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Secretory Leukocyte Protease Inhibitor Binding to mRNA and DNA as a Possible Cause of Toxicity to *Escherichia coli*

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The expression of the positively charged human protein secretory leukocyte protease inhibitor (SLPI) in *Escherichia coli* causes severe cellular toxicity. After induction of SLPI synthesis in a high-level-expression strain, SGE61, the growth of the strain is arrested and total protein and RNA synthesis rates decline by 60 to 70%. The mechanism of SLPI-mediated inhibition of macromolecular synthesis was examined in cell-free transcription-translation systems. SLPI proved to be a potent inhibitor of translation in vitro. When SLPI was added to translation reactions at SLPI/mRNA ratios attained during maximal SLPI accumulation in SGE61, translation of a test mRNA was inhibited by 75%. The mechanism of translation inhibition was deduced from in vitro experiments showing that SLPI bound to mRNA and interfered with the interaction of RNA-metabolizing enzymes, such as RNase. In addition, SLPI bound to DNA in vitro, but transcription was not inhibited as strongly in cell-free reactions as it was in SGE61. Similar nucleic acid-binding and translation inhibition properties were displayed in vitro by another basic protein, chicken egg white lysozyme, but were not displayed by the relatively acidic protein bovine serum albumin. On the basis of these results, we concluded that SLPI binds to nucleic acids via charge interactions and inhibits translation by competing with ribosomes for binding to mRNA. Since SLPI-mRNA and SLPI-DNA binding occurred at SLPI/mRNA and SLPI/DNA ratios existing in SGE61, nucleic acid binding may contribute to the toxicity of SLPI to *E. coli*. These results indicate that, in general, high-level expression of basic recombinant proteins in *E. coli* may be problematic.

A number of recombinant proteins, such as human interferons (11, 19), rat insulin (2), and bacterio-opsin (5), are toxic to *Escherichia coli* when expressed in high amounts. Shortly after the induction of their synthesis, cell growth rates decline, and cultures stop growing before reaching normal stationary phase. As a consequence of growth inhibition, the accumulation of product is suboptimal.

Presumably, there are many mechanisms by which the expression of recombinant proteins in *E. coli* can interfere with essential metabolic processes and growth. For the secreted forms of human interferon (11) and insulin (2), toxicity is caused by the binding of the proteins to the inner membrane and disruption of protein export (6). Intracellularly produced recombinant proteins, such as methionylinsulin (2), can also be toxic to *E. coli*, but in this case the mechanism(s) leading to toxicity is unknown.

We have encountered toxicity problems when the human protein secretory leukocyte protease inhibitor (SLPI) (16, 17) is expressed in *E. coli*. SLPI (M_r = 11,726) is a highly basic protein that is composed of two partially homologous domains that are internally cross-linked via sulfhydryl bonds (8, 17). SLPI is a potent inhibitor of serine proteases and as an inhibitor of human leukocyte elastase may be of therapeutic value in the treatment of emphysema (17).

As reported herein, intracellular expression of SLPI causes severe reduction of the growth rate and rates of protein and RNA synthesis of an *E. coli* expression strain. The mechanism by which SLPI disrupts macromolecular synthesis was examined by using cell-free protein synthesis systems and nucleic acid-binding assays. The data show that SLPI binds to both DNA and mRNA in vitro and inhibits translation by interfering with ribosome-mRNA interaction. Since experiments were conducted under conditions closely resembling those in *E. coli*, we propose that SLPI-nucleic acid interactions also occur in vivo and contribute to the toxic effects of SLPI on *E. coli*.

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MATERIALS AND METHODS

Strains. The SLPI expression strain SGE61 is strain JM109 (20) that has been transformed with an SLPI expression vector, pCJX-1-mSLPI-3. The construction of pCJX-1-mSLPI-3 will be described in detail elsewhere. In summary, the vector is derived from the *tac* promoter expression vector pKK223-3 (Pharmacia, Inc.) and contains a synthetic SLPI structural gene cloned into the polylinker site located downstream of the *tac* promoter. The synthesis of methionyl-SLPI can be induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and is translationally coupled to a peptide coding for the proximal half of the *ompA* signal sequence. The complete *tet* gene sequence was restored to the vector for selection with tetracycline. In some experiments, we used JM109 transformed with a vector (pCJX-1) that lacks the SLPI structural gene but is otherwise identical to the SLPI expression vector.

Measurement of protein and RNA synthesis rates. One-hundred-microliter samples of cultures (1.4×10^8 cells per ml) growing in M9 medium plus 12.5 μg of tetracycline per ml were transferred to prewarmed culture tubes containing 10 μl of a mixture of L-[^{35}S]methionine (3.2 Ci/mmol; 0.12 mM) and [^{3}H]uridine (17.5 Ci/mmol; 0.053 mM) and were aerated at 37°C for 1 min. One milliliter of 10% trichloroacetic acid (TCA) was added, and the TCA-precipitated culture was immediately divided into two parts which were chilled on ice for 20 to 30 min. One sample was filtered directly using Whatman GF/C filters, and radioactivity bound to the filter was measured by scintillation counting (cold TCA-precipitable radioactivity was ^{32}P-labeled mRNA). The other sample was placed in a boiling water bath for 8 to 10 min and was then returned to ice for 5 min before being filtered (hot TCA-precipitable radioactivity was ^{35}S-labeled protein). The

incorporation of radioactivity into precipitated protein and mRNA was determined by double-label liquid scintillation counting.

Preparation of SLPI mRNA in vitro. The SLPI mRNA used in translation, mRNA recovery, and protein-binding experiments was synthesized in vitro from a T7 RNA polymerase promoter-driven SLPI expression vector, pT6-SLPI, by using the Riboprobe T7 RNA polymerase transcription system (Promega Biotec). pT6-SLPI is derived from a T7 RNA polymerase promoter vector, pET-3b (13), by cloning the SLPI structural gene downstream of the T7 promoter. pT6-SLPI was linearized by digestion at a *PvuI* restriction site located downstream of the SLPI gene for preparation of runoff transcripts of 836 nucleotides. [α-^{32}P]CTP (specific activity, >400 Ci/mmol; Amersham Corp.) was added to some reactions for synthesis of low-specific-activity (2.2×10^5 dpm/μg) ^{32}P-labeled mRNA used in mRNA recovery experiments and protein-binding assays.

Transcription-translation assays. Experiments were performed with procaryotic DNA-directed translation kits (Amersham) by the method of Zubay (21). Reactions (18 μl) contained 3 μl of S30 cell extract, 4.5 μl of protein synthesis supplements (e.g., tRNA, energy regeneration system, etc.), 1.8 μl of amino acids minus methionine, and either 1.2 μl of ~10.5-μCi/μl L-[^{35}S]methionine (specific activity, ~1,150 Ci/mmol; Dupont, NEN Research Products) for synthesis of ^{35}S-labeled proteins or 0.6 μl of 18.4 μM unlabeled L-methionine (in place of radiolabeled methionine) and 0.6 μl of ~10-μCi/μl [α-^{32}P]CTP (>400 Ci/mmol) for synthesis of ^{32}P-labeled mRNA. Samples (1 to 6.5 μl) of concentrated solutions of recombinant SLPI, bovine serum albumin (BSA) (nucleic acid enzyme grade; Bethesda Research Laboratories, Inc.), or chicken egg white lysozyme (Miles Scientific) in 10 mM Tris–1 mM EDTA buffer, pH 7.0, were added to test for their effects on transcription and translation. Either 1 μl (0.5 μg; 10 nM) of the *tac* promoter-driven galactose kinase assay vector pDR540 (14) or 1 μl (0.6 μg; 91 nM) of in vitro-derived SLPI mRNA was added to start the reactions. Incubations were performed for 55 min at 37°C, and 3 μl of 50 mM L-methionine chase solution was added for 5 min before TCA precipitation of products. The amounts of ^{32}P-labeled mRNA in dried precipitates and ^{35}S-labeled proteins solubilized in 5% sodium dodecyl sulfate (SDS)–10 mM Tris–1 mM EDTA (pH 8) buffer were determined by scintillation counting.

mRNA recovery experiments. Protein synthesis reaction mixtures containing 91 nM (0.6 μg; 18,000 cpm) ^{32}P-labeled SLPI mRNA were prepared as described above except that radiolabeled CTP and methionine were omitted from the reactions. Samples were incubated for 1 h at 37°C, and the amount of TCA-precipitable ^{32}P-labeled mRNA remaining after incubation was measured by scintillation counting.

Gel retardation assays. The binding of SLPI, BSA, and lysozyme to mRNA and plasmid DNA was assayed by examining the mobilities of mRNA and DNA bands on agarose gels after incubation with the proteins (7). Binding mixtures (18 μl) were prepared by combining 91 nM (0.6 μg; 67,000 cpm) ^{32}P-labeled SLPI mRNA or 10 nM (0.5 μg) pDR540 and samples of SLPI, BSA, or lysozyme in 22 mM Tris-acetate (pH 8.2) buffer. Samples were placed at 37°C for 10 min to permit association of protein and nucleic acids. Immediately after incubation, 4 μl of 6× concentrated tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol, 40% [wt/vol] sucrose) was added, and 3-μl (9,000 cpm of ^{32}P-labeled mRNA) or 15-μl (340 ng of plasmid DNA) samples were loaded on 0.6% agarose (plasmid DNA) or 2% agarose (mRNA) gels cast in 40 mM Tris-acetate–2 mM EDTA (pH 8.0) buffer. After electrophoresis, mRNA band positions were located by autoradiography and plasmid DNA bands were located by staining with 0.5 μg of ethidium bromide per ml.

TABLE 1. Measurement of total RNA and protein synthesis in SGE61 and JM109(pCJX-1)[a]

Time (min)[b]	RNA synthesis (kcpm)		Protein synthesis (kcpm)	
	JM109 (pCJX-1)	SGE61	JM109 (pCJX-1)	SGE61
−10	88.8	90.7	18.5	22.5
−6	96.0	90.8	20.3	22.4
−4	106	99.6	20.2	24.2
−2	104	93.8	20.8	25.2
+1	106	106	19.7	17.0
+3	110	48.3	20.4	10.2
+5	102	37.4	20.9	7.3
+8	102	31.7	20.3	6.3

[a] Cultures were grown at 37°C in M9 medium plus 12.5 μg tetracycline per ml. At the indicated times, samples were taken for determination of the incorporation of [^{3}H]uridine into RNA and L-[^{35}S]methionine into protein (see Materials and Methods).

[b] IPTG (1 mM) was added to the cultures at 0 min.

RESULTS

SLPI expression inhibits growth and macromolecular synthesis. The SLPI expression strain SGE61 was made by transforming strain JM109 (20) with an SLPI expression vector, pCJX-1-mSLPI-3. SLPI synthesis is controlled by the *tac* promoter and is translationally coupled to an *ompA* signal peptide fragment. When a culture of SGE61 growing in Luria-Bertani medium containing 12.5 μg of tetracycline per ml was induced at mid-log phase by the addition of IPTG to 1 mM, there was an immediate and marked reduction of its growth rate (data not shown). In fact, the optical density of the induced culture increased by only 75% during the next 3 h, whereas an uninduced culture will undergo ~2 further doublings and enter stationary phase. If IPTG was added to a culture of a control strain, JM109(pCJX-1), which contains a vector that lacks the SLPI gene but is otherwise identical to pCJX-1-mSLPI-3, the growth rate of the culture was unaffected (data not shown).

Experiments were performed to assess the effects of SLPI induction on the synthesis of RNA and protein in SGE61. As shown in Table 1, the incorporation of [^{3}H]uridine into RNA and L-[^{35}S]methionine into protein declined below preinduction levels within minutes after SLPI induction. The inhibition of protein synthesis ($t_{1/2}$ = 1.5 min) occurred more rapidly than inhibition of RNA synthesis ($t_{1/2}$ = 2.5 min). However, by 5 min postinduction the effects were comparable, namely, protein synthesis was reduced by 60% and RNA synthesis was reduced by 70%. In contrast, protein and RNA synthesis rates were not affected by addition of IPTG to a control strain, JM109(pCJX-1) (Table 1). These results established that the expression of SLPI, and not induction of the *tac* promoter per se or production of an *ompA* signal peptide fragment, causes inhibition of macromolecular synthesis and reduction of growth rate in SGE61.

Design of experiments performed in vitro. The mechanism of SLPI-mediated inhibition of RNA and protein synthesis was examined by using a cell-free, coupled transcription-translation system (21). To accurately model conditions in SGE61, SLPI was added to cell-free reactions at ratios to

TABLE 2. Comparison of the concentrations of SLPI, SLPI mRNA, and components of the transcription-translation apparatus in SGE61 and in cell-free reactions

Cell component	SGE61[a]		In vitro[b] concn	Relative concn[c]
	No./cell	Concn (μM)		
Ribosomes	18,700	33	0.66 μM	50/1
tRNA	205,000	360	6.8 μM	53/1
DNA	1,030	1.7	10 nM	170/1
mRNA				
Cell	1,500 (un)[d]	2.5	91 nM	30/1 (un)
SLPI	80 (un)	0.2		102/1 (in)
	4,000 (in)[d]	6.7		
Total	5,600 (in)	9.3		
SLPI	6×10^4 (un)	105	4.6 to 300	23/1 (low)[e]
	2.2×10^6 (in)	3.8[f]	μM	13/1 (high)[g]

[a] Concentrations were calculated assuming a volume of 0.95 μm^3 for the average SGE61 bacterium in a culture growing at 1.25 doublings per hour (4). The number of ribosomes, tRNA molecules, and mRNA molecules per cell were obtained from the data in reference 9. The concentrations of SLPI mRNA were calculated from dot blot hybridization experiments. The concentrations of SLPI protein were calculated from measurement of the micrograms of SLPI per milliliter at A_{600} by quantitative Western blot analysis and determination of 8×10^8 cells per ml at A_{600} for this strain. The concentration of DNA was expressed in units of 4-kilobase plasmid equivalents, assuming total cellular DNA consists of 4,000 kilobases of chromosomal DNA (4,000 kilobases/4 kilobases per plasmid = 1,000 copies) plus 30 copies of plasmid expression vector per cell (i.e., 1,000 + 30 = 1,030 copies per cell).

[b] The concentration of ribosomes in cell-free reactions was determined from measurement of the A_{260} of the S30 extract, assuming 1 A_{260} unit of RNA = 40 μg/ml and an RNA mass per ribosome of 1.7×10^6 daltons. The concentrations of tRNA, plasmid DNA, SLPI mRNA, and SLPI per reaction were obtained by assuming molecular weights of 2.5×10^4 daltons for tRNA, 2.68×10^6 daltons for the pDR540 vector, 3.63×10^5 daltons for SLPI mRNA, and 11,726 daltons for SLPI.

[c] Ratios are expressed as the concentrations in SGE61 divided by those in cell-free reactions.

[d] un, Uninduced; in, induced with 1 mM IPTG.

[e] Low expression levels, such as in the uninduced state (105 μM/4.6 μM [23/1]).

[f] Concentration in millimolar.

[g] High expression levels (3.8 mM/300 μM [13/1]).

DNA, mRNA, and components of the protein synthesis apparatus which are found in vivo. Therefore, we measured the levels of SLPI and its mRNA in SGE61.

SLPI protein expression levels were determined by quantitative Western (immuno-) blot analysis (data not shown). Before induction, SLPI accounted for ~0.3% of the total cell protein in SGE61. By 3 h postinduction, SLPI levels had increased and plateaued at ~10%. The level of SLPI mRNA in SGE61 was determined by dot blot hybridization analysis (data not shown). SLPI mRNA accounted for 4 to 5% of the total RNA, excluding tRNA, present in SGE61 by 30 min postinduction and remained at this level through 3 h postinduction. We calculated that the SLPI message level was ~50-fold lower before induction than after induction.

On the basis of the measurements of SLPI protein and mRNA levels, we calculated the intracellular concentrations of SLPI, its mRNA, and components of the protein synthesis machinery in SGE61 (Table 2). The concentrations of ribosomes, etc., in SGE61 were calculated by using data given in reference 9 for *E. coli* growing at 1.5 doublings per h. Since the doubling rate of SGE61 (~1.25 doublings per h; data not shown) was approximately the same, we assumed that the values in reference 9 are appropriate for SGE61. However, it should be kept in mind that owing to the toxic effects of SLPI, the intracellular volume, number of ribosomes, etc., may be altered from those in healthy *E. coli*.

TABLE 3. Ratios of components in SGE61 and in cell-free reactions[a]

Components	Ratio in:	
	SGE61[b]	In vitro[c]
Ribosomes/mRNA	13/1 (un)	7.2/1
	3.5/1 (in)	
tRNA/ribosomes	11/1	10.3/1
SLPI/tRNA	0.3/1 (un)	0.7/1 (4.6 μM SLPI)
	10.5/1 (in)	44/1 (300 μM SLPI)
SLPI/mRNA	38/1 (un)	50/1 (4.6 μM SLPI)
	405/1 (in)	505/1 (46 μM SLPI)
		3,300/1 (300 μM SLPI)
SLPI/DNA	62/1 (un)	460/1 (4.6 μM SLPI)
	2,220/1 (in)	4,600/1 (46 μM SLPI)
		30,000/1 (300 μM SLPI)

[a] Ratios were calculated from the data in Table 2.

[b] Ratios of SGE61 components were calculated before and after induction of SLPI synthesis with 1 mM IPTG. un, Uninduced; in, induced.

[c] Ratios of components in cell-free reactions were calculated for several concentrations of SLPI.

We also calculated the concentrations of components present in the cell-free protein synthesis system (Table 2) so that SLPI, plasmid DNA, and mRNA could be added to reactions at appropriate ratios to the translation apparatus. The SLPI/mRNA ratios, etc., in SGE61 and the ratios that were used in cell-free reactions are summarized in Table 3. While the ratios of components in vitro were physiologically accurate, the absolute concentrations of components in cell-free reactions were lower because the translation machinery was diluted >50-fold during preparation of cell extracts (Table 2).

SLPI inhibits DNA-directed protein synthesis in vitro. First, we tested the ability of SLPI to inhibit DNA-directed protein synthesis from the galactose kinase assay vector, pDR540 (14). Since SLPI is highly basic (17), a basic control protein, chicken egg white lysozyme (pI = 10 to 11.5 [1]) was also tested for its ability to inhibit protein synthesis. SLPI and lysozyme have similar charge properties; namely, SLPI contains 20 basic and 8 acidic residues of 107 total (16, 17) and lysozyme contains 17 basic and 9 acidic residues of 129 total (3). We also examined the effects of a negatively charged protein, BSA (pI = 4.9 [12]) on protein synthesis.

SLPI strongly inhibited DNA-directed protein synthesis from 10 nM pDR540 (Fig. 1). At 4.6 μM SLPI (SPLI/pDR540, 460/1), inhibition was 45%, and at 45 μM SLPI and above protein synthesis was almost completely blocked. In contrast, the addition of 4.5 μM BSA to reactions (BSA/pDR540, 450/1) did not inhibit protein synthesis. At BSA concentrations of 45 to 266 μM, a modest inhibition of 40 to 60% was observed. Lysozyme caused inhibition that was intermediate between that observed for SLPI and BSA. Protein synthesis was reduced by 20 and 45% at 4.3 and 43 μM lysozyme, respectively, and was completely inhibited at lysozyme concentrations of 129 μM and higher.

SLPI and lysozyme inhibit translation in vitro. To compare their effects on translation, SLPI, BSA, and lysozyme were added to protein synthesis reactions in which translation was directed by 91 nM SLPI mRNA (Fig. 2). The amount of mRNA added to the reactions gave a ribosome-to-mRNA ratio (7.2/1) that was within the range of ratios observed in SGE61 (3.5/1 to 13/1) (Table 3).

SLPI was highly effective in blocking translation (Fig. 2). Inhibition was >80% at 46 μM SLPI, a concentration at which the SLPI/mRNA ratio (505/1) in the reactions was

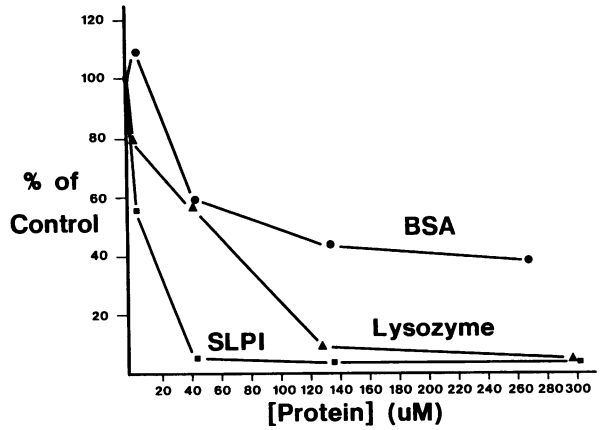

FIG. 1. DNA-directed protein synthesis from pDR540 in the presence of SLPI, BSA, and lysozyme. Reactions contained 0.5 μg (10 nM) of pDR540 and the indicated concentrations of the test proteins. Data are plotted as percentages, compared with a control incubation (no added protein, 2.23×10^6 cpm per reaction) of incorporation of L-[^{35}S]methionine into TCA-precipitable ^{35}S-labeled protein. Background synthesis of ^{35}S-labeled protein in the absence of plasmid was 2.4×10^5 cpm per reaction.

similar to the ratio (405/1) found in SGE61 during maximum SLPI accumulation (Table 3). Lysozyme caused less inhibition of translation than SLPI at low concentrations (4.3 to 43 μM) but also caused complete inhibition at high levels (~300 μM). In contrast, the inclusion of BSA in the reactions improved translation slightly. Thus, the modest inhibition of DNA-directed protein synthesis by BSA (Fig. 1) was not caused by translation inhibition.

Modulation of transcription in vitro by SLPI, BSA, and lysozyme. A series of in vitro reactions containing 10 nM pDR540 and [α-^{32}P]CTP were performed to assess the effects of the proteins on transcription (Fig. 3). When SLPI was added to the reactions, the amount of ^{32}P-labeled mRNA recovered by TCA precipitation was reduced by 30% at 4.6 μM SLPI, increased above the control level at intermediate SLPI concentrations (46 and 138 μM), and was 50% lower at

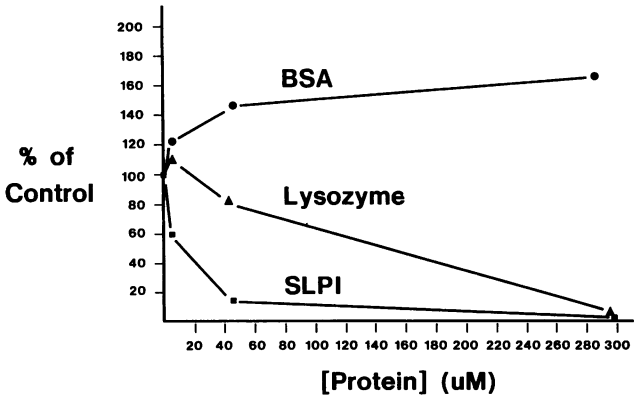

FIG. 2. Translation of SLPI mRNA in the presence of SLPI, BSA, and lysozyme. Reactions contained 0.6 μg (91 nM) of SLPI mRNA and the indicated concentrations of the test proteins. Data are plotted as percentages of incorporation of L-[^{35}S]methionine into TCA-precipitable ^{35}S-labeled proteins compared with a control incubation containing 91 nM mRNA and no added protein (7.2×10^5 cpm). Note that the amount of SLPI synthesized de novo in the translation reactions was insufficient to cause translation inhibition.

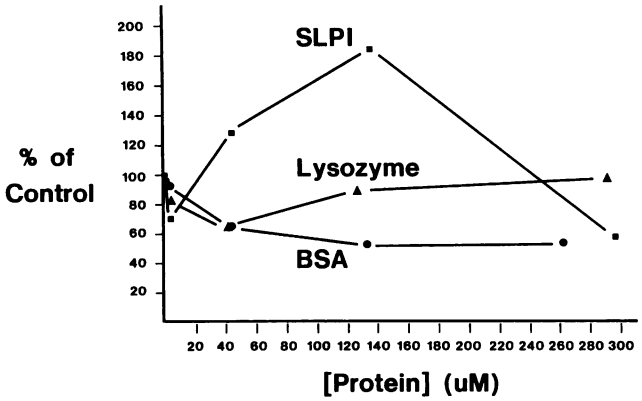

FIG. 3. Transcription of mRNA from pDR540 in the presence of SLPI, BSA, and lysozyme. Reactions contained 0.5 μg (10 nM) of pDR540 and the indicated concentrations of the three proteins. The incorporation of [α-^{32}P]CTP into mRNA was determined by TCA precipitation and scintillation counting. Data are plotted as percentages of the control level of incorporation into mRNA for a reaction containing 10 nM pDR540 but no added protein (1.6×10^5 cpm).

the highest SLPI concentration (300 μM). The addition of 4.3 and 43 μM lysozyme to reactions reduced ^{32}P-labeled mRNA recovery, whereas the amount of ^{32}P-labeled mRNA was restored to control levels at higher concentrations of lysozyme. In contrast, the recovery of ^{32}P-labeled mRNA from transcription assays was reduced at all concentrations of BSA. Since BSA did not inhibit translation (Fig. 2) and the percentages of inhibition of transcription (Fig. 3) and DNA-directed protein synthesis (Fig. 1) were comparable for a given BSA concentration, we concluded that BSA inhibits DNA-directed protein synthesis primarily at the level of transcription.

SLPI and lysozyme protect mRNA added to cell-free reactions from RNase degradation. On the basis of the results obtained from transcription experiments, it appeared that transcription can be stimulated at some SLPI concentrations. However, these results could be obtained if SLPI protected ^{32}P-labeled mRNA made in vitro from degradation by endogenous RNase. To test this hypothesis, SLPI, BSA, and lysozyme were added to mock transcription reactions containing ^{32}P-labeled SLPI mRNA, and the amount of TCA-precipitable mRNA remaining after a 1-h incubation at 37°C was measured. The addition of ≥46 μM SLPI and ≥43μM lysozyme to reactions increased the recovery of ^{32}P-labeled mRNA above the level recovered in the absence of added proteins (Fig. 4). However, BSA was completely ineffective in improving mRNA recovery at all concentrations tested (up to 266 μM). These results indicate that SLPI and lysozyme protected the mRNA in the reactions from RNase degradation and did not actually stimulate transcription.

SLPI and lysozyme bind to mRNA and DNA in vitro. SLPI and lysozyme could prevent mRNA degradation by inactivating RNase or by binding to mRNA and blocking its access to the enzyme. Therefore, we assayed the mRNA binding capacity of the proteins by performing gel retardation experiments (7). The concentrations of proteins and ^{32}P-labeled mRNA used in these experiments were similar to those used in cell-free protein synthesis reactions.

mRNA that had been incubated with ≥1.38 μM SLPI (SLPI/mRNA ≥ 15/1; Fig. 5A, lanes 3 to 7) exhibited altered mobility on agarose gels. Similar changes in mobility were observed at concentrations of ≥2.8 μM lysozyme (lyso-

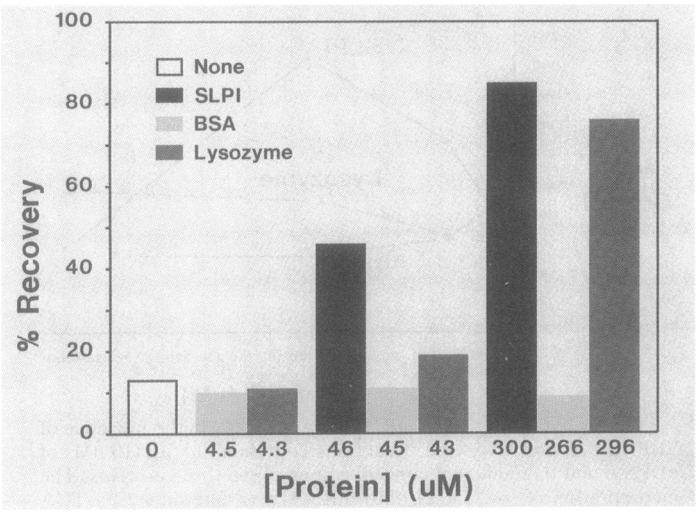

FIG. 4. Recovery of SLPI mRNA from in vitro reactions containing SLPI, BSA, and lysozyme. The cell-free reactions contained 0.6 μg (91 nM) of ^{32}P-labeled SLPI mRNA (17,000 cpm) and the indicated concentrations of SLPI, BSA, and lysozyme. After incubation, the radioactivity remaining in TCA-precipitable mRNA was determined by scintillation counting.

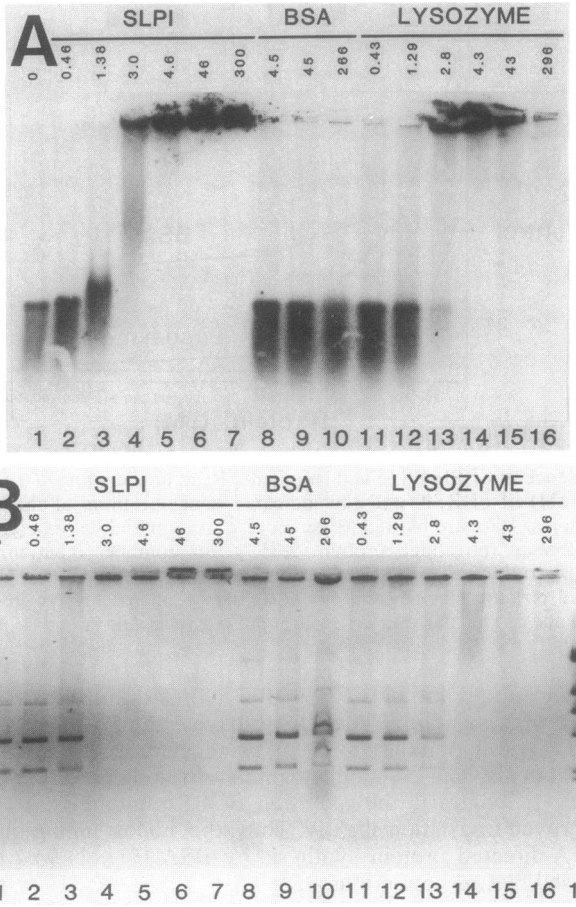

FIG. 5. Gel retardation analysis of SLPI, BSA, and lysozyme binding to mRNA (A) and plasmid DNA (B). Mixtures containing 91 nM ^{32}P-labeled SLPI mRNA or 10 nM pDR540 were incubated in the presence of the indicated (micromolar) concentrations of SLPI, BSA, or lysozyme. Samples containing 9,000 cpm of ^{32}P-labeled mRNA or 340 ng of pDR540 were analyzed by electrophoresis on agarose gels. Bands were visualized by autoradiography (mRNA) or ethidium bromide staining (DNA). Lambda *Hin*dIII digestion fragments were applied to lane 17 of the DNA gel (B).

zyme/mRNA ≥ 31/1; Fig. 5A, lanes 13 to 16). At sufficiently high concentrations of SLPI (≥3 μM) and lysozyme (≥2.8 μM), mRNA did not enter the gel. In contrast, the mobility of mRNA incubated with BSA at concentrations up to 266 μM (BSA/mRNA = 2,920/1) was not significantly affected (Fig. 5A, lanes 8 to 10).

Similar alterations in the mobilities of plasmid DNA species were observed when 3 to 300 μM SLPI (Fig. 5B, lanes 4 to 7) and 2.8 to 296 μM lysozyme (Fig. 5B, lanes 13 to 16) were incubated with 10 nM pDR540. In fact, DNA bands did not enter the gel at these concentrations of SLPI and lysozyme. However, pDR540 that was incubated with 4.5 to 266 μM BSA (up to a BSA/pDR540 ratio of 26,600/1) migrated normally (Fig. 5B, lanes 8 to 10). Slight irregularities in the appearance of the bands for the incubation conducted at 266 μM BSA (Fig. 5B, lane 10) may have been caused by the large mass (~220 μg) of BSA loaded per lane. In conclusion, SLPI and lysozyme but not BSA form complexes with mRNA and DNA.

Translation inhibition by SLPI in vitro is reduced by increasing the mRNA/SLPI ratio. We examined whether translation inhibition could be overcome by increasing the ratio of mRNA to SLPI in reaction mixtures (Table 4). The two concentrations of mRNA selected for study (0.9 and 1.9 μM) were saturating for translation in the cell-free system. Consequently, the amounts of ^{35}S-labeled protein synthesized in the absence of SLPI were similar. However, when a strongly inhibitory concentration of SLPI (138 μM) was present in the reactions, the level of residual protein synthesis was fourfold higher with 1.9 μM mRNA than it was with 0.9 μM mRNA. Thus, the extent of inhibition of translation by SLPI was dependent on the relative amounts of SLPI and mRNA present in the reactions and was less at higher mRNA/SLPI ratios.

DISCUSSION

The mechanism by which SLPI and lysozyme inhibit translation in vitro was deduced from mRNA stabilization (Fig. 4) and nucleic acid-binding (Fig. 5) experiments. On the basis of these experiments, we concluded that these basic proteins inhibit translation by binding to mRNA and interfering with the normal functioning of ribosomes during translation. Presumably, SLPI and lysozyme bind to mRNA via electrostatic interactions between their basic amino acid

TABLE 4. Translation inhibition by SLPI overcome by increasing the mRNA/SLPI ratio

mRNA concn	SLPI concn (μM)	Ribosome concn	mRNA/ ribosome	mRNA/ SLPI	^{35}S-protein (cpm, 10^6 [%][a])
0.9	0	0.66	1.4/1		2.3
1.9	0	0.66	2.9/1		2.0
					2.15[b] (100)
0.9	138	0.66	1.4/1	1/153	0.2 (9)
1.9	138	0.66	2.9/1	1/73	0.8 (37)

[a] Protein synthesis was averaged for the two reactions without SLPI since translation is saturated at these concentrations of mRNA.

[b] The average counts per minute of ^{35}S-labeled protein obtained in the absence of SLPI was set at 100%.

residues and the phosphate groups of mRNA. Since they have no known physiological functions requiring recognition of mRNA, it is unlikely that they bind to specific sequences of mRNA. Thus, SLPI and lysozyme could inhibit either translation initiation by blocking the access of ribosomes to the Shine-Dalgarno region (15) of the message or elongation by binding to coding sequences. There are precedents for the occurrence of the first of these mechanisms in *E. coli*. For example, ribosomal proteins (10) and the T4 phage *regA* protein (18) repress translation of their own mRNAs by binding to their Shine-Dalgarno regions.

While SLPI-mRNA binding causes translation inhibition in vitro, careful comparison of conditions in cell-free reactions and in SGE61 should be made before concluding that similar events could cause inhibition in vivo. Prior to the induction of SLPI synthesis in SGE61, the SLPI/mRNA ratio was ~38/1 (Table 3). According to cell-free translation experiments, translation was inhibited by ~25% at an SLPI/mRNA ratio of 38/1 (i.e., 3.5 μM SLPI [Fig. 2]). However, protein synthesis rates in SGE61 before induction and in a control strain were approximately equal (Table 1). Thus, the in vitro model system does not accurately predict the percentage of translation inhibition occurring in SGE61, at least at the low SLPI/mRNA ratios existing prior to induction. Perhaps at these SLPI/mRNA ratios, ribosomes in vivo are better able to compete with SLPI for binding to mRNA.

However, excellent agreement between the percentages of translation inhibition occurring in cell-free reactions and in SGE61 was found at higher SLPI/mRNA ratios. For example, by 3 h postinduction of SLPI synthesis in SGE61, the SLPI/mRNA ratio attained a value of 405/1 (Table 3) and protein synthesis was inhibited by 65 to 70% (Table 1). We calculate that translation would be inhibited by 75% in cell-free reactions at an SLPI/mRNA ratio of 405/1 (i.e., 37 μM SLPI [Fig. 2]). Since the data obtained in the model systems and SGE61 were similar at higher SLPI/mRNA ratios, we concluded that the most probable mechanism by which SLPI expression causes translation inhibition late after induction is by formation of untranslatable SLPI-mRNA complexes.

We also attempted to study the mechanism by which SLPI inhibits transcription in SGE61 by performing cell-free transcription assays. The effects of SLPI on transcription in vitro were complex (Fig. 3). At the lowest (4.6 μM) and highest (300 μM) SLPI levels transcription appeared to be inhibited, whereas at intermediate concentrations of SLPI (46 and 138 μM) transcription seemed to be stimulated. These results can be explained on the basis of the finding that SLPI can protect mRNA from RNase degradation (Fig. 4). Thus, the variable effects of SLPI on transcription actually indicate that at some SLPI concentrations, e.g., 46 and 138 μM, SLPI has a relatively stronger ability to protect mRNA from degradation than to inhibit transcription. Because it is difficult to determine the relative contributions of these two effects, we cannot compare transcription inhibition at a given SLPI/DNA ratio in vitro and in SGE61.

We conclude, however, that the SLPI/DNA ratio in SGE61 is high enough after induction to allow SLPI to bind to DNA (Table 3; Fig. 5B). So, if SLPI competes with RNA polymerase for DNA binding, then it could inhibit transcription directly. Alternatively, transcription inhibition in vivo may result from the disruption of translation, since transcription and translation are tightly coupled in *E. coli*. In fact, the onset of transcription inhibition in SGE61 lagged slightly behind the onset of translation inhibition (Table 1).

In summary, our findings indicate that basic recombinant proteins, when overexpressed in *E. coli*, can bind to DNA and mRNA and inhibit transcription and translation. Likewise, basic proteins may bind to other negatively charged molecules, e.g., tRNA. We have tried to prevent translation inhibition by SLPI in vitro by supplementing reactions with large amounts of tRNA, but these measures have failed to relieve translation inhibition (data not shown). Thus, the function of tRNA probably is not impaired by SLPI. However, we have found that translation inhibition by SLPI can be partially relieved by increasing the mRNA/SLPI or mRNA/ribosome ratio (Table 4). Apparently, the number of translatable mRNA molecules is greater at higher mRNA/SLPI ratios. We are currently performing experiments to determine whether RNA and protein synthesis can be improved in SLPI expression strains under conditions in which SLPI-nucleic acid interactions are reduced.

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