Escherichia coli Poly(A)-binding Proteins That Interact with Components of Degradosomes or Impede RNA Decay Mediated by Polynucleotide Phosphorylase and RNase E*

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The multifunctional ribonuclease RNase E and the 3′-exonuclease polynucleotide phosphorylase (PNPase) are major components of an Escherichia coli ribonucleolytic “machine” that has been termed the RNA degradosome. Previous work has shown that poly(A) additions to the 3′ ends of RNA substrates affect RNA degradation by both of these enzymes. To better understand the mechanism(s) by which poly(A) tails can modulate ribonuclease action, we used selective binding in 1 M salt to identify E. coli proteins that interact at high affinity with poly(A) tracts. We report here that CspE, a member of a family of RNA-binding “cold shock” proteins, and S1, an essential component of the 30 S ribosomal subunit, are poly(A)-binding proteins that interact functionally and physically, respectively, with degradosome ribonucleases. We show that purified CspE impedes poly(A)-mediated 3′ to 5′ exonucleolytic decay by PNPase by interfering with its digestion through the poly(A) tail and also inhibits both internal cleavage and poly(A) tail removal by RNase E. The ribosomal protein S1, which is known to interact with sequences at the 5′ ends of mRNA molecules during the initiation of translation, can bind to both RNase E and PNPase, but in contrast to CspE, did not affect the ribonucleolytic actions of these enzymes. Our findings raise the prospect that E. coli proteins that bind to poly(A) tracts may link the functions of degradosomes and ribosomes.

3′ Polyadenylation is now known to occur in a variety of bacterial species as well as in eukaryotes (1, 2). In Escherichia coli, poly(A) tails can dramatically promote the decay of structural RNA and mRNA both in vitro and in vivo (e.g. Refs. 3–6, for recent reviews, see Refs. 7 and 8), at least in part by facilitating circularization of mRNA molecules (Ref. 17; for reviews, see Refs. 18–23). PABPs also influence the shortening of poly(A) tails, which in eukaryotic cells is a crucial initial step in mRNA decay. Currently, there is substantial evidence for yeast and mammalian cells that the biological effects of poly(A) tails on mRNA decay and mRNA translation are mediated through the actions of proteins that bind to them. In prokaryotes, however, comparable information does not exist for protein binding to poly(A) tails.

Here we report the results of experiments aimed at isolating and identifying E. coli proteins that interact with poly(A) tracts. Using methods similar to those employed to obtain yeast poly(A)-binding proteins, we purified and characterized two E. coli proteins that bind tightly to poly(A) tracts: one of these is the ribosomal protein S1, as has been found independently (24), while the other is CspE (25), a member of a family of bacterial proteins that contains a “cold-shock” RNA-binding domain and has been implicated as a transcription anti-terminator (26, 27). We show that CspE binding impedes poly(A) tail removal by PNPase and RNase E, and that S1 interacts with both PNPase and RNase E as well as with poly(A) tails. Our findings raise the prospect that E. coli proteins that bind to poly(A) tails may link the functions of degradosomes and ribosomes.

**Experimental Procedures**

Bacteria Strains and Plasmid—Bacteria strain E. coli CA244, which contains a Tn5 insertion in the PNPase gene, was used as the source of PABPs in order to reduce digestion of poly(A) tracts during the purification (28). To overexpress His-tagged PABPs and N-truncated RNase E polypeptides, BL21(DE3) was used as host. Plasmids expressing His-tagged PABPs under control of bacteriophage T7 RNA polymerase expressed from an isopropyl-β-D-thiogalactopyranoside-inducible promoter were constructed by polymerase chain reaction amplification of E. coli chromosomal DNA using the primers 5′-CATATGACT-GAATCTTTTGCTCAACTC and 5′-AGCTCTTACTGCCTTGTGGCATTTGAAGC for the S1 gene and 5′-CATATGCTCAAGATTAAAGGTAACGTTAGG and 5′-GAGCTCGCCCTACAAGGACACC for the CspE gene. The polymerase chain reaction products were

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cleaved with NdeI/SacI and cloned into plasmid pET28 (Novagen) cut with the same enzymes. Plasmids used for overexpression of truncated RNase E (peptide 2, peptide 5, peptide 8) have been described (29).

**Purification of PABPs from E. coli**—The entire purification procedure was carried out in 1 M NaCl. Cultures were grown in Luria broth (LB) at 37 °C until the A600 reached 0.8, harvested, washed, resuspended in 4 ml of buffer A containing 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100. The cells were broken by French Press and treated with RNase A and RNase T1 to digest endogenous RNA sequences other than poly(A) (30). The mixture was kept at 4 °C for 10 min, centrifuged at 5,000 × g for 10 min, fractionated on a 5 to 30% sucrose gradient in buffer A, and centrifuged at 31,000 rpm for 48 h. Fractions were collected and the poly(A) binding activity was tested using a filter binding assay (see below); the fractions showing high binding activity were pooled and dialyzed against buffer A overnight. Exogenous poly(A) was added to a final concentration of 80 μg/ml and the mixture was incubated at 4 °C for 20 min and then applied to an oligo(dt)-cellulose column (height, 3.0 cm; diameter, 1.75 cm), which had been washed as described by the manufacturer and equilibrated in buffer A. The column was then washed with 40 ml of buffer A containing 1 μg/ml poly(C), followed by 10 ml of buffer A. Poly(A)-binding proteins were finally eluted with buffer A containing 1.1 to 1.4 M guanidine hydrochloride. The eluted fractions were dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride, and analyzed by SDS-PAGE.

**Determination of Poly(A) Binding Activity for PABPs**—Poly(A) (Amersham Pharmacia Biotech) was labeled at the 5’ end with [α-32P]ATP by T4 polynucleotide kinase to 107 cpm/μg. Assay mixtures contained 20 ng of 32P-labeled poly(A), 10 μg of cold poly(C) (Amersham Pharmacia Biotech), the protein fraction, and 1 μl of RNase inhibitor (Life Technologies, Inc.) in 100 μl of buffer A. Mixtures were kept for 10 min at room temperature, diluted to 1 ml with ice-cold buffer A, and passed through 13-mm nitrocellulose filters (HAWP; Millipore Corp.). The filters were washed five times with 1 ml of ice-cold buffer A, dried, and counted. Background was determined by substituting unlabeled poly(A) for poly(C). To further determine the RNA-binding specificity of purified PABPs, RNA homopolymer attached to Sepharose beads (Sigma) was used to test binding at different salt concentrations, as previously described (31); purified recombinant CspE and S1 were incubated with poly(A)+, poly(U)+, poly(G)+, and poly(C)-Sepharose beads in binding buffer containing buffer A with indicated NaCl concentration. Sepharose beads were then washed three times with binding buffer to remove unbound protein. Protein that remained bound to Sepharose beads was eluted by SDS-sample buffer, analyzed on SDS-PAGE, and stained with Commassie Blue.

**Overexpression and Purification of His-tagged PABPs, His-tagged RNase E, and Truncated RNase E Polypeptides**—50 ml of cells of BL21(DE3) containing plasmids encoding His-tagged proteins were grown in LB at 37 °C until the A660 reached 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a concentration of 1 mM to induce expression and cultures were grown for another 30 min. Cells were harvested and His-tagged proteins were purified using non-denaturing immobilized metal affinity chromatography (32) as described in the pET System Manual (Novagen). Following chromatography, His-tagged S1, RNase E, and truncated RNase E polypeptides were dialyzed three times against storage buffer (29) and then stored at −70 °C. The N-terminal His-tag of recombinant CspE was removed by treatment with thrombin (Novagen) and the protein was further purified according to the pET System Manual instructions.

**Gel Mobility Shift Assay**—Gel mobility shift assays were performed in binding buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM KC1, 10 mM K2HPO4, 1 mM dithiothreitol, and 7.5% glycerol using RNA I-40 A and RNA I as probes. RNA I and RNA I-40A were synthesized in vitro as described in Ref. 6 and universally labeled with [α-32P]ATP. Serial dilutions of purified His-tagged PABPs (200 to 3.13 pmol) were mixed with 100 fmol of RNA probe in a total volume of 10 μl and incubated for 15 min at room temperature. For competition experiments, the unlabeled homopolymer competitor was mixed with labeled RNA probe before the addition of PABPs and the RNA-protein complexes were analyzed electrophoretically on 4% polyacrylamide gels.

**Far-Western Assay**—The His-tagged S1 and RNase E proteins were purified as described (29). Rabbit polyclonal antibodies to S1 were raised using purified proteins. Antibody raised using purified poly(A) binding and metal affinity chromatography, as described above. Anti-RNase E monoclonal antibody was described previously (29). Electrophoresis was carried out for 40 min at 200 V in 8% SDS-PAGE gels containing Tris glycine, as described (34). After separation, proteins were blotted onto Schleicher & Schuell nitrocellulose membranes, which were incubated in TEN buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 50 mM NaCl) (35) at 4 °C for 4 days for protein renaturation. Membranes were blocked for 1 h in HEPES hybridization buffer and then incubated either with probe proteins or, for negative control, with bovine serum albumin in hybridization buffer as described (33). Western blotting using antibody against probe protein was performed and positive signals were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). All membranes were stained with 1% Ponceau S after ECL.

**RESULTS**

**Isolation and Purification of Poly(A)-binding Proteins from E. coli**—Multiple proteins that bind to poly(A) tails (PABPs) at a high (1 M) salt concentration have been purified from yeast and higher eukaryotes (16, 31, 36). As shown in Fig. 1, use of conditions employed to obtain PABPs from eukaryotic cells (e.g. Refs. 31 and 36) enabled us to purify poly(A) stretches and attached PABPs from E. coli cell extracts by chromatography on oligo(dt)-cellulose columns as described under “Experimen
tal Procedures.” SDS-PAGE analysis of the concentrated sample obtained by elution from oligo(dt)-cellulose using a gradient containing 1.1 to 1.4 M guanidinium HCl showed two major bands having apparent molecular masses of 75 and 8 kDa (Fig. 1, lane 5). The N-terminal amino acid sequence determined for p75 (i.e. MTEFSALFQF) matched exactly with the sequence of S1, a major component of the 30 S ribosomal subunit that previously has been reported to bind to poly(A) tails (24). While the S1 nucleotide sequence predicts a protein of 61 kDa, SDS-PAGE analyses have shown that S1 has an apparent molecular mass of 65–76 kDa (37), consistent with the observed migration properties of p75. The sequence of a tryptic-generated peptide isolated from p8 was DVFVHFSAIQTNQF. This sequence plus the migration properties observed during both SDS-PAGE and two-dimensional gel electrophoresis, indicate that p8 is CspE, a small basic protein identified for its ability to suppress mutations in the chromosome partitioning gene mukB and then found to show major homology with members of the cold-shock protein family (25).
Proteins that remained bound to homopolymer-Sepharose beads at the indicated salt concentration (0.1 to 2 M) were resolved in SDS-PAGE and detected by Coomassie Blue staining of the gel. Lane 9, gel shift assays of CspE binding to RNA substrates. Left panel, gel shift assay showing that CspE interacts with polyadenylated RNA I (RNAI-40A) but not with non-adenylated RNA I. Lane 1, RNA I-40A (100 fmol); lanes 2–8, 100 fmol of RNA I-40A was incubated with CspE in the amount of 200, 100, 50, 25, 12.5, 6.25, and 3.13 pmol, respectively. Lane 9, RNA I (100 fmol); lanes 10–16, 100 fmol of RNA I incubated with CspE in the amounts indicated above. Right panel, RNA I-40 A was used for gel shift assays with serial dilutions of polymer as competitor. Each reaction contained 200 pmol of CspE and 100 fmol of RNA I-40A. The $K_d$ value, which was defined as the concentration of protein at which 50% of maximum binding (48.7 fmol in this experiment) (41), was 8.4 M, whereas there was no detectable binding of CspE to non-adenylated RNA I. 40A indicates RNA I-40A; C, RNA I-40A mixed with CspE. Lanes 1–4, contained the indicated polymer as competitor in the amount of 0.02, 0.2, 2, and 20 pmol, respectively. Molecular weights of polymers were calculated based on an average length of 20 nucleotides.

\[ \text{Binding Specificity of CspE and S1} \]

Although CspE contains a cold-shock domain (CSD) present in all members of the bacterial cold-shock protein family as well as in eukaryotic Y-box transcription factors (38), CspE is not itself inducible by cold (25, 39). Within the CSD is the RNA-binding sequence motif, RNP1 (40), enabling CspE and other CSD proteins to interact with single stranded RNA (41). In contrast to the nonspecific RNA binding properties of the prototype cold shock protein CspA (42), recent studies of sequence-binding preferences for the CspB, -C, -D, and -E proteins showed an AAAUUU “consensus” binding sequence for CspE (41). We found that CspE protein purified from *E. coli* cells following overexpression from the pET28 vector interacted highly specifically with poly(A) homopolymer at 1 M NaCl and remained bound in reactions containing up to 2 M NaCl (Fig. 2A, left panel). At low salt concentration (0.1 M), binding of CspE to poly(U) and poly(G) homopolymers also was observed.

Preferential binding of CspE to RNA molecules having poly(A) tails was also shown by gel mobility shift assays with RNA I, a 108-nucleotide antisense RNA that has been used extensively as a substrate in studies of mRNA decay (43–46). RNA I normally is adenylated in *vitro* and the addition of a poly(A) tail to RNA I promotes decay of the primary transcript (5, 6). Under the conditions we used for gel-shift assays (standard PNPase assay conditions; see below), CspE caused a prominent shift in migration of a polyadenylated RNA I variant containing a 40-nucleotide 3' tract of A-residues (RNA I-40A), whereas there was no detectable binding of CspE to non-adenylated RNA I (Fig. 2B, left panel). At the salt concentration used for gel shift assays, both poly(A) and poly(U) homopolymers competed effectively for the binding of CspE to RNA I-40A; however, poly(C) or poly(G) showed no detectable ability to compete (Fig. 2B, right panel).

Ribosomal Protein S1 Has Two Distinct Domains—The short N-terminal domain (amino acids 1–195) links S1 to the 30 S ribosomal subunit while the long C-terminal domain (amino acids 196–591), which contains four repeats of an RNA-binding motif, accounts for S1's strong RNA-binding properties (37). However, unlike CspE, whose interaction with poly(A) homopolymer was highly specific through a wide range of NaCl concentrations, S1 interacted similarly with different RNA homopolymers in 0.1 to 2 M NaCl (Fig. 3A, right panel). S1 previously has been reported to contain separate binding sites for different homopolymers (24). This conclusion is supported by our observation that concurrently present excess poly(C), which can bind to S1, did not prevent purification of S1 as a poly(A)-binding protein (see above) and the finding that S1 can interact independently with poly(A) and poly(C) with similar affinity. Mixing S1 and CspE at different ratios did not affect their individual RNA-binding properties (data not shown).

Effects of S1 and CspE on mRNA Decay—The eukaryotic proteins that bind to poly(A) tails are known to be able to modulate RNA decay (36, 47, 48). Earlier work has shown that in *E. coli* poly(A) tails facilitate 3'-5' degradation of primary transcripts by PNPase, both *in vivo* and *in vitro* (5, 9, 10, 49). Movement of PNPase through primary transcripts normally is slowed by pausing of the enzyme at distally located stem-loop structures (Refs. 4, 15, 49, and 50); CspE protected the polyadenylated RNA from PNPase-mediated decay by both impeding the initiation of PNPase digestion and enhancing the slowing of this exonuclease at or near the junction of the poly(A) tail with the primary transcript sequence (Fig. 3A). Protection by CspE against poly(A)-promoted digestion by PNPase was observed also for an mRNA species that contains the 3' terminal 330 nucleotides of the lacZ transcript, indicating that the ability of CspE to inhibit the action of PNPase is not restricted to RNA I (Fig. 3B).

In addition to protecting RNA I-40A from PNPase, CspE interfered with both the poly(A) tail shortening activity of RNase E (11) (Fig. 4) and its ability to endonucleolytically cleave RNA I-40A and, less efficiently, RNA I, near the 5' end (45, 50) (Fig. 4). The previously reported poly(A) tail-mediated
inhibition of endonucleolytic digestion by RNase E also was increased by the addition of CspE, which at a concentration of 25 nM interfered with the conversion of RNA-I-40A to RNA-I-S. Protection of non-polyadenylated RNA-I from RNase E endonucleolytic digestion was not apparent at this CspE concentration.

In contrast to the observed effects of CspE on RNA decay, an equimolar amount of S1 (i.e., ~10 times the CspE protein concentration in w/v) did not significantly affect the degradation of RNA-I-40A by PNPase (Fig. 3C, left panel). As S1 also has strong RNA binding properties under the conditions of the PNPase assay (Fig. 3C, right panel, and Ref. 24), its inability to block exonucleolytic progression of PNPase through poly(A) tails implies that the action of CspE on PNPase mediated degradation of RNA is specific and not a reflection of its poly(A) binding ability per se.

Interaction between Proteins Acting on Bacterial Poly(A) Tails—The results described above, together with previous work showing that poly(A) tails can be attacked and shortened by RNase E and PNPase (5, 9, 11) led us to investigate possible interaction between these degradosome components and the poly(A)-binding proteins we isolated. We overexpressed His-tagged S1, CspE, and RNase E in E. coli strain BL21(DE3) and tested for interaction by both immunoprecipitation and Far-Western blotting analysis using antibodies against the individual proteins. Immunoprecipitates brought down by monoclonal antibody to the His-tag showed only the previously reported interaction between RNase E and PNPase (12–14). However, Far-Western blotting using antibodies against purified RNase E, PNPase, CspE, or S1 to detect interactions with the electrophoretically separated His-tagged proteins showed binding of S1 to RNase E and PNPase (Fig. 5), but no detectable interaction between CspE and either S1 or RNase E. The interaction between S1 and RNase E was shown reciprocally (Fig. 5A, lane 6, and Fig. 5B, lane 6), and neither RNase E nor S1 showed cross-reaction with antibody to the other protein (Fig. 5A, lane 4, and Fig. 5B, lane 4). Using truncated RNase E peptides, we mapped the S1-binding site in RNase E to a region (amino acids 499 to 765) that also includes the sites of interaction with enolase and the RhlB helicase (13, 33) (Fig. 5A).

PNPase antibody, which was shown by concurrent Western blotting control experiments not to cross-react with S1 (Fig. 5D, lane 4), detected PNPase bound to S1 immobilized on nitrocel-
nylated RNA I from both the poly(A) tail shortening activity of RNase E and the endonucleolytic cleavage that ordinarily occurs near the RNA I 5′ end.

Ribosomal protein S1, the second poly(A)-binding protein detected by our screen, is a weakly held 30 S subunit protein that interacts with sequences at the 5′ ends of mRNA molecules (37) as well as with poly(A) tails (this work; see also Ref. 24). Binding of S1 to an “enhancer” located near the Shine-Dalgarno (SD) sequence at the 5′ end of E. coli mRNA molecules promotes interaction between the SD and 16 S rRNA, as well as correct positioning of the translational start codon (e.g. Ref. 37 and 51). In contrast with the results observed for CspE, binding of S1 to poly(A) had no detectable effect on the ribonucleolytic activity of PNPase or RNase E. However, the ability of this poly(A)-binding protein to interact with degradosome protein S1 may functionally link the processes of mRNA degradation and translation in E. coli cells: by binding to sites located at both ends of mRNA molecules, protein complexes containing S1 may “bridge” 3′ and 5′ mRNA termini, as has been proposed for the eIF4E/eIF4G/Pab1p complex in yeast and for its mammalian cell counterpart (e.g. Refs. 17 and 18). Consistent with the notion of communication between the two ends of RNA molecules in E. coli cells are the results of earlier work showing that events taking place at one end of a transcript can influence ribonucleolytic attack in vivo at the other terminus (5, 6, 45).

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