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Biochemistry

Role of the SulB (FtsZ) protein in division inhibition during the SOS response in *Escherichia coli*: FtsZ stabilizes the inhibitor SulA in maxicells

(cell division control/induced division inhibitor/inner membrane proteins)

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ABSTRACT **Induction of the SOS response in *Escherichia coli* by DNA-damaging treatments results in the synthesis of the SulA polypeptide, and this is sufficient to cause the resulting inhibition of cell division. Mutations at either *sulA* (*sfiA*) or *sulB* (*sfiB*) suppress this division inhibition. The SulB protein is identical to FtsZ, a protein required for normal division in *E. coli*. In the presence of FtsZ, the half-life of SulA synthesized in maxicells is ≈12 min. In contrast, in the absence of FtsZ or in the presence of a mutant form of FtsZ (SulB114) that prevents division inhibition *in vivo*, SulA is extremely unstable with a half-life of only 3 min. Both FtsZ and SulA are isolated with the inner membrane of *E. coli* maxicells in the presence of $MgCl_2$. We propose that the SulA inhibitor interacts directly with FtsZ *in vivo* to block the essential division function of this protein.**

Cell division in UV-irradiated *Escherichia coli* is initially inhibited and then resumes as DNA repair is completed and blocked replication forks resume elongation. Inhibition of cell division under these conditions was proposed by Witkin (1) to be an inducible function related to recovery from DNA damage. Subsequently, Huisman *et al.* (2) obtained direct evidence for an inducible inhibitor of division under control of the SOS *recA/lexA* damage-repair system. In addition, three major classes of mutant affected in this division-inhibition response have been isolated. Mutations in *lon* (3) largely prevent recovery from division inhibition, whereas *sulA* (*sfiA*) or *sulB* (*sfiB*) mutations (4, 5), which suppress the Lon^- phenotype, relieve or reduce division inhibition following DNA damage. However, careful analysis of UV- and nalidixic acid-treated (6) or thymine-starved cells (2) has revealed that division inhibition under these conditions is blocked by at least two independent systems, the "SOS" *sulA/sulB* pathway and a second pathway which Burton and Holland (6) have suggested is due to failure to terminate DNA replication. Recently, D'Ari and Huisman (7) have reported the presence of a third, *sfiC*-dependent pathway present in some *E. coli* K-12 strains. In this paper, we are concerned specifically with the *sulA/sulB* pathway.

The *sulA* gene product was identified as an 18-kDa polypeptide with a half-life of 1–2 min in UV-irradiated cells infected with λ bacteriophage carrying *sulA* (8). Moreover, Mizusawa and Gottesman (8) showed that the half-life of the SulA protein was increased to 19 min in a *lon* host. Taken together these data provided strong evidence that the SulA protein is responsible for division inhibition in response to DNA damage. This has now been confirmed by the demonstration that the synthesis of SulA independent of DNA damage is sufficient to block cell division (9, 10).

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The *sulB* gene, which maps among a cluster of division and related genes at 2 min on the *E. coli* chromosome, has been postulated by several groups to be the target for the SulA inhibitor. Moreover, in contrast to *sulA* (11), *sulB* appears to be part of the normal cell division machinery. We have recently shown (12) that *sulB114* maps to a locus identical with the *ftsZ* gene in *E. coli*. Similarly, Luktenhaus (13) has mapped other *sulB* alleles (*sulB25* and *sulB9*) to *ftsZ*. The *ftsZ* gene was shown previously to be essential for cell division in *E. coli* (14, 15). In this study, we used *sulA* and *sulB* genes cloned in the same "maxicell" host to obtain evidence for a direct interaction between the SulA and SulB polypeptides. The results obtained indicate that both polypeptides associate with the *E. coli* inner membrane and that the product of $sulB^+$ stabilizes SulA in maxicells.

MATERIALS AND METHODS

Strains. The *E. coli* strain used in this study was CSH26 (F6)(F′ $lacI^Q$) [*ara* Δ(*lac–pro*) Δ(*recA–srl*)F6 *rpsL thi*].

Cell Number. Bacterial cells were counted with a Coulter Counter model ZB1 fitted with a 30-μm orifice.

Construction of Plasmids pLG552, pLG554, and pLG558. Plasmid DNA restriction enzyme digests, ligation, and preparation were done according to Maniatis *et al.* (16). DNA restriction fragments were purified by agarose gel electrophoresis and isolated from the gel by the "freeze/squeeze" method (17).

Synthesis of Plasmid-Encoded Polypeptides in Maxicells. Plasmids of interest were transferred into the maxicell strain CSH26ΔF6, and proteins were labeled by incubation of the maxicells with [^{35}S]methionine as described (18). The labeled cells were lysed in $NaDodSO_4$ solution and analyzed by $NaDodSO_4$/PAGE followed by autoradiography (19). To determine stability of polypeptides, we incubated [^{35}S]methionine-labeled maxicells with excess (150 μg/ml) unlabeled methionine and then removed samples at intervals. After the addition of 0.25 volume of ice-cold 50% (wt/vol) trichloroacetic acid, precipitated proteins were analyzed by $NaDodSO_4$/PAGE and autoradiography.

Subcellular Fractionation of Maxicells. Maxicells (20 ml; 10^9 per ml) containing the plasmids of interest were labeled as above, and membrane samples were prepared by sonication and centrifugation (20). This maxicell-membrane fraction was mixed with unlabeled carrier membranes and then separated into inner and outer membrane fractions by the sarkosyl (*N*-dodecanoylsarcosine) method (21). Cell equivalents of each sample were analyzed by $NaDodSO_4$/PAGE and autoradiography. Polyacrylamide gels were stained for protein (with Coomassie blue) to monitor the efficiency of the membrane fractionation.

Abbreviation: kb, kilobase(s).

Quantitation of Radioactivity in Gel Bands. Autoradiographs of radioactively labeled proteins separated by NaDodSO$_4$/PAGE were scanned with an LKB 2202 Ultrascan laser densitometer, and the relative amount of radioactivity in each protein band was quantified.

RESULTS

Construction of *sulA* and *sulB* Plasmids. Both the *sulB*$^+$ and the mutant *sulB114* genes were previously inserted into recombinant plasmids by the construction of gene banks from *sulB*$^+$ and *sulB114* chromosomal DNA. The libraries were screened for recombinant plasmids capable of complementation of the nearby temperature-sensitive mutation, *ftsA*, (12). In the current study, 13.5-kilobase (kb) *Bam*HI fragments carrying *sulB*$^+$ or *sulB114* were subcloned from their original plasmids (pLG551 and pLG550) into the low copy number (6–8 per cell) vector pLG339 (18). The two resulting plasmids were named pLG554 and pLG552, respectively (Fig. 1). Both plasmids also encode resistance to kanamycin, which can be used as a convenient genetic marker and for maintenance of the plasmid.

The *sulA* gene was cloned fortuitously by Bremer *et al.* (22) and then sequenced (23) in experiments designed to clone the adjacent *ompA* gene. To obtain expression of the *sulA* gene in *E. coli* under appropriate conditions but without inducing other SOS functions (including enzymes involved in DNA repair), we inserted a 1.78-kb *Bam*HI fragment from plasmid pTU201 (22) into a position downstream of the *lac* UV5 promoter of *lac* expression vector pPM60 (24). This plasmid is derived from pAT153, which has a copy number of 50–60 per cell. In strains containing this recombinant plasmid pLG558 (Fig. 1), an F′ plasmid carrying the mutant *lacI*^{Q} gene, which produces higher levels of LacI repressor, is also required to prevent the titration of the wild-type Lac repressor by the *lac* promoter (*Plac*) on pLG558. Following these constructions, it was found that addition of isopropyl β-D-thiogalactopyranoside to a culture of a wild-type strain carrying pLG558 caused immediate cessation of a cell division. This showed that inhibition of cell division could result solely from induction of the synthesis of the SulA protein, as found by others (9, 10). Moreover, this inhibition could be suppressed by a *sulB114* mutation on the chromosome of the host strain (data not shown).

Effect of SulA on the Synthesis of SulB. The product of the *sulA* gene has been widely postulated to act through a target gene, *sulB*. The latter has now been identified as *ftsZ* (12, 13), which appears to be essential for cell division in *E. coli*. It has been assumed that *sulB* mutations act by rendering *ftsZ* or its product insensitive to the action of SulA. By constructing maxicells that harbor both *sulB*$^+$ (*ftsZ*) or *sulB114* plasmids and the *sulA* plasmid pLG558 and inducing maximum production of SulA by the presence of isopropyl β-D-thiogalactoside, we have investigated directly the effects of SulA on the synthesis of FtsZ/SulB.

Fig. 2 shows the proteins produced in maxicells containing pLG554, pLG552, pLG554 and pLG558, or pLG552 and pLG558. First, the results indicated that in the absence of SulA, SulB (FtsZ) is expressed reproducibly at a level ≈1.8 higher than that for SulB114, compared to the expression of the EnvA (or SecA) polypeptides as an internal reference standard. However, the presence of SulA increased the relative abundance of both SulB$^+$ and SulB114, compared to EnvA, by factors of 2.5 and 1.8, respectively, as determined by densitometric scanning of autoradiographs. Second, the abundance of the SulA protein was found reproducibly to be considerably higher in the presence of SulB$^+$ compared to SulB114.

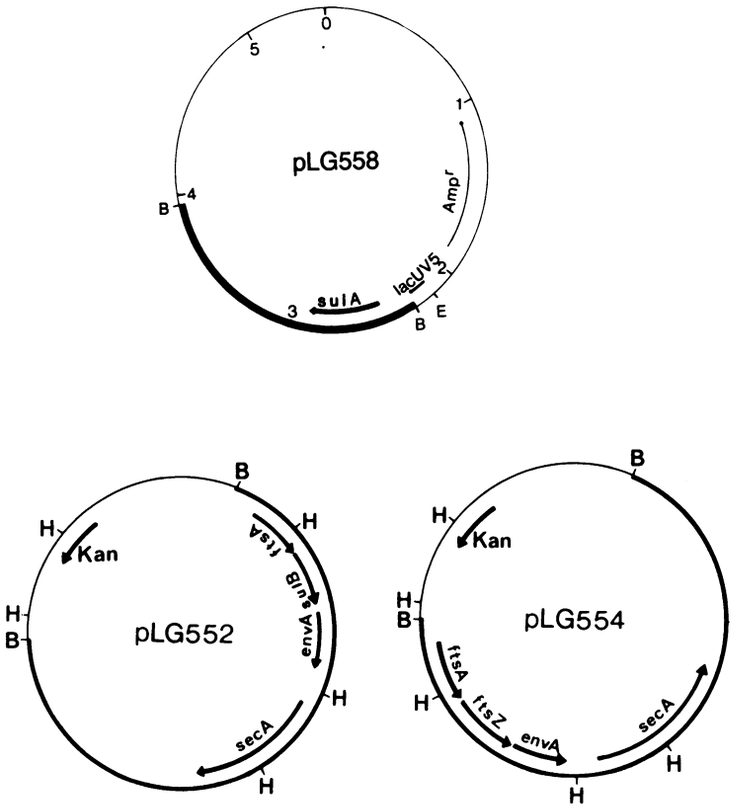

FIG. 1. Composition of major plasmids used in this study: pLG558 (*Plac sulA*$^+$), pLG552 (*sulB*114), and pLG554 (*sulB*$^+$/*ftsZ*$^+$). Arrows show direction of transcription. Amp^r and Kan, ampicillin-resistance and kanamycin-resistance genes, respectively. Restriction enzyme cleavage sites: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. Scale numbers 0–5 inside the pLG558 schematic represent kb.

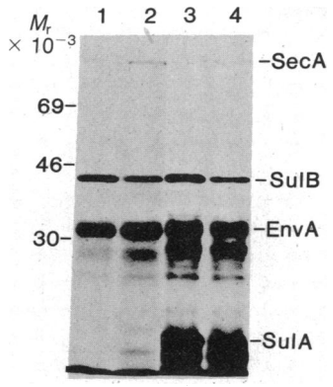

FIG. 2. Effects of SulA on the expression of SulB in maxicells. The maxicell strain CSH26ΔF6 containing different plasmids was labeled with [^{35}S]methionine for 30 min at 37°C. After irradiation and overnight incubation as described (18). Samples were analyzed by NaDodSO$_4$/11% PAGE and autoradiography (19). Lanes: 1, pLG554 (*sulB*$^+$/*ftsZ*$^+$); 2, pLG552 (*sulB*114); 3, pLG554 plus pLG558 (*sulA*); 4, pLG552 plus pLG558. The positions of the major polypeptides are indicated.

These results seem to exclude the possibility that SulA acts to block either the transcription or the translation of *sulB*$^+$ (*ftsZ*). In contrast, the presence of SulA seems to stimulate the relative rate of synthesis of SulB under these conditions. Alternatively, we considered the possibility that SulA might be affecting the stability of SulB.

Stability of SulA, SulB$^+$ and SulB114 in Maxicells. To test the possible effects of SulA on SulB stability, maxicells containing pLG558 alone, pLG558 and pLG554, or pLG558 and pLG552 were pulse-labeled with [^{35}S]methionine for 2 min and then incubated with unlabeled methionine. Samples were removed at intervals and analyzed by NaDodSO$_4$/PAGE (Fig. 3 *a* and *b*). SulB$^+$, SulB114, and EnvA proteins were all stable for the duration of the chase (30 min) and therefore differences in the ratio between SulB$^+$ and EnvA and between SulB114 and EnvA must be due to differences in rates of synthesis.

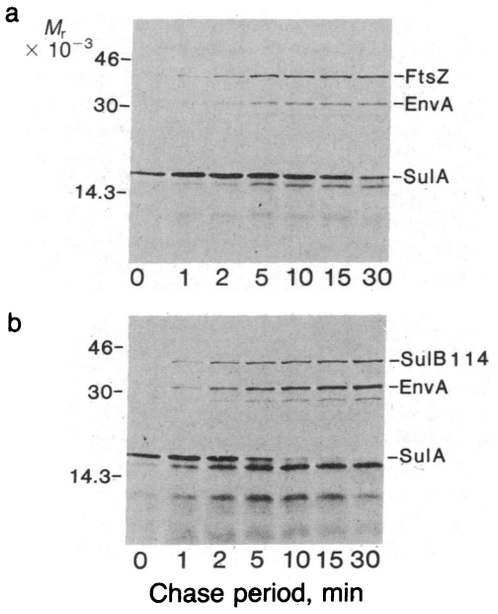

FIG. 3. Stability of SulA in the presence of FtsZ (*a*) or SulB114 (*b*) in maxicells. Maxicells were pulse-labeled with [^{35}S]methionine for 2 min at 37°C and then incubated with an excess of unlabeled methionine during the chase period. Samples were analyzed by NaDodSO$_4$/15% PAGE and autoradiography. (*a*) Maxicells with pLG554 and pLG558. (*b*) Maxicells with pLG552 and pLG558.

SulA Is Stabilized in the Presence of SulB$^+$. The results in Fig. 4 show that the SulA protein is extremely unstable, as previously shown using UV-irradiated bacteria infected with a λ *sulA* phage (8). The half-life of SulA in maxicells was calculated to be ≈3 min from the data shown in Fig. 4. Surprisingly, however, the presence of the *sulB*$^+$ plasmid pLG554 resulted in a significant increase in the stability of SulA synthesized in maxicells (Fig. 3 *a* and *b*). SulA under these conditions had a half-life of 10–14 min (Fig. 5), and this was confirmed in several experiments. This result indicated that the relative abundance of SulA observed in the presence of SulB$^+$ (Fig. 2) was due to increased stability of SulA rather than its increased rate of synthesis. Moreover, the apparent increase in SulB synthesis in the presence of SulA might be explained in terms of autoregulation by free SulB as reported recently by Donachie *et al.* (25).

The results in Fig. 3*b*, furthermore, showed that in the presence of the mutant *sulB114* allele, the increased stability of SulA was not observed (the half-life of SulA staying at ≈3 min). We conclude that the SulB and SulA proteins must interact directly to result in increased SulA stability. SulA either fails to bind to the mutant form of SulB or the interaction, if it takes place, fails to protect SulA from proteolytic digestion.

Under conditions of maximum SulA instability, we always observed additional bands at lower molecular weight (17,000; 12,000; and possibly 7000) that represented polypeptides not encoded by the vector alone and not detected when SulA was synthesized *in vitro*, where it appears quite stable (unpublished data). Moreover, the M_r 17,000 polypeptide in particular increased in intensity during the chase period in a manner almost reciprocal with the disappearance of the SulA polypeptide. These properties indicate that this polypeptide is the primary degradation product of SulA as a result of proteolytic cleavage. Such degradation products were not observed previously in experiments involving infection of UV-irradiated cells with λ *sulA*, indicating that levels of the relevant protease may be fortuitously reduced in maxicells. Unfortunately, we were not able to construct *lon*$^-$ maxicell derivatives carrying the *sulA* plasmid, presumably because the levels of SulA were too high (despite the presence of *sulB*114) under these conditions, and so were unable to test the effect of *lon*$^-$ on the breakdown pattern.

Cellular Location of SulA and SulB$^+$. To obtain additional evidence for direct protein–protein interaction between SulA and SulB, the cellular location of the two proteins was investigated. Detailed studies in this laboratory have shown that several inner-membrane, periplasmic, and outer-mem-

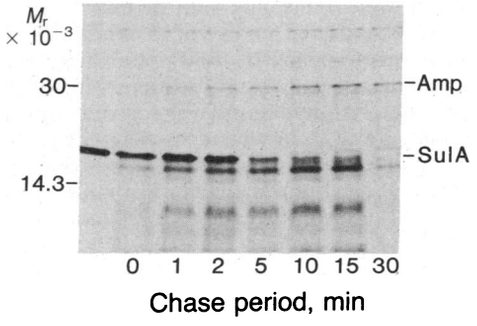

FIG. 4. Stability of SulA in maxi-cells. A pulse–chase experiment was carried out as described for Fig. 3, with maxi cells containing pLG558 (*Plac-sulA*$^+$). Lane at left shows the *in vitro* translation product of the 1.78-kb *Bam*HI restriction fragment from pLG558 encoding SulA.

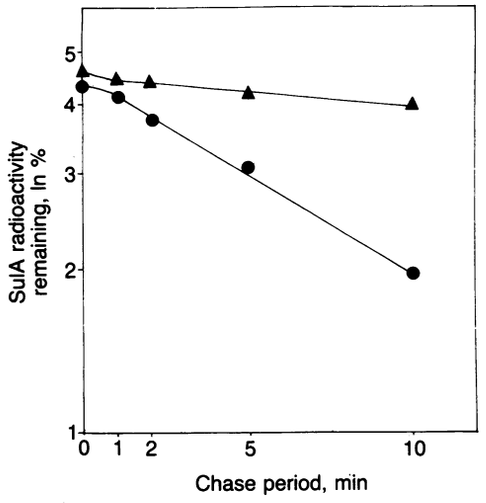

FIG. 5. Decay curves for the SulA polypeptide in maxicells. The SulA radioactivity present in the samples shown in Fig. 3 was determined by densitometric scanning. Data are presented as the natural logarithm of the percentage total protein radioactivity found in the SulA band. SulA in the presence of FtsZ$^+$ ▲, (see Fig. 3*a*); ●, SulA in the presence of SulB114 (see Fig. 3*b*). The stability of SulA calculated from the data in Fig. 4 (not shown) was identical to that for SulA in the presence of SulB114.

brane proteins are recovered in the corresponding fractions when maxicells are fractionated (unpublished data). Maxicells containing pLG554 alone or pLG554 plus pLG558 were labeled with [^{35}S]methionine and separated into cytoplasmic, inner membrane, and outer membrane fractions. This fractionation was performed either in the absence or in the presence of 10 mM Mg^{2+}. Mg^{2+} stabilizes the specific binding of many extrinsic proteins [for example the ATP synthetase complex (26)] to the cytoplasmic membrane. Consequently, by performing cellular fractionation in the presence or absence of 10 mM Mg^{2+}, it is possible to distinguish between integral membrane proteins and a particular class of membrane-binding proteins. The results presented in Fig. 6 *a* and *b* show that both SulB$^+$ (FtsZ) and SulA displayed Mg^{2+}-dependent binding to the inner membrane, whereas only trace amounts of either protein were detected in the outer membrane. The SecA protein, which is encoded by plasmid pLG554, also was recovered with the inner membrane in the presence of Mg^{2+}, as previously reported (27). In fact, substantial amounts of SulA could be recovered with the inner membrane even in the absence of Mg^{2+}, suggesting that this protein has a greater affinity for the membrane. As can be seen in Fig. 6, some SulA, SulB, and SecA still fractionated with the soluble or cytoplasmic fraction even in the presence of Mg^{2+}. This may indicate that the membrane binding sites are saturated under these conditions but also may reflect the inefficiency of the fractionation procedure following breakage of cells by sonication. Membrane fractionation in these experiments was carried out by the sarkosyl procedure (21), in which the detergent solubilizes the inner membrane polypeptides in isolated envelopes whilst the outer membrane proteins remain insoluble. It was not possible to confirm the inner membrane location of SulA and SulB by the Osborn procedure (28) because the essential first step, the formation of osmotically stable spheroplasts, could not be achieved with the maxicells used in this study (unpublished data).

In the previous section, we presented evidence for a specific interaction between SulA and SulB. We therefore looked for an effect of the presence of SulA upon the affinity of SulB for the inner membrane in fractionated maxicells (Fig. 6), but no effect was observed.

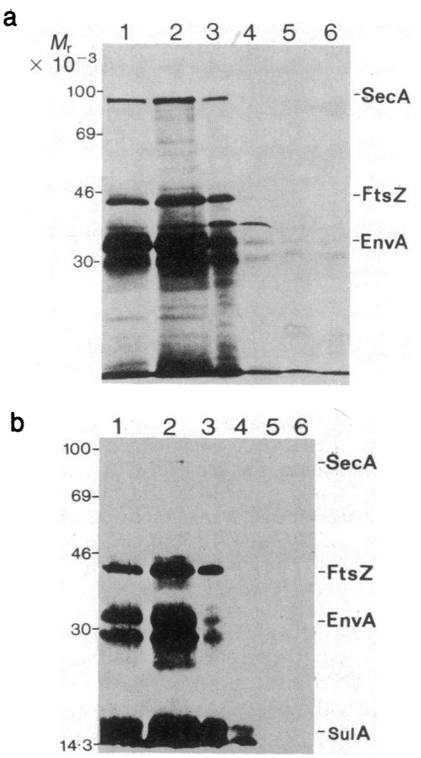

FIG. 6. Fractionation of maxicells: location of SulA and SulB. Maxicells containing appropriate plasmids were labeled with [^{35}S]methionine and then were fractionated into cytoplasmic and envelope fractions in either the presence or the absence of 10 mM $MgCl_2$. Envelopes were separated into inner and outer membrane proteins by use of sarkosyl (21). Samples, which contained identical cell equivalents,, were analyzed by $NaDodSO_4$/11% PAGE and autoradiography. (*a*) pLG554 (*ftsZ*$^+$). (*b*) pLG554 plus pLG558 (*sulA*$^+$). Lanes: 1, cytoplasm (+Mg^{2+}); 2, cytoplasm; 3, inner membranes (+Mg^{2+}); 4, inner membranes; 5, outer membranes (+Mg^{2+}); 6, outer membranes.

DISCUSSION

Recent studies (9, 10) have shown that the product of the *sulA* gene is sufficient to arrest division even in the absence of DNA-damaging treatments. A similar result was obtained in this study by placing *sulA* under the control of the *lac* promoter and thereby rendering it inducible by isopropyl β-D-thiogalactoside. In addition, Huisman *et al.* (9) provided genetic evidence for an interaction between SulA and its putative target SulB. In this study we primarily sought to demonstrate some direct effects of SulA upon SulB or its genetic determinant by using maxicells as the test system. Since *sulB* is identical to *ftsZ* (12, 13), a gene essential for normal division in *E. coli*, decrease of the activity of SulB or its cellular concentration promoted by SulA should be sufficient to block division.

The synthesis of SulA in maxicells had no effect on the stability or the properties of FtsZ as determined by $NaDodSO_4$/PAGE analysis. Moreover, SulA did not inhibit the synthesis of FtsZ and did not affect the cofractionation of FtsZ with the inner membrane in the presence of $MgCl_2$. In this latter respect, FtsZ displays the same properties as polypeptides like ATP synthetase and SecA (26, 27), which bind tightly to the inner membrane in the presence of $MgCl_2$. The half-life of SulA in maxicells is 2–3 min compared to 1–2 min reported by Mizusawa and Gottesman (8) in a UV-irradiated host infected with λ carrying *sulA*. Remarkably, in the presence of FtsZ in maxicells, the stability of SulA was increased 3- to 4-fold. This effect was not observed when an

otherwise identical plasmid carrying the *sulB114* allele, which suppresses SulA-promoted inhibition *in vivo*, was present. This result demonstrated that stabilization of SulA was specifically due to FtsZ and not to other polypeptides encoded by pLG554.

Since FtsZ is apparently essential for division in *E. coli*, it seems quite unlikely that the observed effect of FtsZ on SulA stability is simply due, for example, to inhibition of the protease activity of the *lon* gene product (see ref. 8) by nonspecific binding to FtsZ. Indeed, *sulB* mutations, although suppressing the inhibition of division promoted by *lon* mutations after DNA damage, do not suppress other pleiotropic effects associated with *lon* strains, such as mucoidy. It seems more reasonable to conclude from our data and the genetic evidence indicated above (9) that the protective effect of FtsZ represents a specific molecular interaction between FtsZ and SulA. This interaction presumably would prevent access to the proteolytic cleavage sites of SulA. We propose that such an interaction also occurs *in vivo* and simultaneously inactivates the division function of FtsZ. The ability of mutant forms like *sulB114* to suppress the effect of SulA *in vivo* could therefore be ascribed to a failure of the mutant FtsZ to bind SulA or an incorrect binding that fails to affect the division function of the FtsZ polypeptide. The precise stoichiometry of the above reactions may, however, be different *in vivo* than in maxicells. The relative concentrations of SulA and FtsZ in maxicells are determined in part by the copy number of the corresponding vectors, promoter strengths under these quasi-*in vivo* conditions, and (above all) the relative stabilities of the two polypeptides as they accumulate during the several hours preincubation before labeling. Despite attempts to ensure an excess of SulA, as expected to be the case *in vivo*, by inserting *sulA* in a high copy number plasmid (copy number 40 compared to 6 for *ftsZ*), the greater stability of FtsZ may in fact lead to the accumulation of an excess of this polypeptide. Consequently, the number of SulA molecules in stable complexes with FtsZ might be unusually high and therefore lead to an overestimation of the average half-life compared to that *in vivo*.

We repeatedly recovered FtsZ with the inner membrane when maxicells were fractionated in the presence of $MgCl_2$; this is consistent with preliminary observations that FtsZ remains with the cytoplasmic fraction when cells are osmotically shocked in order to release the periplasmic fraction (unpublished data). SulA similarly was recovered almost exclusively in the inner membrane (sarkosyl-soluble envelope fraction) when envelopes were prepared in the presence of $MgCl_2$. In the absence of $MgCl_2$, however, substantial amounts of SulA protein were located in the soluble fraction. All localization experiments with SulA were carried out in the presence of SulB because in the absence of SulB, SulA is too unstable to permit satisfactory cell-fractionation studies. Consequently, we cannot rule out the possibility that SulA associates with the inner membrane as a result of its binding to SulB. In contrast, Schoemaker *et al.* (10) have reported that SulA associates with the outer membrane in equilibrium sucrose gradients. This result was obtained with cells that greatly overproduced SulA, and the possibility was not ruled out that insoluble aggregates of SulA coincidently sedimented with the outer membrane. The presence of SulA in the soluble fraction observed in this study in the absence of $MgCl_2$ and the absence of a signal-like sequence in the DNA sequence of *sulA* (23) indicates to us that SulA is not in fact located in the outer membrane and that SulA associates with the cytoplasmic face of the inner membrane *in vivo*. Under these conditions, it would therefore be able to form a complex with FtsZ sufficient to promote division inhibition promptly and reversibly during the SOS response. As we have argued elsewhere (29), this finding is consistent with a pivotal role for FtsZ in the normal cell cycle.

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