was retained as a total cell fraction. The other fraction was centrifuged at 4°C for 30 min at 13, 000g, and 3 ml of the supernatant carefully transferred to a new tube, the remainder poured off and the pellet resuspended in 5 ml of the above buffer. A 100 µl portion of each fraction were precipitated with TCA for SDS-PAGE analysis.

Fluorescence microscopy - Bacteria expressing the S100B constructs were grown overnight in LB medium containing 40 µg/ml carbenicillin. Following this, bacteria were diluted 50- to 100-fold and grown to an OD$_{600}$ of 0.1. Protein expression was induced by IPTG added to final concentrations of 1 µM, 10 µM, 20 µM, 100 µM, 400 µM, and 1 mM. In cases of extended induction periods
bacteria were diluted at intervals ("pseudochemostat") to maintain cells in mid-log phase. Induced bacteria were harvested at intervals and stored at 4° C for microscopy later that day or for use up to one week later, with no apparent change in viability or phenotype. Bacteria were also fixed by 4% formaldehyde in PBS and stored overnight at 4 °C. The fixed bacteria were pelleted, resuspended in PBS and incubated several hours at 4°C with 2.5 µg/ml Hoechst 33342 to stain the bacterial DNA. Bacteria (10 µl) were mounted on slides coated with a dried film of 2% agarose. Co-localization experiments using polyclonal anti-S100B (ICN) were performed on poly-L-lysine coated slides as described by Mukherjee and Lutkenhaus (56). Using goat anti-rabbit Alexa594 (Molecular Probes) as the fluorescent secondary antibody, images were captured using a 100x Neoplan oil immersion objective on a Zeiss Axioskop 2 MOT microscope equipped with a QImaging Retiga 1300™ cooled CCD (Mississauga, Canada). Images were processed using Northern Eclipse, version 6.0 (Empix Imaging, Inc., Mississauga, Canada) and Adobe Photoshop™ LE (Adobe Systems Inc.).

Affinity chromatography - Human S100B (10 mg) was cross-linked to a 1 ml HiTrap™ NHS-activated HP column (Pharmacia) according to the manufacturer’s directions. The column was equilibrated with TKC buffer prior to loading the FtsZ protein. After overnight washing with 70-80 ml of the TKC buffer (0.15 ml/min), in which 2 ml fractions of flow-through were collected for analysis, bound protein was eluted with TKE buffer (50 mM Tris-Cl, pH 7.9; 50 mM KCl; 10% glycerol, 10 mM EDTA). For the peptide binding competition assay, peptide TRTK-12 was prepared at 2 mg/ml in TKC buffer and 2 ml were loaded onto the column and washed extensively with TKC buffer prior to loading FtsZ.

Western blotting - Bacterial proteins eluted from the S100B affinity column were precipitated with TCA and prepared for SDS-PAGE and transblotting as described previously (58). To correlate FtsZ expression levels to induced production of S100B-EGFP, 0.2 ml portions of bacterial cultures were pelleted in a microcentrifuge and resuspended in 30 µl of 1.5X Laemmli buffer for SDS-PAGE and western blotting. The proteins were probed with 1:2000 diluted rabbit anti-FtsZ (kindly
Human S100B interacts with *E. coli* FtsZ provided by W. Margolin) and detected by enhanced chemiluminescence ECL kit (Pharmacia-Amersham).

**Results**

*Filamenting phenotype* - The N99 strain of *E. coli* used in this study is typical of the species in terms of its morphology. When grown in rich medium, the bacteria have a diameter of 0.6 µm and range in length between 2 and 3 µm, depending on their stage in the division cycle. Wild-type human S100B expression induced by 1 mM IPTG causes a filamenting phenotype in the bacteria, (Fig. 1, panel A). This filamenting phenotype is also observed at lower induction levels (20 µM IPTG) over longer periods for expression of S100B and S100B-EGFP (Fig. 1, panels B and C). The control protein, sol-EGFP, which contains two short segments of S100B fused in frame to EGFP (see Experimental procedures), does not cause the filamenting phenotype (Fig. 1, panel D), even at 1 mM IPTG induction (not shown) and the fluorescence is uniformly distributed in the cytoplasm (not shown), indicating the EGFP moiety is a passive marker. This is consistent with previous reports of uniform EGFP localization throughout the bacterial cytoplasm (26). Many of the elongated cells show no signs of constrictions associated with septation. However, within the population there are bacteria of the normal size distribution indicating either that some proper septation does occur or some bacteria have been cured of the plasmid because of its deleterious effects. The filamentation caused by S100B and S100B-EGFP is similar to that observed when FtsZ is dysfunctional or under-expressed (24-26) including the presence of some normal-length bacteria. This suggests that S100B may be recognizing the tubulin ancestor, FtsZ, and interfering with its normal function.

*Co-localization studies*- To test the *in vivo* interaction between S100B and FtsZ, the intracellular localization of S100B was monitored by using the S100B-EGFP fusion protein. Previous studies showed the utility of FtsZ-GFP fusions to monitor septum assembly is strongly dependent on the expression level of the fusion protein (59). With this in mind, and because we wanted to test the
tunability of S100B-EGFP expression, N99 cells bearing the S100B-EGFP plasmid were induced for 4 hours with various concentrations of IPTG. Between 0 - 100 µM IPTG, there is a positive correlation between S100B and S100B-EGFP expression levels and severity of the filamenting phenotype (not shown). Induction with 10 µM IPTG yielded a population of cells with a nice distribution of lengths from normal size to 4-, 8-, and 16-times longer than normal. In the filaments the fluorescent foci of S100B-EGFP localization occur at the poles and along the cell with a spacing of 2 - 3 µm with interspersed daughter nucleoids (Fig. 2, panel A). The fluorescence from the S100B-EGFP protein forms foci close to the membrane and broad bands that span the cell diameter. This banded pattern within elongated filaments is similar to that observed by immunofluorescence studies of FtsZ dynamics and localization (59-62), and is in fact, indistinguishable from the FtsZ-EGFP fusion protein distribution (see Figure 3 in Ref. (26)). The polar localization of S100B-EGFP is consistent with FtsZ mislocalization to bacterial poles under certain conditions (26,27). The pattern of S100B-EGFP expression at increasing IPTG expression concentrations argues against an inclusion body explanation (see below), since in a small fraction of the uninduced bacteria, low level (“leaky”) expression of the S100B-EGFP fusion protein from the plasmid's strong tac promoter results in very small, distinct fluorescent foci at one or both poles of the normal-length cells with occasional mid-cell positioning in cells that are long enough to be dividing (not shown). Polar localization of S100B-EGFP occurs at all levels of IPTG induction but between 1 - 100 µM the polar foci do not become larger, rather foci become more numerous and spaced along the bacterial filaments. Further, protein structures within bacteria can be observed under visible light by careful manipulation of light intensity, gain, and offset parameters during digital DIC microscopy. The fluorescent foci of S100B-EGFP within the filament in Fig. 2, panel A, are visible by light microscopy (Fig. 2, panel B) and the pattern is indistinguishable from that formed by unlabeled FtsZ over-expressed in the N99 cells (Fig. 2, panel C).

Further evidence of S100B-EGFP and FtsZ co-localization is the observation that in some bacteria expressing S100B-EGFP, helical structures are visible due to the fluorescence of the EGFP moiety (Fig. 2, panel D-F). These structures are similar to those formed and containing FtsZ...
polymers in *E. coli* (26,59,63-65) and *B. subtilis* (66) indicating S100B and FtsZ occupy similar locations in the cell.

Finally, S100B localization in the bacteria was detected by immunolabeling permeabilized bacteria with anti-S100B and a fluorescent secondary antibody. The bacteria were induced by low levels of IPTG to allow for normal bacterial division to occur and to have a normal distribution of bacterial lengths. The helical structures observed in bacteria expressing S100B-EGFP are not the result of the EGFP fusion because similar helices are detected by the anti-S100B immunolabeling of bacteria expressing S100B (Fig. 2, panel G-I). More importantly, a number of bacteria exhibit a single bright, sharp midcell band consistent with S100B co-localization at the septum (Fig. 3). In some cases of advanced cell division, fluorescent immunolabeling is observed in the elongated constriction between the dividing cells (Fig. 3, inset).

*Intracellular levels of FtsZ*—The levels of FtsZ in *E. coli* have been estimated to be between 5000 (67) and 15,000 copies per bacterium (40), with little apparent fluctuation during the division cycle (29,68,69). Over-expression of FtsZ causes either toxic effects (56) or the phenotype of minicells and filamentation (27), whereas under-expression of FtsZ results in filamentation only (25). While the above experiments are consistent with an interaction of S100B with FtsZ, it is possible that expression of S100B (or S100B-EGFP) might alter FtsZ protein levels. This was tested by inducing S100B-EGFP expression with IPTG and comparing the levels of FtsZ and S100B-EGFP at intervals after the induction (Fig. 4). S100B-EGFP expression increases significantly over the time of induction, whereas there is little change in the amount of intracellular FtsZ. Further, the small fluctuations observed in FtsZ levels (<15%) are well below the 2- to 7-fold fluctuations considered necessary to alter the timing and precision of division (27,70). These observations show that FtsZ protein levels in *E. coli* are neither increased nor decreased upon S100B expression and can not be responsible for the observed filamenting phenotype.

Over-expressed proteins can cause a filamentation phenotype because of the formation of inclusion bodies (71). Typically, the inclusion is a single, large insoluble aggregate located near a
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cell pole and distending the cell wall (72). In the case of S100B, we thought inclusion body formation highly unlikely, particularly because of our experience in purifying the protein and because the protein is very soluble (>20 mg/ml), having been named for its solubility even in 100% ammonium sulfate (73). Nonetheless, we tested for the presence of inclusion bodies after 4h of high level induction with 1 mM IPTG (Fig. 5). No evidence for S100B and S100B-EGFP inclusions was found in the pellet fraction after IPTG induction and cell lysis, whereas large amounts of S100B and S100B-EGFP are found in the total and soluble fractions.

Interaction of FtsZ and S100B – The binding of S100B to a target protein such as tubulin, is calcium-dependent, requiring about $10^{-5}$ M Ca$^{2+}$. Binding of calcium ions to S100B triggers a conformational change in S100B, exposing residues required for protein interactions (74). The interaction between FtsZ and S100B was characterized biochemically by using an affinity column to which S100B had been covalently linked. A purified preparation of FtsZ protein in calcium buffer (TKC) was loaded onto the S100B affinity column and washed thoroughly with calcium buffer. The excess FtsZ loaded on the column passes through in the first few wash fractions (Fig. 6A, lanes 1-3) and there is no detectable FtsZ by wash fraction 32 (Fig. 6A, lane 4). The FtsZ retained by the column is eluted in the first EDTA fraction (lane 5). A very minor amount of FtsZ is detectable in lane 6 of longer blot exposures (not shown). When the S100B affinity column is presaturated with TRTK-12 peptide which specifically and tightly binds the S100B target recognition site, all of the loaded FtsZ passes in the first two wash fractions (Fig. 6B, lanes 1, 2) and no protein remains to be eluted by EDTA (Fig. 6B, lane 5). This demonstrates FtsZ binding is calcium-dependent and specific to the normal target binding site on S100B.

Discussion

The high level expression of human S100B in E. coli causes a filamenting phenotype lacking constrictions at normal division sites in the filaments. This phenotype resembles that observed when FtsZ is either dysfunctional or not expressed (24-26). Coincident with this, S100B has little affect
on the FtsZ expression levels and is able to bind specifically in a calcium-sensitive manner to FtsZ. These observations indicate the interaction between S100B and FtsZ cause the filamentation phenotype, likely due to an inhibition of FtsZ function.

Interaction of S100B with FtsZ is supported by its intracellular localization. Fluorescence microscopy of normal length bacteria expressing low levels of S100B shows a single sharp band mid-cell (Fig. 3) that matches the pattern of FtsZ distribution in normally dividing bacteria (26,60), including extended constrictions in actively dividing cells, as previously observed (60). We suspect that at higher induction levels, filamentation occurs because S100B (or S100B-EGFP) interferes with FtsZ assembly and the 2 - 3 µm spacing of punctate fluorescence shown in Fig. 2A indicates aborted septum assembly sites. FtsZ assembly nucleates at the inner surface of the cytoplasmic membrane and polymerizes bi-directionally to encircle the cell as a Z-ring (63). When FtsZ ring completion is thwarted, the remaining soluble FtsZ in the cytoplasm can override the Min protein localization apparatus and nucleate new septation sites at the cell poles and at intervals along filaments, an explanation previously offered to account for FtsZ-GFP effects on septum assembly (26). The polar localization of FtsZ (Fig. 2, panel C) and S100B-EGFP (Fig. 2, panel A) is consistent with observations that FtsZ has an affinity for polar locations (perhaps a remnant from previous divisions) that the Min complex of proteins is responsible for preventing (28). Alternatively, S100B-EGFP may be localized at the poles after a recent division, preventing the depolymerization of some of the residual FtsZ ring.

The co-localization of S100B and S100B-EGFP to FtsZ is further supported by the observation of helical structures in a small proportion of cells (Fig. 2, panels D-I). These spirals are known to contain FtsZ because they have been observed for mutants of FtsZ (63,65), with GFP fusions to FtsZ and FtsA proteins (26,64), in E. coli over-expressing FtsZ (44), and in E. coli with altered membrane composition (75). In the current work the helical structures are visible due to the S100B-EGFP fluorescence indicating S100B is co-localized with the FtsZ-containing spirals.

Affinity chromatography showed the interaction between S100B and FtsZ is specific and calcium-dependent. This is consistent with the S100B calcium-dependent target recognition
mechanism since the interaction is inhibited by the well-characterized target peptide TRTK-12. This peptide, which has the highest affinity for S100B of any known target (76), contains a consensus motif for S100B-binding that is conserved in several mammalian cytoskeletal proteins including tubulin, GFAP, and vimentin (13). Further, S100B and S100B-EGFP both produce the filamenting phenotype and show similar co-localization patterns, indicating that the EGFP fusion does not interfere with the target binding regions in S100B located at its C-terminus (helix IV) and linker (76). Sedimentation equilibrium studies have shown the EGFP moiety neither interferes with S100B dimerization nor causes oligomerization or aggregation of S100B-EGFP (Ferguson and Shaw, unpublished results).

The role of calcium in E. coli metabolism

Human S100B binds calcium and undergoes a conformational change in response to rises in intracellular calcium from the resting level of $10^{-7}$ M to $10^{-5}$ M. In this work, the calcium–dependent binding of FtsZ to S100B is consistent with the known mode of S100B activation. In bacteria, the co-localization of S100B with FtsZ and the resulting filamentation suggests that S100B is calcium-saturated, at least periodically. Such a possibility exists since the cytoplasm of E. coli experiences transient increases in calcium concentration, particularly near the membrane (77). A calcium flux has been suggested to coordinate events of the cell cycle in a manner similar to that observed in eukaryotes (78-80). This proposal is supported by experiments showing that, in vitro, calcium enhances FtsZ polymerization (35), the formation of higher order structures (37,81,82) and alters its GTPase activity (81). In addition, the growing list of calcium-binding proteins in prokaryotes (reviewed by (83)), including several EF-hand proteins, indicates that calcium signalling in bacteria may be an important part of modulating protein activity, including cytoskeletal and septum assembly. Thus, S100B may be a useful probe to improve our understanding of calcium metabolism in bacteria.
Structural similarities between FtsZ and tubulin

In eukaryotes, tubulin has the capacity to form microtubules and interact with motor proteins. *In vitro* experiments show the polymerization of tubulin is inhibited by the calcium-signaling protein S100B, a protein that is restricted to vertebrates (1). The polymerization of FtsZ and its molecular interactions with septal proteins is less well characterized.

The evolution of tubulin from FtsZ is strongly supported by their structural similarity (Figure 7) and conservation of functional sites. Both proteins have the GTP binding site (GGGTGTG in *E. coli* FtsZ, GGGTGSG in human β-tubulin) located in the T4 loop between β-sheet S3 and helix H4 (33,84) and share a conserved mechanism of GTPase activity (85) that requires a second site, the T7 (synergy) loop, located between helix H7 and helix H8 in both FtsZ and tubulin. The T7 loop interacts with the bound GTP in the neighboring subunit within the protofilament and activates the neighbor's GTPase activity (86,87). Contacts within the T7 loop between FtsZ monomers are proposed to mediate assembly of the FtsZ protofilaments. A detailed comparison of the sequences of FtsZ and tubulin has revealed the totally conserved sequence GXXNXD in the T7 loop as well as residues near the N-terminus of helix H8 that would be buried at the FtsZ or tubulin protofilament interface (32). It might be expected that S100B interaction with tubulin, and by analogy FtsZ, would occur near this site and lead to an inhibition of polymerization. The current work shows that the target peptide TRTK-12 can compete with FtsZ for S100B binding in a calcium-sensitive manner. Similarly, TRTK-12 is able to compete with the inhibitory action of S100B for the assembly of microtubules and intermediate filaments (4). Together, these results suggest that FtsZ, like tubulin, may be interacting with S100B via a region containing a version of the TRTK-12 recognition motif. Since the T7 loop in both FtsZ and tubulin is proposed to mediate assembly of protofilaments it would follow that the TRTK-12 recognition sequence lies in or near this region. In tubulin, one candidate sequence (KLAVNMVP) occurs within helix H8 (13) immediately following the T7 loop. A similar sequence in FtsZ (KGAVQGIA) is found nearby at the C-terminus of helix H7 prior to the T7 loop. These observations indicate that in principle S100B could be interacting with a region near the T7 loop, inhibiting protofilament assembly and that
competition with TRTK-12 is able to relieve this effect. One influence that inhibition of FtsZ protofilament assembly might have would be to prevent completion of the mid-cell Z-ring as the first step for cell division (39, 40). Alternatively, S100B could be interacting with FtsZ on a surface necessary either for the lateral contacts between FtsZ to form the Z-ring bundle, or for subsequent addition of another septal protein.

It has been pointed out previously that there is currently no genetic test to identify thus-far undetected proteins involved in septum site selection or septum assembly (43). Using a foreign protein such as S100B to detect FtsZ has an advantage over antibodies in that it can be co-expressed with FtsZ in vivo and the levels of S100B can be tuned to adjust the impact on septum assembly. We have expressed S100B at low levels to prevent filamentation and to observe the midcell septum. GFP fusions to FtsZ and other proteins of the septum (such as FtsA) have been useful in localization studies but have some potential drawbacks such as causing mislocalization or interference in protein-protein contacts. For example, Sun and Margolin (59) found cloned FtsZ-GFP to be partially functional in vivo only when expressed at low levels, where it could form mixed polymers with endogenous FtsZ. Using S100B protein as a marker to study bacterial division may complement the use of tagged septal proteins.

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Figure Legends

Fig. 1. Filamenting phenotype of *E. coli* strain N99 caused by various expressed proteins. *Panel A*, bacteria expressing S100B after 2h induction with 1 mM IPTG exhibits a wide distribution of cell lengths. The filamentous cell is 30 times longer than a single bacterium suggesting it has missed 5 rounds of division. A pair of daughter cells completing division is indicated by the arrow. Because of the length of the bacterium at the magnification used, the image is a composite of three fields of view. Scale bar = 3µm. *Panels B-D*, Phenotypes of N99 cells expressing cloned proteins induced by 20 µM IPTG and grown for 7h under "pseudochemostat" conditions (see "Experimental Procedures"). *B*, S100B; *C*, S100B-EGFP; and *D*, sol-EGFP control.

Fig. 2. Expression of S100B causes phenotypes similar to those observed when FtsZ is under-expressed or dysfunctional. *Panel A*, false color merged image of GFP fluorescence and Hoechst 33342-stained nucleoids (blue) of S100B-EGFP induced 2h with 10 µM IPTG, shows fluorescent foci at the poles and at intervals in the filamented bacterium; *B*, DIC image of the same bacteria in panel A shows the green fluorescent foci under visible light; *C*, bacteria expressing cloned FtsZ induced 3 h with 100 µM IPTG; *D-F* fluorescent helical structures present in bacteria expressing S100B-EGFP fusion protein. In *panel D*, the helical structure in the right half of the bacterium is partially obscured by bright foci of the S100B-EGFP. *Panel F* is an interpretive drawing of the helical structure in *panel E*; *G-I*, bacteria expressing S100B and detected with anti-S100B antibodies and fluorescent secondary antibodies also show S100B co-localizes with a helical structure. Scale bars: 3 µm in panels A-D; 1µm in panels E-G; 2 µm in panel H.

Fig. 3. Low-level expression of cloned S100B (3h with 20 µM IPTG) results in mostly normal size bacteria with sharp mid-cell bands detected by anti-S100B. *Panel A*, fluorescent antibodies reveal anti-S100B localization to single mid-cell bands (filled arrows). Open head arrow points to a cell with a helical structure inside. *Inset*, the constricted waist between two bacteria in a late division stage is also stained with anti-S100B; *B*, DIC micrograph of the same field of bacteria.
Fig. 4. S100B-EGFP expression has no effect on intracellular levels of FtsZ. Samples of bacteria were collected at 30 minute intervals after induction by 1 mM IPTG. S100B-EGFP levels (closed bars) were determined by digital processing of Coomassie-stained gels and normalized to total protein levels to correct for variable cell density and cell length distributions. FtsZ levels (open bars) were determined by densitometry scanning of X-ray film exposures of western blots and similarly normalized. Bar values represent average error of duplicate measurements. Direct comparison of the relative concentrations between S100B-EGFP and FtsZ is not feasible from this figure because the proteins are measured by different methods.

Fig. 5. Test for the presence of inclusion bodies as the cause of the filamenting phenotype. Panel A, S100B-EGFP and S100B expression induced by 1 mM IPTG was tested for inclusion bodies by SDS-PAGE. Lanes are labeled P (pellet), S (soluble), T (total lysate). Asterisk indicates the S100B-EGFP band position. Panel B, western blot with anti-S100B. Arrows indicate S100B band position on the blot. The chemiluminescence image shown is an over-exposure to demonstrate that low level cross-reactivity to other protein bands in lanes S and T is detectable, whereas S100B in lane P is not detected.

Fig. 6. Western blots of purified FtsZ analyzed by S100B affinity chromatography. Panel A, 1 ml of FtsZ protein in TKC buffer (see Experimental procedures) is loaded on an S100B-affinity column pre-equilibrated with the calcium-containing TKC buffer. The column is washed with approximately 70 column volumes of TKC buffer and 2 ml fractions were collected for analysis. Lanes 1 - 3 are the first three TKC washes fractions. Lane 4 is wash fraction 32. Lanes 5 - 7 are the first three fractions after EDTA elution. Panel B, the same S100B affinity column is loaded with 2 ml of TRTK-12 peptide (2mg/ml) to presaturate the column prior to loading 1 ml of purified FtsZ. Washes and elutions are as in panel A except lane 4 represents wash fraction 36. Arrow indicates FtsZ.
Fig. 7. Ribbon drawing showing the architecture of the bacterial division protein FtsZ. Helices (H1-H10) and β-sheets (S1-S10) are labeled according to the three-dimensional structures of *M. jannaschi* FtsZ (33), αβ-tubulin dimer (85) and structural alignment between the two proteins (32). The N-terminal helix H0 from FtsZ is not shown as this region is non-existent in tubulin. Likewise, tubulin contains two C-terminal α-helices (H11, H12) not found in FtsZ and the loop connecting H1 and S2 in tubulin is much longer than in FtsZ, containing approximately 35 extra residues. The nucleotide binding domain (blue) and C-terminal domain (magenta) are shown separated by the intermediate domain (yellow) where the T7 loop involved in FtsZ and tubulin polymerization is found. In tubulin a sequence with similarity to the TRTK-12 recognition motif for S100B interaction is found near the N-terminus of H8 in tubulin and near the C-terminus of H7 in FtsZ.
Figure 1
Human S100B protein interacts with E. coli FtsZ

Figure 2

A
B
C
D
E
F
G
H
I
Human S100B protein interacts with E. coli FtsZ

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Figure 4
Human S100B protein interacts with E. coli FtsZ

Figure 5

A  
|    | S100B-EGFP | S100B |
|----|------------|-------|
| P  |            |       |
| S  |            |       |
| T  |            |       |

B  
|    | S100B |
|----|-------|
| P  |       |
| S  |       |
| T  |       |
Figure 6

A

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|

wash | elution

B

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|

wash | elution

Arrow indicates the interaction of Human S100B protein with E. coli FtsZ.
Figure 7
Human S100B protein interacts with the escherichia coli division protein FtsZ in a calcium-sensitive manner
Peter L. Ferguson and Gary S. Shaw

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