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Cellular Localization of the *Escherichia coli* SpoT Protein

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The SpoT protein of *Escherichia coli* serves as a source of degradation as well as an apparent source of synthesis of (p)ppGpp. Since the subcellular localization of SpoT might be a clue to its function, we have used SpoT-specific antisera to analyze cell extracts fractionated on sucrose gradients. We find that the SpoT protein is not bound to ribosomes or to either inner or outer membrane fractions. Although the SpoT protein is found in large aggregates, its localization is probably cytosolic.

The (p)ppGpp nucleotides are involved in the regulation of gene expression in prokaryotes (2, 5). Intracellular levels of (p)ppGpp rise under a number of conditions, including starvation for amino acids as well as for sources of energy, nitrogen, and phosphate (2, 13, 14, 16). The function of (p)ppGpp is to serve as an intracellular signal for nutritional deficiency since gene expression is affected in response to (p)ppGpp accumulation but (p)ppGpp is not essential for cell growth (31). The synthesis and degradation of (p)ppGpp are mediated largely by two proteins, RelA and SpoT. The RelA protein is functionally ribosome associated (2). The intracellular location of SpoT is uncertain. The goal of the work reported here is to clarify conflicting reports of the intracellular localization of SpoT as an approach toward understanding the regulation of this protein.

Amino acid deficiency is detected by the ribosomally bound RelA protein, which monitors the ratio of charged to uncharged tRNA available for binding to acceptor sites of actively translating ribosomes. Codon-specified uncharged tRNA binding to ribosomes activates the (p)ppGpp synthetic activity of RelA (6, 9, 10, 21).

In addition to the RelA protein, a second (p)ppGpp synthetase exists. A *relA*-deleted strain is no longer capable of synthesizing (p)ppGpp in response to amino acid starvation but does accumulate these nucleotides during glucose limitation (17). The genetic source of the second (p)ppGpp synthetase has been deduced to be the *spoT* gene because simultaneous deletions of both the *relA* and *spoT* genes give strains in which (p)ppGpp is undetectable (31). The conclusion that SpoT shares with RelA an ability to synthesize (p)ppGpp is supported by the observation that RelA and SpoT are homologous proteins (18).

The discovery that SpoT is a likely ppGpp synthetase was surprising given that it was identified initially as being required for the main pathway of (p)ppGpp breakdown (15). The SpoT ppGppase activity was shown to consist of a manganese-dependent (p)ppGpp 3′-pyrophosphohydrolase in vitro (1, 11, 12, 28). Carbon source starvation was also shown to result in inhibition of SpoT-mediated (p)ppGppase and thereby account for (p)ppGpp accumulation under these conditions (16). In addition, (p)ppGppase activity is inhibited in vivo by osmotic shock (8), fatty acid synthesis inhibition (26), oxidative phosphorylation uncouplers (30), or long-chain alcohols (19). These effects can be dissociated from the effects of ATP levels (30), leading to the suggestion that the (p)ppGppase activity of SpoT is sensitive to membrane integrity in a manner that might involve membrane association of SpoT (29, 30).

The dual ability of SpoT to mediate both (p)ppGpp synthesis and (p)ppGpp degradation is not shared by the RelA protein; the latter does not catalyze (p)ppGpp degradation under physiological conditions (27). Nevertheless, additional parallels between RelA synthetic and SpoT degradative activity come from reports of uncharged tRNA effects on each of these reactions and the putative association of SpoT with ribosomes. The activity of SpoT-mediated (p)ppGppase is inhibited by uncharged tRNA both in permeable cells (23) and in vitro (1, 4, 24), while RelA synthetic activity is stimulated by uncharged tRNA. The association of SpoT with ribosomes was inferred from its tendency to sediment with crude ribosomes (see below).

Previous attempts to localize SpoT relied on enzyme assays of various fractions. Such an approach is difficult given the low abundance of the SpoT enzyme and its tendency to aggregate (25). An attempt to circumvent this problem by a 10-fold overexpression of SpoT with a multicopy *spoT* plasmid gave easily assayable activities and led to the conclusion that SpoT is cytosolic (1). This conclusion is uncertain given that the overexpression may have obscured its true localization and that the fractionation procedure involved conditions that lead to the disruption of ribosomal subunits and associated proteins. To circumvent the various problems involved in detecting SpoT enzyme activity, we have taken the approach of using antibodies raised against SpoT to identify SpoT in fractionated extracts of wild-type strains.

Antibodies to SpoT were raised in rabbits by use of SpoT purified from an overproducing strain (4) and affinity purified on a SpoT-Sepharose column. Before use, the affinity-purified antibodies were preabsorbed with extracts from a Δ*spoT* strain to reduce residual background reactivities. As shown in Fig. 1, the antibodies are specific to SpoT and do not recognize the homologous RelA protein. Also shown in Fig. 1 is an extract of a strain in which the chromosomal copy of *spoT* has been deleted. This was included to show that, in addition, the antibody does not react with RelA or other peptides that may migrate with SpoT. To provide a ppGppase activity that is required in *relA*$^+$ backgrounds, a plasmid encoding only the amino terminus of SpoT, which retains ppGppase activity, is present in this strain. The mutant SpoT is detected by the antibody and is labeled SpoT*. Also seen migrating faster than SpoT* is an apparent degradation product of SpoT*.

Previous reports suggested that SpoT is ribosome associated mainly because of its location in high-speed pellets (100,000 ×

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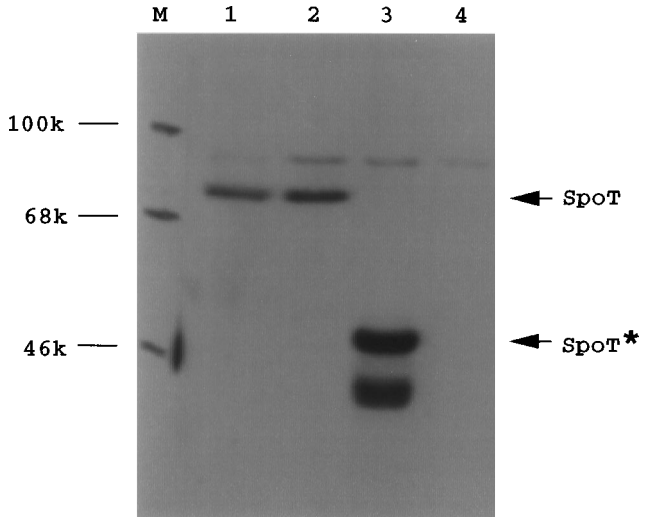

FIG. 1. Specificity of anti-SpoT antibodies as shown by Western blot of whole-cell extracts of various *E. coli* strains. Lanes: M, markers (prestained high molecular weight [Bethesda Research Laboratories]); 1, strain MG1655 (wild type); 2, strain CF1651 (Δ*relA*); 3, strain GN238 (Δ*spoT* with a plasmid encoding the amino terminus of the SpoT protein, indicated here as SpoT*, which retains ppGpp degradase activity [the band migrating below SpoT* is an apparent degradation product of the mutant peptide]); 4, strain CF1693 (Δ*relA* Δ*spoT*). The minor band migrating above SpoT is not RelA or SpoT specific given that it is present in the Δ*relA* Δ*spoT* strain. Molecular weight markers (k, thousand) are shown on the left.

g) of *Escherichia coli* lysates which are enriched for ribosomes (11, 28). Using conditions indicated in previous reports, we confirmed by Western blotting (immunoblotting) that it is present in such pellets and that it could be extracted with high salt concentrations (data not shown). This, in our view, does not indicate with certainty that SpoT binds ribosomes, given that other proteins that do not associate with ribosomes, such as RNA polymerase, are also found in the pellet. To test more rigorously for ribosome association, we fractionated extracts on sucrose gradients. Extracts were prepared by resuspending 1.0 g of frozen *E. coli* paste (University of Alabama at Birmingham Biotechnology Center) in 3.0 ml of ribosome buffer (10 mM Tris-acetate, 60 mM KCl, 14 mM Mg acetate, 1.0 mM dithiothreitol) containing DNase (10 U), RNasin (12.5 U), and phenylmethylsulfonyl fluoride (150 μg/ml) and then subjecting the suspension to sonication. After clarification by centrifugation for 30 min in a microcentrifuge at 4°C, a sample of the supernatant (0.5 ml) was layered onto the top of a 12-ml 5 to 30% sucrose gradient made in ribosome buffer and centrifuged for 2.5 h at 39,000 rpm in a Beckman SW41 rotor. Fractions were collected from the bottom and assayed for SpoT by Western blotting. The location of ribosomes was determined by the A_{260} of fractions diluted 1/10. To fractionate dissociated ribosomal subunits, the extracts were made in the absence of added Mg^{2+}. As a control, fractions were also probed with anti-RelA antibody. As can be seen in Fig. 2, while RelA sediments with ribosomes, most SpoT does not. In the presence of Mg^{2+}, RelA seems to bind preferentially to the 50S subunit, as found by others (22), and so sediment on the shoulder of the 70S peak. As also found by others (22), RelA is readily lost from ribosomes during centrifugation as indicated by its presence at intermediate densities. The sedimentation of SpoT in the sucrose gradients is, like that of RelA, fairly broadly distributed and this is exacerbated by the presence of Mg^{2+}. While it may be argued that because of this sedimentation pattern, SpoT binds to ribosomes but is released from them at a much higher

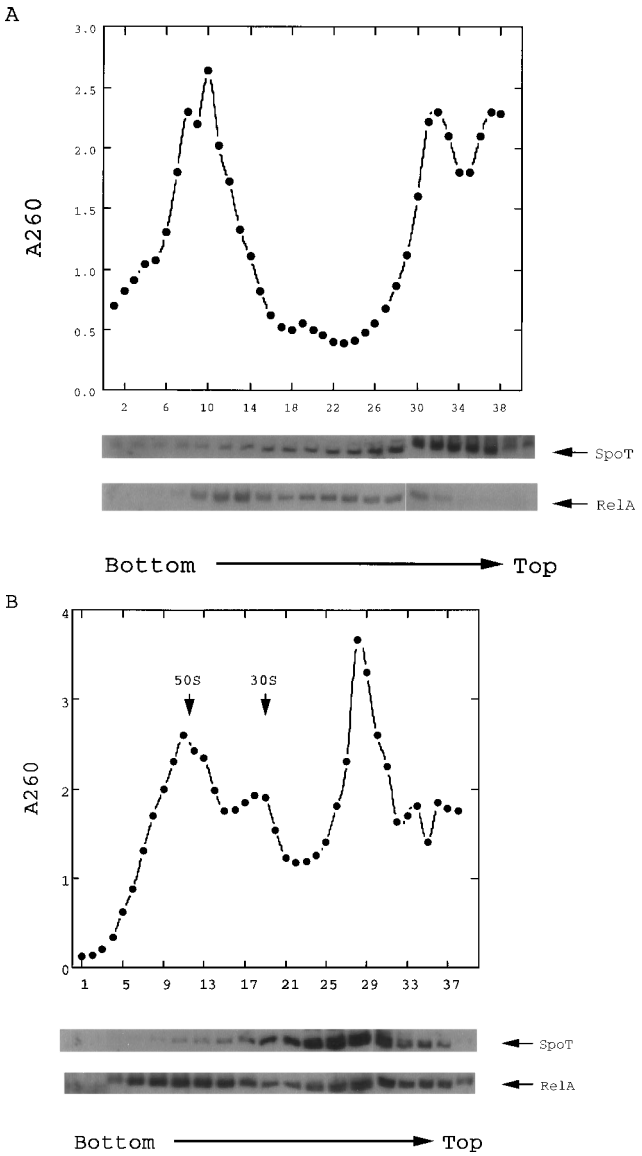

FIG. 2. SpoT does not sediment with ribosomes. Fractions from sucrose gradients (5 to 30%) with (A) or without (B) Mg^{2+} were assayed for SpoT and RelA by Western blotting. Fraction numbers are depicted on the *x* axis. Below the *x* axis are strips from relevant regions of Western blots probed with either anti-SpoT or anti-RelA. Analysis of the material in the peak centered around fraction 12 in panel A by SDS-PAGE indicates that it consists of partially overlapping peaks of 70S and 50S ribosomal subunits (data not shown).

rate than RelA, the simpler interpretation is that the somewhat broad distribution of SpoT in the sucrose gradients is due to aggregation. Purified SpoT exhibits a strong tendency to aggregate (4, 25), and in our hands, this tendency is increased in the presence of Mg^{2+} (4).

In addition to detecting SpoT in high-speed pellets, we also found that the low-speed pellet (30,000 × *g*) of crude extracts also contained substantial amounts of the SpoT protein. Low-speed pellets consist primarily of crude membranes, and the localization of SpoT to this fraction could indicate membrane association, as suggested previously (29, 30). To determine if SpoT is membrane associated, we fractionated crude membranes as described by Osborn and Munson (20). Briefly, crude membranes were isolated from protoplasts of freshly grown

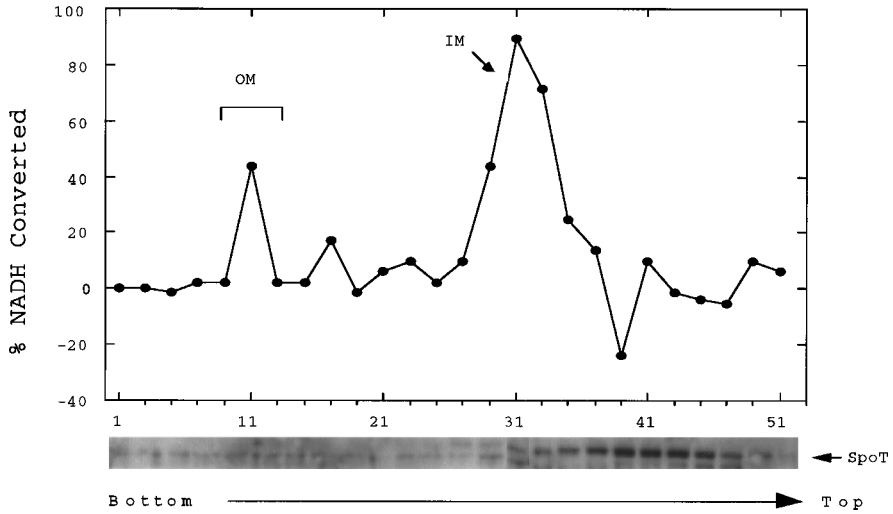

FIG. 3. SpoT is not associated with membranes. Membranes from strain MG1655 (wild type) were fractionated on sucrose step gradients as described in the text. Inner membranes (IM) were located by the presence of NADH oxidase activity (fractions 29 to 37). Outer membranes (OM) were localized by Coomassie blue staining of SDS-polyacrylamide gels (fractions 9 to 13 [data not shown]). The presence of SpoT was determined by Western blotting. Below the plot of NADH activity versus fraction number is the portion of the blot corresponding to the position of SpoT migration.

cells by lysis in a cell disruption bomb followed by pelleting. Inner and outer membranes were separated on sucrose step gradients, and fractions were collected from the bottom. Fractions containing inner membranes were determined by assaying for NADH oxidase (7). Fractions containing outer membranes were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to localize the position of porin proteins. SpoT was localized by Western blot analysis. As can be seen in Fig. 3, most SpoT does not sediment with inner or outer membranes. Because glucose starvation may induce SpoT to associate with membranes, thereby inactivating it, we tested whether SpoT became associated with membranes following glucose starvation. We found that glucose starvation by the addition of *methyl*-α-D-glucopyranoside in a defined medium, under conditions that lead to increased ppGpp levels, failed to induce membrane association by SpoT (data not shown). While association with inner membranes is not apparent, SpoT does sediment close to the position of inner membranes. Analysis of the SpoT-containing fractions by SDS-PAGE and agarose gel electrophoresis indicates the presence of substantial amounts of nucleic acids and ribosomal proteins (data not shown). We interpret this to mean that the SpoT-containing fractions are enriched for protein and protein-nucleic acid aggregates. Nonspecific association of SpoT with nucleic acids is not unexpected, given its predicted pI of 9.1. Indeed, the major difficulty in purifying SpoT is its insidious tendency to aggregate and its tight association with nucleic acid (4). It is likely that these properties led previous investigators to conclude that SpoT is ribosome associated because such aggregates may cosediment with ribosomal subunits. While we cannot discount transient association with ribosomes or membranes, our data indicate that SpoT does not interact strongly with either structure.

The implication of this finding is that SpoT probably does not directly monitor tRNA charging like RelA does nor is it likely to directly monitor energy availability by associating with membranes. This implies that SpoT monitors energy availability through an intermediate or that a soluble signal exists for carbon or energy deficiency. One attractive possibility, suggested by Seyfzadeh et al. (26), is that SpoT is regulated not by energy availability but by fatty acid synthesis. Further work is in progress to address this issue.

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