RNase/Anti-RNase Activities of the Bacterial parD Toxin-Antitoxin System

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Programmed cell death in bacteria is mediated by specialized chromosomal modules called the toxin-antitoxin (TA) systems (11, 17, 33). These systems, formerly discovered in bacterial plasmids, were shown to contribute to plasmid maintenance by preventing the growth of plasmid-free progeny through a mechanism called postsegregational killing (13, 18). Later, it was discovered that TA systems were widely conserved in bacterial and archaeal chromosomes (2, 12). Generally, TA systems consist of two genes that encode a stable and potent toxin and an unstable antitoxin, respectively (10, 12). The antitoxins neutralize the toxin by direct protein-protein interaction but are degraded by cellular proteases (12).

Among TA systems, one of the best characterized is the parD operon of plasmid R1 (4), also found in plasmid R100 and called pem (38). Its toxin and antitoxin pair of proteins, respectively Kid (PemK) and Kis (PemI), has been shown to form a tight nontoxic complex that autoregulates parD at the level of transcription (34). The Kid toxin inhibits cell growth in Escherichia coli, while the Kis antitoxin and were not displayed by the KidR85W variant, which is nontoxic in E. coli. Moreover, while Kid cleaved single- and double-stranded RNA with a preference for UAA or UAC triplets, KidR85W maintained this sequence preference but hardly cleaved double-stranded RNA. Kid was formerly shown to inhibit DNA replication of the ColE1 plasmid. Here we provide in vitro evidence that Kid cleaves the ColE1 RNA II primer, which is required for the initiation of ColE1 replication. In contrast, KidR85W did not affect the stability of RNA II, nor did it inhibit the in vitro replication of ColE1. Thus, the endoribonuclease and the cytotoxic and DNA replication-inhibitory activities of Kid seem tightly correlated. We propose that the spectrum of action of this toxin extends beyond the sole inhibition of protein synthesis to control a broad range of RNA-regulated cellular processes.

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shown to cleave only single-stranded RNA preferentially at UAH sequences, where H is C, A, or U (40). Thus, the mode of action of the Kid toxin and how it accounts for the known in vivo activities of the protein are still an unsolved question.

In this paper we approach this issue by providing evidence that the Kid toxin promotes RNA cleavage and inhibits protein synthesis in mammalian cell-free systems. We show that the Kis antitoxin specifically reverses these effects and that a specific Kid variant that is nontoxic in E. coli fails to degrade RNA and to inhibit protein synthesis in the eukaryotic cell-free system. Moreover, we illustrate the inhibition of ColE1 DNA replication by Kid, a phenomenon that was previously described (32, 35), by presenting clues that link this effect to the RNase activity of the toxin. In addition, we provide an independent analysis of RNA cleavage by Kid that further documents and clarifies the mechanisms underlying this activity.

MATERIALS AND METHODS

Protein purification. The Kid toxin and the His6-Kis antitoxin were purified as described previously (15). The KidR85W mutant was purified by an identical procedure (36). Briefly, lysates were prepared from induced cultures of E. coli C600 strains overproducing the toxin protein together with the His6-tagged antitoxin protein. Lysates were clarified, and the soluble fraction was loaded onto an Ni affinity column. The complexes between the toxin and the His6 antitoxin remained tightly bound to the affinity column. To release the strong interaction between the proteins, it was necessary to denature with 5 M guanidine-HCl. Refolding of the toxin was accomplished by a dialysis procedure (14). Finally, the refolded sample was loaded onto an SP-Sepharose column and the bound toxin was eluted using a KCl gradient. The His6 antitoxin retained in the Ni affinity column after elution of the toxin with 5 M guanidine-HCl was eluted from the Ni column under denaturing conditions by the addition of 50 mM EDTA. The eluted protein was refolded basically as described for the toxin, and then it was further purified by Q-Sepharose chromatography using the same loading buffer and linear gradient used for chromatography of the toxin. The purified proteins were concentrated in the same elution buffer using a 50-ml Amicon cell fitted with a Diaflow PM-10 membrane. The proteins were diluted in 50 mM KCl, 20 mM HEPES, pH 7.8, 100 μg/ml BSA before use.

Protein synthesis in E. coli. In vitro protein synthesis in E. coli was monitored by using the E. coli S30 Extract System for Circular DNA (Promega). Initial reaction mixtures (10 μl) contained pH5 E. coli plasmid DNA (400 ng), 3 μl of S30 extract, 4 μl of S30 premix, and 1 μl of a mixture of amino acids minus methionine (1 mM). The assay was started by adding 3 μCi of [35S]methionine and 0.4 μl of dilutions of the purified protein Kid, Kis, or KidR85W followed by incubation for 30 min at 37°C. The reactions were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%).

Protein synthesis and mRNA stability in rabbit reticulocyte lysates. Protein synthesis was monitored using the Rabbit Reticulocyte Lysate system (Promega). Initial reaction mixtures (10 μl) contained 7 μl of rabbit reticulocyte lysate, 0.2 μl of amino acid mixture minus methionine (1 mM), 0.1 μl of luciferase control RNA, and 0.3 μl of KCl (70 mM, final concentration), complemented with 0.2 μl of RNAGuard RNase Inhibitor (Amersham Pharmacia Biotech). The assays were started by adding 4 μl of [35S]methionine and 3 pmol of the purified proteins and incubating for 1 h at 30°C. The reactions were analyzed using SDS-PAGE (10%).

For RNA stability analyses, uniformly labeled luciferase mRNA was obtained from in vitro transcription with T7 RNA polymerase (3) in the presence of [α-32P]UTP and using as a DNA template a PCR-generated product based on the LTRW-LUC plasmid (1) and the pair of primers 5′-TATAGCTCTTACAATTTGAGCTTTC3′ and 5′-TATACGACTCACTATAGTTGTG GATATAGAAGACG3′. The RNA was fractionated by electrophoresis on a 4% polyacrylamide gel containing 7 M urea in TBE buffer.

In vitro RNA cleavage analysis. The CopA, CopT, and TAR RNAs were prepared and 5′ end labeled using [γ-32P]ATP as described previously (29). The cleavage reactions were carried out using 1,000 cpm of 5′-end-labeled RNAs in 10 mM KCl, 10 μg/ml bovine serum albumin, 2 mM HEPES, pH 7.8, in the presence of 4 U of SUPERase-In (Ambion). The purified Kid, KidR85W, and His6-Kis were added when appropriate, and the reaction mixtures were incubated for 2 min at 37°C. The reactions were stopped by adding formamide loading buffer and chilling quickly in dry ice. The RNAs were separated on 6% polyacrylamide gels containing 7 M urea in TBE buffer. The control reactions (alkali ladders and digestions with RNase T1) were performed as described above (29).

RESULTS

Correlation between inhibition of protein synthesis and toxicity in E. coli. Since the Kid toxin inhibits protein synthesis in E. coli (40), it was intuitively assumed that this activity accounted for the toxicity of the protein. According to this view, a Kid mutation that would specifically disrupt the toxicity of the protein should also prevent its inhibitory effect on protein synthesis. To test this hypothesis, we used KidR85W, a highly specific nontoxic variant of Kid that has been thoroughly characterized at the genetic, biochemical, and biophysical levels (16, 25, 36). This mutant bears a single R85W amino acid change that completely inactivates the toxicity of the protein in E. coli (25) without significantly affecting its state of association, thermal stability, or overall structure in solution (36). Moreover, the KidR85W protein efficiently interacts with the Kis antitoxin in vitro (see Materials and Methods) and in vivo (as monitored in transcriptional autoregulation assays) (36). To monitor protein synthesis, we used an E. coli cell-free system which allows detecting the products of the coupled transcription/translation of a DNA template expressing the firefly luciferase gene (see Materials and Methods). This yielded several protein products, including the full-length luciferase protein (Fig. 1A). Addition of increasing concentrations of the purified wild-type Kid protein resulted in a marked inhibition of protein synthesis that was particularly evident in the case of luciferase and of other high-molecular-weight protein products. As a control of specificity, we used a His6-tagged version of the Kis antitoxin (referred to as Kis throughout this study) which, as the native protein does, forms a tight complex with the Kid toxin in vitro (see Materials and Methods) and neutralizes its toxicity in vivo (our unpublished results). Indeed, addition of the Kis antitoxin abolished the inhibition of protein synthesis by Kid, thus indicating that this activity was specific (Fig. 1A). Furthermore, when the purified KidR85W protein was tested at a protein concentration that was severely inhibitory in the case of wild-type Kid, no detectable effect on
protein synthesis was observed (Fig. 1A). Consistent with this, KidR85W failed to inhibit protein synthesis in *E. coli* cells in vivo (data not shown). Thus, these results indicated that the inhibition of protein synthesis and the toxicity of the Kid protein in *E. coli* cells were tightly correlated.

Kid inhibits protein synthesis in rabbit reticulocyte lysates. Considering that Kid was toxic in eukaryotic cells (9), we examined whether protein synthesis would also be affected by Kid in a eukaryotic cell-free system. We used a rabbit reticulocyte lysate translation system and a purified mRNA encoding the firefly luciferase as a template. In the presence of Kid concentrations that were inhibitory in *E. coli* extracts, the synthesis of the luciferase protein was dramatically impaired in rabbit reticulocyte lysates (Fig. 1B). This effect was completely neutralized by the Kis antitoxin, and no inhibition was observed when the KidR85W mutant was used. Therefore, Kid inhibited protein synthesis both in prokaryotic and eukaryotic cell-free systems, and these activities were not only specifically counteracted by Kis but were also disrupted by the R85W mutation, which abolished Kid toxicity in *E. coli* cells. This provided strong evidence that the mechanism of action of Kid was conserved in eukaryotes and prokaryotes.

RNA cleavage by Kid is modified in cell extracts. Since Kid is an endoribonuclease (40) and this activity was proposed to account for both the inhibition of protein synthesis and the in vivo toxic effects of Kid, we analyzed whether RNA cleavage by Kid would be affected by (i) the nontoxic R85W mutation and (ii) the presence of cell extracts. When Kid was added directly to gel-purified 32P-labeled luciferase mRNA, several cleavage products were produced (Fig. 1C). These products were absent when Kid and Kis were added together. When the KidR85W mutant protein was used, fewer cleavage products were observed, whose size corresponded to products obtained with the wild-type Kid protein. This suggested that KidR85W retained a specific RNA cleavage activity, albeit markedly reduced compared to the wild-type Kid toxin. Since the toxicity of KidR85W is completely abolished in *E. coli* cells (25, 36) and this protein has no detectable effects on protein synthesis (see above), these results suggested either that there was no strict correlation between RNA cleavage and protein synthesis inhibition or that the RNA cleavage by KidR85W was inhibited in the presence of cellular factors. To address this issue, gel-purified luciferase mRNA was incubated with rabbit reticulocyte lysates before the cleavage reaction was initiated in the presence of Kid or KidR85W (Fig. 1D). Surprisingly, while the cleavage by Kid was clearly apparent, the activity of KidR85W was dramatically inhibited in the rabbit reticulocyte lysates (Fig. 1D). Incubation with the Kis antitoxin prevented the degradation of RNA by Kid and by KidR85W, irrespective of the presence of cell extracts (Fig. 1C and D; see also results below). Similar results were obtained when *E. coli* S30 extracts were used instead of rabbit reticulocyte lysates (data not shown). Altogether, the RNA cleavage pattern in cell extracts was consistent with the results obtained when protein synthesis was monitored, indicating that both activities were tightly correlated.

The R85W mutation affects but does not abolish RNA cleavage by Kid in vitro. To further analyze the endoribonuclease activities of Kid and of its nontoxic variant KidR85W, we performed an RNA cleavage analysis using a panel of three
small model RNA molecules (TAR, CopA, and CopT RNAs) that were previously used to characterize the endoribonuclease activity of the MazF toxin (29). The three RNAs were 5′ end labeled with 32P, purified, and challenged with increasing amounts of the purified Kid toxin prior to being fractionated by denaturing polyacrylamide gel electrophoresis. Also included were RNA samples treated either with alkali or with RNase T1 to allow the precise mapping of the cleavage sites. TAR, CopA, and CopT RNAs were present as single RNA species that were cleaved in the three cases by the Kid toxin (Fig. 2A). The cleavage products were absent when Kid was provided together with the Kis antitoxin, thus indicating that the activity was specific. Kid cleaved the TAR RNA at four positions, with a clear preference for three sites in which cleavage occurred at the 5′ or 3′ side of the A residue in the dinucleotide sequence 5′-UA-3′ (Fig. 2). Of these sites, the one that was situated in the 3′ single-stranded region of TAR RNA was cut at the lowest concentration of Kid, indicating a preferential accessibility to cleavage (Fig. 2, empty squares, corresponding to TAR). This general tendency in cleavage preference was confirmed by the analysis performed on the CopA and CopT RNAs. In both cases, the most intense bands corresponded to cleavage at UAU, but it can be argued that the UAU sites that were present in the RNAs that we analyzed are all situated in single-stranded regions (Fig. 2). In fact, out of the 12 cleavage sites detected in the overall analysis, only 2 did not involve a 5′-UA-3′ sequence (Fig. 2C). Interestingly, 9 out of the 10 cleavage sites at 5′-UA-3′ corresponded to UAA or UAC triplets and only one to UAG. When KidR85W was used, RNA cleavage was observed at specific sites that were also cleaved by wild-type Kid (Fig. 2A). Moreover, the cleavage by KidR85W was inhibited by Kis, thereby indicating that the mutant protein formed an efficient complex with Kis and thus was properly folded (Fig. 2A). Compared to wild-type Kid, the patterns obtained using KidR85W differed in that the cleavage activity was (i) from a quantitative point of view reduced, as apparent in TAR and CopT analysis, and consistent with the results obtained with the luciferase mRNA (Fig. 1C); and (ii) restricted to cuts between the first and second nucleotides of UAA and UAC sites, predominantly in single-stranded regions, which correspond to the preferred cleavage sites for the wild-type Kid protein.

Kid processes the ColE1 RNA II primer and inhibits ColE1 replication, while KidR85W lacks both activities. The endoribonuclease activity of the Kid toxin not only provided a molecular basis for Kid toxicity but also gave clues to the mechanisms underlying other described activities of Kid, such as the inhibition of the initiation of DNA replication of the ColE1 plasmid (32, 35). Initiation of ColE1 replication requires the formation of an RNA-DNA hybrid between the RNA II precursor primer molecule and its template sequence around the origin (22). The precursor is then processed by RNase H to yield a mature 550-nucleotide primer that is subsequently elongated by DNA polymerase I to initiate ColE1 replication (22). We first asked whether the R85W mutation, which affects toxicity and RNase activities, would also affect the inhibition of ColE1 replication. We addressed this question by means of in vitro DNA replication assays using the ColE1 plasmid as a template (see Materials and Methods). While Kid inhibited ColE1 replication, consistent with former results (35), addition of KidR85W protein did not affect the replication of the ColE1 plasmid (Fig. 3A). These results revealed a correlation between the inhibition of ColE1 replication and the cytotoxicity of Kid. We then asked whether the processing of the mature RNA II primer by the Kid toxin could account for this phenomenon. Indeed, RNA II was a target for Kid, as revealed by the extensive cleavage observed upon incubation of the purified RNA II primer with Kid (Fig. 3B). This cleavage was not observed when Kis was provided together with Kid. Interestingly, the R85W mutation dramatically inhibited the cleavage of RNA II by Kid (Fig. 3B). Thus, while Kid cleaved the RNA II primer and inhibited the replication of ColE1, these activities were severely affected in the KidR85W nontoxic mutant. Altogether, these results strongly supported the hypothesis that the endoribonuclease activity of Kid was the underlying mechanism of the Kid-dependent inhibition of ColE1 plasmid replication.

**DISCUSSION**

In this work we have approached the mechanism of action of the Kid toxin at two levels. The first one, essentially mechanistic, deals with the assessment of the specificity of RNA cleavage by Kid. The second one broadens this analysis to address the question of whether the endoribonuclease activity may account for other known activities of the protein.

Formerly, the specificity of RNA cleavage by Kid was addressed by Zhang and collaborators in an independent work to show that (i) Kid cleaves at the 5′ or 3′ side of the A residue in UAC, UAA, or UAU and (ii) only the sites situated in single-stranded regions are cleaved (40). Our results are fully consistent with their first conclusion, with the sole nuance that we also see cleavage at UAG (Fig. 2C). We did not observe cleavage at UAU, but it can be argued that the UAU sites that are present in the RNAs that we analyzed are all situated in double-stranded regions (Fig. 2C). Thus, we cannot rule out that UAU in single-stranded RNA regions are target sites for Kid. We also observed cleavage either at the 5′ or 3′ side of the first A in UAA (see the cleavages symbolized respectively by a square and a triangle in TAR, Fig. 2C), again consistent with the first conclusion of Zhang et al.
interaction, which the R85W mutation disrupts, is needed for the protein. Rather, it seems that a specific type of Kid-RNA for the inhibition of protein synthesis and the cytotoxicity of sites in single-stranded RNA regions does not account by itself indicates that the capacity to cleave canonical UAA and UAC sites was generally conserved in the KidR85W nontoxic mutant. This suggests that the Kid toxin cleaves RNA with a preference for UAA or UAC sites present in single-stranded regions. How-ever, the same sequences situated in double-stranded RNA were efficiently cleaved by Kid (Fig. 2). Interestingly, the KidR85W mutant generally conserved the ability to cleave single-stranded RNA sites but was severely affected in the cleavage of sites situated in double-stranded RNA (Fig. 2C). The fact that KidR85W failed to inhibit protein synthesis in E. coli and rabbit reticulocyte cell-free systems (Fig. 1) indicates that the capacity to cleave canonical UAA and UAC sites in single-stranded RNA regions does not account by itself for the inhibition of protein synthesis and the cytotoxicity of the protein. Rather, it seems that a specific type of Kid-RNA interaction, which the R85W mutation disrupts, is needed for Kid to efficiently degrade RNA and inhibit protein synthesis in vivo. Thus, the cleavage of double-stranded RNA by Kid exists, and it is correlated with the cytotoxicity of the protein. In addition, the observation that the presence of rabbit reticulocyte lysates influences the cleavage pattern of the toxin opens the possibility that particular host factors modulate the activity of Kid in vivo. These factors, which remain unidentified, may either influence the accessibility of the Kid to the target cleavage sites or, alternatively, modify the architecture of the Kid-RNA complex to alter its activity. Addressing these mechanistic issues awaits further structural studies aimed at the resolution of such complexes, as well as direct approaches towards the identification of the host factors that influence the activity of Kid.

Since Kid inhibited the initiation of replication of the ColE1 plasmid and overexpression of the E. coli DnaB helicase neutralized the toxicity of Kid (35), it was suggested that DnaB was the target of Kid. In this paper, we show that the ColE1 RNA II primer, which is required for initiation of ColE1 replication, is efficiently cleaved by Kid in vitro. In addition, the nontoxic KidR85W mutant, which inefficiently cleaves this primer, fails to inhibit this replication. These data correlate the cleavage of the RNA primer by Kid and the inhibition of ColE1 replication. However, testing the effects of the toxin on the nascent RNA primer will be required to directly evaluate this correlation, as natural inhibition of ColE1 replication by the antisense RNA occurs during synthesis and folding of the preprimer (37). Thus, it is possible that the RNase activity of Kid accounts for its inhibitory effect on this and other RNA-dependent processes other than translation. Alternatively, DnaB overexpression could lead to indirect titration of Kid toxicity, perhaps through massive stimulation of primer synthesis, as reported for in vitro studies (23). Although the involvement of DnaB in the mechanism of action of Kid cannot be formally ruled out, we believe that there is increasing evidence, to which this work contributes, that this link is due to circumstantial, indirect effects.

In addition, we have shown that Kid inhibits protein synthesis in prokaryotic and eukaryotic cell extracts. Both activities were counteracted by the Kid antitoxin and were not displayed by the KidR85W mutant, which is nontoxic in E. coli cells. Thus, our results show that inhibition of protein synthesis and toxicity in E. coli cells are two tightly linked phenomena. Our data also provide a hint on the possible mechanism that accounts for the cytotoxicity of Kid in eukaryotes. There are other bacterial cytoxic RNases that inhibit protein synthesis and provoke the death of eukaryotic cells (reviewed in reference 26), such as RNases Sa2 and Sa3 of Streptomyces aureofaciens (19, 20, 26), colicin E3 of E. coli (8), barnase of Bacillus amyloliquefaciens (21), and binase of Bacillus intermedius (21). Although there is no available evidence of structural or functional similarities between these toxins and Kid, it is interesting that in general small, positively charged RNases like Kid have the most potent cytoxic effect in tumor cell lines (26). Conditional death by apoptosis induced by Kid in such cell lines has been described (9). Whether Kid, MazF, or other toxins of this family will play a valuable role in the field of antitumoral research remains to be tested. However, inasmuch as the RNA cleavages by Kid and MazF are not extremely specific in terms of primary and secondary structure requirements (29; this

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FIG. 3. ColE1 plasmid replication and Kid. (A) DNA replication assays. The ColE1 plasmid was allowed to replicate in type II E. coli extracts in the presence of [32P]dCTP and of increasing concentrations of Kid or KidR85W. Proteins were added at 175 nM (lanes 3 and 6), 350 nM (lanes 4 and 7), and 700 nM (lanes 5 and 8). Also shown is the replication in the absence of exogenous proteins (lane 1) and in the presence of rifampin (Rif, 25 g/ml, lane 2). The reactions were analyzed by agarose gel electrophoresis. The gels were stained with ethidium bromide (upper panel) prior to being dried and subjected to autoradiography (lower panel). RFI and RFI indicate the positions of covalently closed and open circular DNA, respectively. (B) Stability of the ColE1 primer RNA II. The uniformly labeled RNA II (indicated by the “R” arrow) was incubated for 10 min at 37°C without added proteins (lane 1) or with purified Kid (lane 2), Kid and Kis (lane 3), or KidR85W (lane 4). The proteins were added at 300 nM. A 5’-labeled DNA fragment (“D” arrow) was included in the reaction mixtures as an internal control.

In contrast, our results do not support their second statement. Using three different small, natural RNA substrates, we observed that the Kid toxin cleaves RNA with a preference for UAA or UAC sites present in single-stranded regions. However, the same sequences situated in double-stranded RNA regions were efficiently cleaved by Kid (Fig. 2). Interestingly, the KidR85W nontoxic mutant generally preserved the ability to cleave single-stranded RNA sites but was severely affected in the cleavage of sites situated in double-stranded RNA (Fig. 2C). The fact that KidR85W failed to inhibit protein synthesis in E. coli and rabbit reticulocyte cell-free systems (Fig. 1) indicates that the capacity to cleave canonical UAA and UAC sites in single-stranded RNA regions does not account by itself for the inhibition of protein synthesis and the cytotoxicity of the protein. Rather, it seems that a specific type of Kid-RNA interaction, which the R85W mutation disrupts, is needed for Kid to efficiently degrade RNA and inhibit protein synthesis in vivo. Thus, the cleavage of double-stranded RNA by Kid exists, and it is correlated with the cytotoxicity of the protein. In addition, the observation that the presence of rabbit reticulocyte lysates influences the cleavage pattern of the toxin opens the possibility that particular host factors modulate the activity of Kid in vivo. These factors, which remain unidentified, may either influence the accessibility of the Kid to the target cleavage sites or, alternatively, modify the architecture of the Kid-RNA complex to alter its activity. Addressing these mechanistic issues awaits further structural studies aimed at the resolution of such complexes, as well as direct approaches towards the identification of the host factors that influence the activity of Kid.

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work), it is doubtful that these enzymes will prove to be useful in the structural mapping of RNA. In contrast, the increased specificity of KidR55W towards single-stranded RNA shows that Kid variants with tight site specificity may be obtained. Together with further structural information on RNA-toxin complexes, this will open the way to the rational design of a second generation of more specific and potent cytotoxic RNases encoded by the parD family.

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