Characterization of ChpBK, an mRNA Interferase from Escherichia coli*

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Escherichia coli contains a number of antitoxin-toxin modules on its chromosome, which are responsible for cell growth arrest and possible cell death. ChpBK is a toxin encoded by the ChpBK antitoxin-toxin module. This module consists of a pair of genes, chpBI and chpBK encoding antitoxin ChpBI and toxin ChpBK, respectively. ChpBK consists of 116 amino acid residues, and its sequence shows 35% identity and 52% similarity to MazF, another E. coli toxin. MazF has been shown to be a sequence-specific (ACA) endoribonuclease that cleaves cellular mRNAs and effectively blocks protein synthesis and is thus termed as an mRNA interferase. Here we demonstrate that ChpBK is another mRNA interferase in E. coli whose induction effectively blocks cell growth in a manner similar to that of MazF. The protein synthesis as judged by incorporation of [35S]methionine was, however, reduced by only 60% upon ChpBK induction. We demonstrate that ChpBK is a new sequence-specific endonuclease that cleaves mRNAs both in vivo and in vitro at the 5′- or 3′-side of the A residue in ACY sequences (Y is U, A, or G). The ChpBK cleavage of a synthetic RNA substrate generated a 2′,3′-cyclic phosphate group at the 3′-end of the 5′-end product and a 5′-OH group at the 5′-end of the 3′-end product in a manner identical to that of MazF.

The existence of toxin or suicidal genes on bacterial chromosomes is intriguing. Escherichia coli contains five such genes, relE, mazF, chpBK, yoeB, and yafQ (1). All these genes exist in an operon together with their cognate antitoxin genes: relB for relE (2), mazE for mazF (3), chpBI for chpBK (4), yefM for yoeB (1), and dinJ for yafQ (1). These antitoxin-toxin operons are often termed as “addiction modules,” since the antitoxins are unstable as compared with their cognate toxins and therefore their continuous expressions are essential to prevent cell growth arrest caused by their respective toxins. Recently, PemK, a homologue of MazF encoded by plasmid R100 (5), was demonstrated to be another sequence-specific endonuclease which preferentially cleaves single-stranded RNA at the 5′- or 3′-side of the A residue in the UAH sequence (where A is C, A, or U) (6). These results suggest that bacteria seem to use a general strategy to block cell growth by interfering with mRNA function by cleaving them at specific sequences. Thus a term, mRNA interferase, was coined for these sequence-specific endonucleases (6).

Among these E. coli toxins, the RelB-RelE and the MazE-MazF systems have been most extensively studied (7, 8). Recently the x-ray structure of the RelB-RelE complex from Pyrococcus horikoshii OT3 has been determined, consisting two molecules of RelB and RelE each (9). RelE has been initially proposed to be a ribosome-dependent endonuclease preferentially cleaving at termination codons in mRNA (10). However, RelE appears to be a factor that stimulates the intrinsic endonuclease activity of ribosomes (11). The x-ray structure of the MazE-MazF complex has also been determined, which consists of two MazF dimers and one MazE dimer (12). The x-ray structure of MazE dimer has also been determined (13–15). When MazF expression was induced using an inducible vector, cell growth was completely blocked as a result of inhibition of protein synthesis (16). Further studies revealed that MazF is a sequence-specific endonuclease that cleaves ACAs sequences in mRNAs or single-stranded RNAs (16). This in turn very effectively reduces cellular protein synthesis. It has been shown that cell growth arrested by MazF can be recovered if MazE is induced in these cells (17). Thus, these authors concluded that MazF toxin is bacteriostatic but not bactericidal. On the other hand, other researchers argue that MazF induction causes programmed cell death in E. coli as MazE induction cannot rescue cell viability after a certain period of time (a point of no return) (8, 18). Recently we demonstrated that MazF-induced cells stay in the quasi-dormant state (19) as these are fully capable of energy metabolism, amino acid and nucleotide biosynthesis, and RNA and protein synthesis, although their growth is completely blocked (19). Indeed, the MazF-induced cells in the quasi-dormant state are able to synthesize a single protein of interest at a high level for a long time if ACA sequences in its coding region are altered to make it resistant to degradation by MazF (19).

In the present study, we showed that ChpBK, which is 35% identical and 52% similar to MazF (see Fig. 1A), also functions as an mRNA interferase by cleaving RNA at the ACY (Y is U, A, or G) sequences.

**EXPERIMENTAL PROCEDURES**

*Strains and Plasmids—E. coli BL21(DE3) and BW25113 cells (20) were used. The chpBK operon was amplified by PCR and cloned into the XbaI-XhoI sites of pET21c (Novagen) by the same method as that used for cloning the mazEF operon (16). This construction created an in-frame translation fusion with a His<sub>6</sub> tag at the ChpBK C-terminal end. The plasmid was designated as pET21c-ChpBK(His<sub>6</sub>). The chpBK gene was cloned into pBAD (21) creating pBAD-ChpBK. The E. coli mazG gene was cloned into the NdeI-BamHI sites of pET11a (New England Biolabs), creating pET11a-MazG (22), which was used for the production of the mazG mRNA. The mazG gene was cloned into a pNIII vector, creating pN-MazG, which was used to identify the in vivo mazG mRNA cleavage sites by the primer extension (16). The E. coli era gene was cloned into the ScaI-XhoI sites of pET28a, creating...
FIG. 1. Amino acid sequence alignments of ChpBK, PemK, and MazF and the effect of ChpBK expression on cell growth. A, sequence alignments of ChpBK (GenBank™ accession number D49339) of E. coli with that of PemK (GenBank™ accession no BAA78898.1) and MazF (GenBank™ accession no P33645). S represents β sheet strand, and H represents α helices. B, characterization of ChpBK toxicity. E. coli BW25113 (ΔaraBAD) cells were transformed with either pBAD-ChpBK or pBAD plasmids. The cells were spread on glycerol-M9-casamino acids agar plates with and without arabinose (0.2%) and these plates were incubated at 37°C for 24 h.

FIG. 2. Effect of ChpBK induction on cell growth and protein synthesis. A, Growth curve of E. coli BW25113 cells containing pBAD-ChpBK. E. coli BW25113 cells transformed with pBAD-ChpBK were grown in glycerol-M9 medium supplemented with an amino acid mixture excluding methionine and cysteine. When the A600 value of the culture reached 0.45, the culture was divided into two equal parts. To one part, arabinose was added to a final concentration of 0.2%, and to the second part, an equivalent volume of water was added. The cell densities were measured at different time points. B, effect of ChpBK on [35S]methionine incorporation in vivo. At the different time intervals indicated, 0.6 ml of the culture was taken into a test tube containing 20 μCi of [35S]methionine, and the mixture was incubated for 1 min at 37°C. After the incubation, 50 μl of the reaction mixture was applied to a filter paper disk (Whatman 3 mm, 2.3 cm diameter). The filter paper disks were treated in 10% trichloroacetic acid solution as described previously (25). The radioactivity on the filter was determined in a liquid scintillation counter. C, SDS-PAGE analysis of [35S]methionine incorporation in vivo. The culture was prepared for the same time points as described for B. 500 μl of the reaction mixture was put into a chilled test tube containing 100 μg/ml non-radioactive methionine and cysteine, and cells were collected by centrifugation and were dissolved in 50 μl of SDS-PAGE loading buffer. The mixtures were incubated in a boiling water bath for 30 min. After removing insoluble materials by centrifugation, the supernatant (10 μl) was analyzed by SDS-PAGE.
pET28a-Era, which was used for the production of the era mRNA (23). The era gene was also cloned into pINIII vector to create pIN-Era, which was used to identify the in vivo era mRNA cleavage sites by the primer extension (23).

Protein Purification—For purification of ChpBK(His)₆, pET21c-Chp-BK(His)₆ was introduced into E. coli BL21(DE3). The co-expression of ChpBI and ChpBK(His)₆ was induced in the presence of 1 mM IPTG for 6 h. The ChpBI-ChpBK(His)₆ complex was purified with use of Ni-NTA¹ (Qiagen). To purify ChpBK(His)₆ from the purified ChpBI-ChpBK(His)₆ complex, ChpBI in the purified ChpBI-ChpBK(His)₆ complex was dissociated from ChpBK(His)₆ in 6 M guanidine HCl. Denatured ChpBK(His)₆ was retrapped on Ni-NTA resin, then eluted and refolded by stepwise dialysis as carried out for purification of MazF (16). ChpBK(His)₆ is referred to as ChpBK in the text.

Assay of Protein Synthesis in Vivo—E. coli BW25113 cells containing pBAD-ChpBK were grown in M9 medium with 0.5% glycerol (no glucose) and all the amino acids except methionine and cysteine (1 mM each). When the A₆₀₀ value of the culture reached 0.45, arabinose was added to a final concentration of 0.2% to induce ChpBK expression. Cell cultures (0.6 ml) were taken at time intervals as indicated in Fig. 2 and mixed with 200 μCi of [³⁵S]methionine. After 1-min incubation at 37 °C, the rate of protein synthesis was determined as described previously (16). To prepare the samples for SDS-PAGE analysis of the total cellular protein synthesis, samples were removed from the [³⁵S]methionine incorporation reaction mixture (500 μl) at time intervals indicated in Fig. 2 and added into a chilled test tube containing 100 μg/ml each of non-radioactive methionine and cysteine. Cell pellets collected by centrifugation were dissolved into 50 μl of loading buffer and subjected to SDS-PAGE followed by autoradiography.

Primer Extension Analysis in Vivo and in Vitro—For primer extension analysis of mRNA cleavage sites in vivo, pIN-MazG or pIN-Era plasmid was transformed into E. coli BW25113 cells containing pBAD-ChpBK. The mazG or era mRNA transcription was induced by the addition of 1 mM IPTG. After 1-h induction, ChpBK was induced by the addition of arabinose (a final concentration of 0.2%). Total RNA was isolated at time intervals as indicated in Fig. 3. Primer extension was carried out using different primers as described previously (16). For in vitro primer extension analysis, the full-length mazG and era mRNAs were synthesized in vitro by T7 RNA polymerase from the DNA fragment containing a T7 promotor sequence and mazG or era ORFs using the RiboMAX™ T7 large-scale RNA production system (Promega). The mazG and era ORFs were cloned into pET-11a and pET-28a, respectively, as described previously (22, 23). The mazG and era DNA fragments were amplified using the forward primer (CCCGCGAATTAATTGACTGACCTAATAG, T7 promotor) and the reverse primers starting from the 3'-end of the mazG or era ORFs, respectively. The mazG

¹The abbreviations used are: Ni-NTA, nickel-nitrilotriacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; ORF, open reading frame; MALDI, matrix-assisted laser desorption ionization.

**Fig. 3.** Primer extension analysis of ChpBK cleavage sites in the mazG and era mRNAs in vivo. The mazG and era mRNAs was prepared by using pIN-MazG and pIN-Era in the presence of 1 mM IPTG, respectively, before and after the induction of ChpBK as described under “Experimental Procedures.” The sequence ladders for the mazG and era mRNAs were obtained using pCR®2.1-TOPO®-MazG and pCR®2.1-TOPO®-Era as template, respectively. The sequences around the ChpBK cleavage sites are shown at the bottom, and the cleavage sites are shown by arrowheads. A–E, primer extension analysis of ChpBK cleavage sites in the era mRNA was carried out with primer E1 (A and B), E2 (D and E) and E3 (C). F–I, primer extension analysis of ChpBK cleavage sites in the mazG mRNA was carried out with primer G1 (F and G) and G2 (H and I).
mRNA thus produced is 860 bases in length and the \textit{era} mRNA 977 bases. The 151-base \textit{mazG} mRNA fragment used as a ChpBK substrate in Figs. 4B and 9 was synthesized in vitro as described previously (16). This fragment consisted of a 71-base sequence from pET-11a and an 80-base sequence from the \textit{mazG} ORF from Met1 to the second base of Gln27. RNA substrates were partially digested with purified ChpBK at

\textbf{FIG. 4.} Primer extension analysis of ChpBK cleavage sites in the \textit{mazG} and \textit{era} mRNAs in vitro. \textit{A}, purification of ChpBK protein. \textit{Lane 1}, total cellular proteins after ChpBIK induction for 6 h; \textit{lane 2}, the ChpBIK complex purified using Ni-NTA resin; \textit{lane 3}, ChpBK protein purified after 6 M guanidine HCl denaturation and renaturation. \textit{B}, primer extension analysis of the ChpBK cleavage site in the 151-base \textit{mazG} mRNA. The experiment was carried out as described under “Experimental Procedures.” The sequence ladder was obtained using pCR®2.1-TOPO®-MazG as template with primer G3. \textit{Lane 1}, control reaction without the addition of ChpBK; \textit{lane 2}, the reaction with ChpBIK; \textit{lane 3}, the reaction with ChpBK. \textit{C–E}, primer extension analysis of ChpBK cleavage sites in the \textit{era} mRNA. The experiment was carried out as described under “Experimental Procedures.” The sequence ladder was obtained using pCR®2.1-TOPO®-Era as template with primers E1 (C and D) and E2 (E). \textit{F} and \textit{G}, primer extension analysis of ChpBK cleavage sites in the \textit{mazG} mRNA. \textit{Lane 1}, control reaction without the addition of ChpBK; \textit{lane 2}, the reaction with ChpBIK; \textit{lane 3}, the reaction with ChpBK. The primers used were identical to those used for primer extension in vitro.

\textbf{FIG. 5.} Effects of mutations in a ChpBK cleavage site on the \textit{mazG} mRNA on the cleavage by ChpBK. \textit{A–D}, site-directed mutagenesis was performed with pET-11a-MazG as template to generate all substitution mutations (23). All mutations were confirmed by DNA sequence analysis. The \textit{mazG} RNA fragment was synthesized in vitro by T7 RNA polymerase from the DNA fragment containing a T7 promoter and the mutated \textit{mazG} DNA fragment using RiboMAX™ T7 large-scale RNA production system (Promega). The resulting 151-base RNA thus consisted of a 71-base sequence from pET-11a and an 80-base sequence from the \textit{mazG} ORF from Met1 to the second base of Gln27. ChpBK (2.5 \mu g) was incubated with 1 \mu g of the \textit{mazG} RNA fragment in a 10-\mu l reaction mixture containing 10 mM Tris-HCl (pH 7.8) at 37 °C for 25 min; partial digestion products were purified with the RNAeasy column (Qiagen) to remove ChpBK protein. Primer G3 (TGCTCTTTATCCCACGGGCA) was used for the primer extension analysis. The primer was 5’-labeled with [\gamma-\text{32P}]ATP using T4 polynucleotide kinase. Primer extension reactions were performed at 42 °C for 30 min. The control experiments were performed under the same conditions except that ChpBK was not added in the digestion reaction mixture.
37 °C for 15 min. The digestion reaction mixture (10 μl) consisted of 2 μg of RNA substrate, 1 μg of ChpBK(His)₆ and 0.5 μl of RNase inhibitor in 10 mM Tris-HCl (pH 7.8). Partial digestion products were purified with the RNeasy column (Qiagen) to remove ChpBK protein. Primer extension was carried out at 42 °C for 1 h in 20 μl of the reaction mixture as described previously. The reactions were stopped by adding the 12 μl of sequence loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol EF). The samples were incubated at 90 °C for 5 min prior to electrophoresis on a 6% polyacrylamide and 36% urea gel. The primers G1 (GCCCAGTTCACCGCGAAGATCGTC), G2 (CATTTCCTCCTCCAGTTTAGCCTGGTC), and G3 (TGCTCTTTATCCCACGGGCAG) were used for primer extension analysis of the mazG mRNA; the primers E1 (CAGTTCAGCGCAGGAAACGCAT), E2 (GATCCCCACAATGCGGTGACGAGT), and E3 (ATGACCATCTTCTTCTGCCCTT CA) were used for primer extension analysis of the era mRNA. The primers were 5'-labeled with [γ-32P]ATP using T4 polynucleotide kinase.

Cleavage of Synthetic RNA by ChpBK—Oligoribonucleotides (11 base in length; see Fig. 6) were commercially synthesized and 5'-labeled with [γ-32P]ATP using T4 polynucleotide kinase. Endoribonuclease activity was assayed in 10 μl of the reaction mixture containing 0.5 μl (20 units) of ribonuclease inhibitor, 1 μg of ChpBK and 32P-labeled oligonucleotides in 10 mM Tris-HCl (pH 7.8). Reactions were carried out at 37 °C for 30 min and stopped by adding the sequence loading buffer as described above. The reaction mixtures were then subjected to 20% sequence gel electrophoresis followed by autoradiography. Note that under the present condition for the cleavage reaction, the RNA substrate concentrations are lower than the enzyme concentration. However, the synthetic RNA substrates were cleaved linearly in an enzyme concentration-dependent manner and also in a time-dependent manner (Figs. 8 and 9 A).

Sample Preparation for MALDI Mass Spectrometry—The saturated 3-hydroxypicolinic acid matrix solution was prepared by dissolving 5 mg of 3-hydroxypicolinic acid (Sigma) in 100 μl of 50 mM diammonium citrate (Sigma) containing 25% acetonitrile. One microliter of an RNA
A (4 μl) sample with or without ChpBK in 10 mM Tris-HCl (pH 7.8) was spotted onto a stainless steel sample plate, and the sample was dried at room temperature. The saturated matrix solution (0.5 μl) was then spotted on the dried sample. After the sample was completely dried, mass measurements were carried out using a Voyager DE PRO MALDI time-of-flight mass spectrometer (Applied Biosystems).

RESULTS AND DISCUSSION

The Effect of ChpBK on Cell Growth and Protein Synthesis in Vivo—The chpBK gene was cloned into an arabinose inducible pBAD plasmid (21). E. coli BW25113 cells carrying pBAD-ChpBK could not form colonies on glycerol-M9-casamino acids agar plates in the presence of arabinose (0.2%) (Fig. 1B). Fig. 2A shows growth curves of E. coli BW25113 cells carrying pBAD-ChpBK in glycerol-M9 medium containing all amino acids except methionine and cysteine (1 mM each). Arabinose was added to exponentially growing cells at a final concentration of 0.2% (closed squares). Addition of arabinose severely inhibited the cell growth. At 30 min after the addition of arabinose, cell growth was almost completely blocked. Next we examined the effect of ChpBK induction on protein synthesis as measured by [35S]methionine incorporation (Fig. 2B). Within 10 min of ChpBK induction, the total protein synthesis was reduced by ~60% (closed squares). Interestingly, there was no significant further reduction of [35S]methionine incorporation after 15 min. These samples were analyzed by SDS-PAGE (Fig. 2C). Consistent with the result in Fig. 2B, ChpBK did not completely block incorporation of [35S]methionine into cellular proteins, and a low level of cellular protein synthesis is observed even at 60 min after ChpBK induction. It should be noted that a new band appeared 5 min after ChpBK induction as indicated by an “arrow a” (Fig. 2C), of which molecular mass was consistent with the calculated molecular mass of ChpBK (12.5 KDa). The production of this protein continued for 60 min, although the rate of its synthesis was reduced after 20 min. Induction of MazF under a similar condition almost completely blocked [35S]methionine incorporation (at 15 min after the addition of arabinose (16)), indicating that ChpBK is less detrimental to the cells than MazF, although the induction of both ChpBK and MazF results in inhibition of colony formation on agar plates.

In Vivo Cleavage of the mazG and era mRNAs by ChpBK—As ChpBK has a sequence very similar to that of MazF (Fig. 1A), we speculate that the observed inhibition of protein synthesis by ChpBK is due to its mRNA interferase function. To test this, the ChpBK-mediated cleavage of the era and mazG mRNAs was examined by primer extension experiments. For this purpose, E. coli BW25113 cells overproducing these mRNAs were used as described previously (6, 16). The primer extension analyses of era (Fig. 3, A–E) and mazG RNAs (Fig. 3, F–I) using different primers clearly demonstrate that distinct bands exhibiting the specific cleavage sites in each mRNA appeared at 5 min after ChpBK induction (Fig. 3, A–I, lane 2). In most cases, the band intensities further increased from 10 to 90 min after ChpBK induction (Fig. 3, B and D and F–I). Importantly, all these bands were very faint or hardly detected at 0 min (Fig. 3, A–I, lane 1). All cleavage sites (XACY) share an AC dinucleotide and the cleavage occurred before or after the A residue, indicating that similar to MazF, ChpBK cleaves mRNAs at specific sequences in vivo. Notably, the C residue was not found in X and Y positions in these cleavage sites.

FIG. 7. Comparison of the cleavage specificity of ChpBK with MazF. A, primer extension analysis of the mazG mRNA in vivo after MazF induction. The mazG mRNA was produced using pIN-MazG in the presence of 1 mM IPTG before the induction of MazF or ChpBK as described under “Experimental Procedures.” The total RNAs were extracted at various time points indicated before (lane 1) and after MazF (lanes 2–5) and ChpBK induction (lanes 2–7). Primer extension analyses were carried out with the same primer for both A and B. Each cleavage site is shown below the gel. C, cleavage sites analysis of the mazG mRNA in vivo after MazF induction.

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In Vitro Cleavage of the mazG and era mRNAs by ChpBK—To further prove that the in vivo cleavage of mazG and era mRNAs observed above was due to the mRNA interferase activity of ChpBK, the in vitro cleavage of these mRNAs was tested with purified ChpBK. To purify ChpBK, the purified ChpBI-ChpBK(His)6 complex was denatured using 6 M guanidine HCl. Denatured ChpBK was then retrapped on Ni-NTA resin, eluted, and refolded by stepwise dialysis described previously for MazF (Fig. 4A, lane 3) (16). Note that the ChpBI-ChpBK complex did not show any endoribonuclease activity (Fig. 4B, lane 2). On the other hand, purified ChpBK cleaved the 151-base mazG mRNA at the UAC sequence as shown by an arrowhead in lane 3, Fig. 4B. The in vitro activity of purified ChpBK on full-length mazG and era mRNAs was also analyzed.
Electrophoresis followed by ethidium bromide staining. The position of RNA molecular weight markers is shown at the left hand side of the gel.

was stopped by the addition of SDS at the final concentration of 0.5%. The reaction products were analyzed by 6% acrylamide native gel electrophoresis followed by autoradiography.

was added at the final concentration of 0.5% to stop the reaction. The reaction products were analyzed by 20% sequence gel electrophoresis followed by primer extension using the same primers used in the

experiment. The primer used in Fig. 3, and was mutated to a U residue, RNA cleavage was observed (Fig. 5, D). However, when A4 was mutated to a C residue, no cleavage was observed (Fig. 5, C).

mRNAs at the ACY sequences (Fig. 3, A). Cleavage of Short RNAs by ChpBK—To further characterize the enzymatic specificity of ChpBK with that of MazF, the in vitro primer extension experiment was carried out using the mazG mRNA. Both ChpBK and MazF were induced using the pBAD vector in BW 25113 cells harboring pIN-MazG. After the addition of arabinose (0.2%), cells were removed at time intervals shown in Fig. 7 for RNA extraction, and primer extension was carried out with the same primer used in Fig. 3, F and G. As seen in Fig. 7, the ChpBK cleavage specificity is distinctly different from that of MazF. The cleavage of mazG mRNA by ChpBK resulted in two bands at sites “a” and “b” (Fig. 7B), which correspond to the bands in Fig. 3, F and G. On the other hand, MazF did not cleave the mazG mRNA at the site “a” at all, while it cleaved the RNA at sites “1” and “2” (Fig. 7, A and C). These results indicate that both enzymes are able to cleave UA ↓ CA (site 2), while UA ↓ CG can be cleaved only by ChpBK but not by MazF. On the other hand, G ↓ ACA can be cleaved by MazF but not by ChpBK.

When the nucleotide sequences of the mazG mRNA and era mRNA were examined within the regions covered by the primer extension experiments (Fig. 3), ACY sequences were not always cleaved (Table I). ACC sequences were not cleaved as predicted from the mutagenesis experiment (Fig. 5), and interestingly a number of supposedly cleavable ACY sequences in mRNAs, there are preferential cleavage sites as found with MazF (23). The reason for this preferential cleavage is not known at present; however, the secondary structures and sequence contexts around the ACY sequences may influence RNA cleavage by ChpBK.

It is interesting to note that complete cleavage of the mazG mRNA by MazF was observed by 5 min after induction as judged from the density of the band of the full-length mRNA (Fig. 7A), while the cleavage by ChpBK was much slower and a substantial amount of the full-length mRNA was visible even 90 min after induction (Fig. 7B). This appears to be consistent with the fact that ChpBK only partially inhibited [35S]methi-
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online incorporation even 60 min after its induction (Fig. 2C) in contrast to MazF, which almost completely inhibited \([\text{35S}]\)methionine within 20 min after its induction (16). The reason for this weaker endoribonuclease activity of ChpBK compared with that of MazF is not clear at present. It is possible that ChpBK is intrinsically a less potent toxin than MazF. Interestingly, the pl value of ChpBK is 5.2 in contrast to basic pl values of MazF (8.3) and PemK (11.0); the basic pl may be more favorable for RNA cleavage enzymes. The weaker toxic effect of ChpBK may be at least partially attributed by its acidic pl and important for its physiology role in the cells as ChpBK may arrest the cell growth in a less damaging manner than MazF.

Quantitative Enzyme Analysis of ChpBK—In our RNAse analysis, the concentrations of RNA substrates are lower than the enzyme concentrations. This is because the RNA substrates are not readily accessible in a lager quantity. However, the cleavage reaction under the condition used is linear to the enzyme concentration (Fig. 8). In this experiment, we used two substrates, RB-11-1 and RB-11-2 (see Fig. 6A), which were labeled with \([\gamma-32P]\)ATP at the 5'-end. The reaction products, which were analyzed by 20% acrylamide gel electrophoresis (Fig. 8A for RB-11-1 and Fig. 8B for RB-11-2). With use of 0.44 \(\mu\)g of ChpBK, about 90% of RB-11-1 was cleaved, while only about 20% of RB-11-2 was hydrolyzed (see Fig. 8, A and B, lane 4, and also Fig. 5C), indicating that there is a preferred base for ChpBK cleavage at the X base in the XACY cleavage site (U > G). This is consistent with the mutational analysis of the cleavage site shown in Fig. 5A.

Fig. 9 shows time courses of the ChpBK cleavage reaction with use of RB-11-1 (Fig. 9A) and the 151-base mazG mRNA (Fig. 9B). Both substrates were cleaved linearly in a time-dependent manner, and the cleavage reaction with a longer RNA substrate is much faster (~5-fold) than that with a shorter RNA substrate. Note that the 151-base mazG mRNA was separated into two bands (zero time in Fig. 9B), and only the lower band was completely digested into two products (84 and 67 bases). At present the nature of the upper band is unknown. As the refolding efficiency of ChpBK used in the present study is unknown, the reaction with both substrates might be faster than that in Fig. 9.

Conclusion—In the present paper, we demonstrated that ChpBK, the toxin encoded by the chpBI-chpBK addiction module, is a sequence-specific endoribonuclease functioning as an mRNA interferase. ChpBK cleaves preferentially at the 5’-side or 3’-side of the A residue in ACY (Y is U, A, or G) sequences and generates a 2’,3’-cyclic phosphate group at the 3’-end of the 5’-end product and a 5’-OH group at the 5’-end of the 3’-end product. This suggests that both ChpBK and MazF (16) cleave single-strand RNAs in a manner similar to that of ribonuclease A. Our results also show that the RNA cleavage by ChpBK is independent of ribosomes, a characteristic distinctly different from RelE (10), the toxin encoded by the relBE addiction module. RelE cannot cleave free RNAs but appears to be a ribosome-associated factor that stimulates mRNA cleavage at the ribosome A site with a high codon specificity (11).

The x-ray crystal structure of the MazE-MazF complex has been determined, which consists of two MazF homodimers and one MazE homodimer (MazF2-MazE-MazF2) (12). The crystal structure of MazE has also been solved, and the mechanism of its interaction with the MazE-MazF promoter region has been proposed (13, 14). Because of high sequence similarities between ChpBK and MazF (52%, in Fig. 1A) and between ChpBI and MazE (54%), ChpBK likely forms a complex with ChpBI in a manner similar to that of the MazE-MazF complex. Interestingly, the conserved loops between \(\beta\) strands S1 and S2 (termed the S1-S2 loops) in the MazE-bound MazF homodimer project into solvent and are mostly disordered (12), while in the Kid (PemK) homodimer the two corresponding loops are in a “closed” conformation (24). The S1-S2 loops of the Kid (PemK) homodimer form a cavity-like structure covering a basic surface and a conserved hydrophobic pocket. The conserved hydrophobic pocket plays an essential role in the recognition of MazE in the MazE-MazF complex formation (12). We have proposed that the highly negatively charged C-terminal extension of MazE may mimic single-stranded RNA substrates (16). We also speculate that the S1-S2 loop in the MazF homodimer may play an essential role in the enzymatic specificity. Notably there are four amino acid residues in the 11-residue loop different between any two enzymes in Fig. 1A, which may be responsible for cleavage specificities.

The present study reveals that \(E.\ coli\) contains at least two different mRNA interferases, MazF and ChpBK. Another toxin, YoeB, has also been reported from \(E.\ coli\) (1). As judged by an in vivo experiment, YoeB may also function as an mRNA interferase. These toxins clearly function as inhibitors for cell growth. Recently, we proposed that MazF expression induces the quasi-dormant state in living cells, a previously unknown physiological state of bacteria, which may play an important role in bacterial survival under stress conditions in nature (19). Further characterization of these toxins and their gene expression will provide clues to how to regulate cell growth of not only \(E.\ coli\) but also pathogenic bacteria.

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