IDENTIFICATION OF AN ESSENTIAL CLEAVAGE SITE IN ColE7 REQUIRED FOR IMPORT AND KILLING CELLS*

Zhonghao Shi1,2, Kin-Fu Chak1 and Hanna S. Yuan1,2*

1Institute of Biochemistry, National Yang-Ming University, Taipei, Taiwan, ROC.
2Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, ROC.

Running title: A specific cleavage at ColE7 during translocation

*Address correspondence to: Hanna S. Yuan, Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan 11529, Tel: 886-2-27884151; Fax: 886-2-27826085; E-mail: hanna@sinica.edu.tw

Colicin E7 (ColE7), a nuclease toxin released from Escherichia coli, kills susceptible bacteria under environmental stress. Nuclease colicins are processed during translocation with only the cytotoxic nuclease domains traversing the inner membrane to cleave tRNA, rRNA or DNA in the cytoplasm of target cells. In this study, we show that the E. coli periplasmic extract cleaves ColE7 between K446-R447 in the presence or absence of its inhibitor Im7 protein. Several residues near cleavage sites were mutated but only mutants of R447 lost completely in vivo cell killing activity. Both the full-length and the nuclease domain of R447 mutants retained their nuclease activities, indicating that failure to kill cells was not a consequence of damage to the enzyme’s endonuclease activity. Moreover, R447E ColE7 mutant was neither cleaved at its 447 site by periplasmic extracts nor transported into the cytoplasm of target cells. Collectively these results suggest that ColE7 is cleaved at R447 during translocation and that cleavage is an essential step for ColE7 import into the cytoplasm of target cells and its cell killing activity. Conserved basic residues aligned with R447 have also been found in other nuclease colicins, implying that the processing at this position may be common to other colicins during translocation.

Protein transport mechanisms across cell or organelle membranes have been studied extensively since these processes are essential for cell survival and defense. Protein toxins provide good opportunities for the study of protein import pathways into eukaryotic or prokaryotic cells because successful transport produces an obvious cell death consequence (1). The protein toxins specifically targeting bacterial cells are classified as bacteriocins. These toxins bind to the respective receptors on target cells and are transported across bacterial outer or, in some cases, inner cytoplasmic membranes, resulting in cell death (2). Presently, Escherichia coli released colicins are likely the best studied sub-family of bacteriocins in terms of outer membrane receptor binding, membrane translocation and cell killing (3-5).

Colicins are SOS-response proteins, expressed under stress that kill sensitive E. coli and other related bacterial strains (6). Most colicins share a similar organization, containing three functional domains, receptor-binding (R), membrane-translocation (T) and cytotoxic (C) domains. After secretion from the host cell, colicins first bind to specific cell surface receptors on target cells, examples include the vitamin B12 receptor BtuB for all the E-group colicins and iron siderophore receptor FepA for colicin B (ColB) and colicin D (ColD) (7).
They are then imported into cells by two different routes, one depending on Ton proteins (ExbB, D and TonB), and the other depending on Tol proteins (TolA, B, Q and R) (8,9). Colicins use a variety of strategies to induce cell death through their C-domains, including pore-forming colicins creating voltage-gated channels in the cytoplasmic membrane (4); nuclease colicins cleaving tRNA or rRNA at specific sites to inhibit protein synthesis (10,11), or degrading nucleic acids non-specifically in target cells (12,13).

The crystal structures of two very different colicins, the Ton-dependent pore-forming Col Ia (14) and Tol-dependent rRNase ColE3 (11), have revealed similarly assembled elongated Y-shaped molecules with R-domains forming a long coiled-coil stalk and the two globular heads of T- and C-domains comprising the two arms. In the ColE3/Im3 structure complex, the immunity protein Im3 is bound to the C-domain to prevent access of ColE3 to the ribosome and inhibits ColE3’s rRNase activity upon ColE3 expression in the host cell. The crystal structure of the ColE3 R-domain in complex with its BtuB receptor further demonstrates how ColE3 interacts with BtuB at its coiled-coil apex to initiate colicin conformational change and translocation (15). However, the crystal structures of the Tol-dependent pore-forming colicin N (16) and the Ton-dependent pore-forming ColB (17) revealed different two-domain architectures. The dumbbell-shaped ColB has its T- and R-domains intertwining into a large single globular structure, suggesting that different colicins may have different mechanisms of translocation even though they use similar transporters.

After import into the periplasm, the nuclease colicins, either containing rRNase, tRNase, or nuclease activities (DNase/RNase), have to transport further across the inner membrane to reach the cytoplasm of target cells. It has been shown that a nuclease colicin, ColE7, is likely processed in the periplasm during translocation with only the C-terminal cytotoxic nuclease domain transported into the cytoplasm (18). The tRNase colicin, ColD, was also reported to be cleaved during translocation and a leader peptidase LepB identified to be required in the processing (19). However, in contrast to ColE7 which is processed in the presence of the immunity protein Im7, the immunity protein of ColD was found to prevent ColD processing. It was thus suggested that the immunity protein may not only inhibit tRNase activity but also protect ColD against LepB-mediated cleavage during export. Based on these results, it is generally accepted that nuclease colicins are processed during translocation but the cleavage sites in colicins and the component proteins involved in the processing have not yet been clearly elucidated. This report identifies the cleavage site at R447 in ColE7 during translocation and demonstrates that this cleavage is essential for ColE7 translocation and its cell killing properties. A conserved basic residue was identified in a number of nuclease colicins, implying that a similar cleavage process may be involved for all these colicins during translocation.

MATERIAL AND METHODS

Protein expression and purification- DNA fragments encoding the full-length ColE7 and Im7 (20) and the C-domain of ColE7 and Im7 were amplified respectively by PCR and subcloned between the SphI and BglII sites of pQE70. All mutants of ColE7/Im7 and C-domain/Im7 were generated using the Quickchange site-directed mutagenesis kits (Invitrogen, Inc). The Escherichia coli strain M15 was used as the host strain for protein expression.

Full-length ColE7- Overnight cultures of E. coli cells were diluted 100-fold in 1 liter of LB containing 50 µg/ml ampicillin. Cells were
grown at 37°C to 0.6 O.D. (A_600) after which IPTG was added to a final concentration of 1 mM to induce protein expression. Crude cell extracts were first loaded onto a Ni-NTA resin affinity column (Qiagen, Germany) followed by a CarboxyMethyl column (Pharmacia). Purified full-length ColE7/Im7 complexes were denatured with 6 M Guanidine-HCl and further separated on a Ni-NTA spin column (Qiagen). The flow through containing full-length ColE7 alone was dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.0) and 10 mM ZnCl_2.

ColE7 C-domain- ColE7 C-domain and Im7 fused with 6 histidine affinity tag at the C terminus were expressed in the same E. Coli M15 strain. After Ni-NTA affinity column purification, the protein complex was denatured by dialysis against 20 mM glycine-HCl buffer (pH 3.0) overnight. The resulting protein solution was loaded onto a Sepharose-SP column (HiTrap SP, Pharmacia) equilibrated with 20 mM glycine-HCl buffer (pH 3.0). The C-domain alone was eluted by a NaCl gradient (0-2.0 M) at pH 3.0 and Im7 was eluted afterward by 20 mM sodium phosphate buffer at pH 7.0. The eluent containing the ColE7 C-domain was dialyzed against 20 mM sodium phosphate buffer (pH 7.0).

Preparation of periplasmic extracts- A published method was used for the preparation of periplasmic proteins by osmotic shock (21) with some modifications. Ten milliliters of fresh overnight Escherichia coli (M15 strain) cell culture was diluted into 200 ml LB and incubated at 37°C for 5 hours. Cells were harvested and suspended in 200 mM Tris-HCl (pH 8.0) buffer, followed by addition of the same volume of the buffer containing 200 ml Tris-HCl (pH 8.0) and 1 M sucrose. A final concentration of 0.5 mM EDTA was then added resulting in a suspension containing 5~20 mg/ml of cells in 0.5 M sucrose. Lysozyme was then added into the cell suspension to a final concentration of 60 µg/ml. The same volume of water was then added to the cell suspension and the reaction kept for 10 minutes at room temperature. MgSO_4 was then added to a final concentration of 20 mM. The osmotic shock fluid containing periplasmic proteins was collected by centrifugation. The periplasmic extracts were further concentrated to 1 mg/ml by centriprep (MILLIPORE) followed by extensive dialysis against buffers containing 20 mM MES (pH 6.0) and 150 mM NaCl.

In vitro processing of ColE7- The purified ColE7/Im7 complex (3 µg) was mixed with 1 µg of the concentrated periplasmic extracts at pH 6.0 and incubated at 37°C for various periods of time (2~12 hours). The processed protein products were fractionated by SDSPAGE and then transferred to a PVDF membrane using semi-dry apparatus (American Bionetics). The membrane was hybridized with polyclonal antibodies raised against the ColE7 C-domain followed by goat anti-IgG antibodies conjugated with alkaline phosphatase (Promega). The blot was visualized by nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (PerkinElmwe™ Life science). The desired fragment was eluted from a PVDF membrane after staining with Coomassie brilliant blue and verified by N-terminal sequencing (492 Protein Sequencer, ABI).

In vivo cell death assay- Purified full-length wild-type or mutated ColE7/Im7 complexes were serially diluted in sodium phosphate buffers. A drop of 20 µl of ColE7/Im7 solution was spotted, at each dilution, onto a disk filter paper placed in freshly prepared bacterial lawns on Ampicillin LB agar plates. Plates were then incubated overnight at 37°C. E. coli M15 containing pQE70 was used for the cell death assay.

CD and tryptophan fluorescence spectroscopy- Circular dichroism measurements were
performed at 25°C using a Jasco J720 spectropolarimeter. The spectra presented in Figure 3 were the average of three scans in units of mean residue ellipticity (θ). The protein concentration was 0.04 mg/ml in 20 mM sodium phosphate (pH 7.0).

Measurements of the intrinsic tryptophan fluorescence emission of full-length ColE7-Im7 complex were performed on a Hitach F4500 fluorescence spectrometer using an excitation wavelength of 295 nm at 25°C. The excitation bandwidth was set at 5 nm and the emission bandwidth at 10 nm. The protein concentrations were 0.04 mg/ml each in 20 mM sodium phosphate (pH 7.0).

DNase activity assay- The purified full-length ColE7 (15 ng) was incubated with pUC18 plasmid in a final volume of 10 µl at 37°C for 2~3 hours. The amount of ColE7 C-domain was first adjusted to between 0.12~120 ng by series dilution and then incubated with the pQE70 plasmid at room temperature for 5 minutes. The plasmid digestion patterns were analyzed on 1 % agarose gels.

DNase activity measured by FRET methods- The dsDNA substrate labeled with fluorogenic material FAM (6-carboxyl-fluorescein) and TAMRA (6-carboxyl-tetramethyl-rhodamine) at the 5’-end was prepared by Biotech (Taiwan):

FAM-5’-CCACAGGTAGCGACAG-3’
3’-GGTGTCCATCGCTGTC-5’-TAMRA

Kinetic measurements were performed in 10 mM Tris (pH 8.0) and 1 mM MgCl₂ at 25°C. Enhanced fluorescence due to cleavage of the fluorogenic dsDNA substrate was monitored at 515 nm, with excitation at 486 nm using a fluorescence plate reader (PerkinElmer 1420 Victor2 Mutilabel Counter). The enzyme concentration used in measuring Kₘ and kcat values was 20 nM with varied substrate concentrations of 9.75, 19.14, 47.85, 71.78, 95.7, 143.55 and 191.4 nM.

Identification of ColE7 translocation products- A previously reported method (18) was used to “fish out” the final processed ColE7 from the cytoplasm of target cells. A cell culture of 200 ml of E. coli M15 containing a plasmid encoding His-tagged Im7 (pQE30-cei) was treated with 5 mg of ColE7/Im7 complex for 4 hours. The treated cell culture was harvested and washed twice with 20 mM sodium phosphate buffer (pH 7.0), followed with 10 mg/ml proteinase K for 1 hour at 37°C to remove residual proteins bound on the cell surface. The proteinase K treated cells were harvested and washed twice with the same buffer and then resuspended in 3 ml sodium phosphate buffer (50 mM, pH7.0) containing 300 mM NaCl and protease inhibitor complex (Roch, Complete, EDTA-free). Cells were disrupted by sonication and the insoluble fractions removed by centrifugation. The soluble fractions were then loaded onto a Ni-NTA spin column (Qiagen). The His-tagged Im7 and any pull-down proteins from the spin column were eluted by 500 mM imidazole. Im7-associated proteins were identified by Western blot hybridization with polyclonal antibodies against the C-domain of ColE7.

RESULTS

Periplasmic proteins cleave ColE7 at R447 in the presence or absence of Im7

In order to ascertain the location of ColE7 processing sites, a full-length ColE7/Im7 complex was incubated with Escherichia coli periplasmic extracts prepared by osmotic shock (21). The quality of the periplasmic extract prepared from the E. coli M15 strain containing a plasmid encoding His-tagged Im7 was checked by Western blot hybridization using the cytoplasmic antibody markers against GroEL and
against His-tags. Both GroEL and His-tagged Im7 were detected in total cell extracts and cytoplasmic fractions but not detected in periplasmic fractions, indicating that the prepared periplasmic fraction was not contaminated by cytoplasmic proteins (see Figure 1A). After incubation with the cell fractions for 12 hours, ColE7 was mostly cleaved by the periplasmic extract, but not cleaved by cytoplasmic fractions (see Figure 1B). The ColE7/Im7 was then further incubated with periplasmic extracts over different periods of time (2 to 12 hours) in the presence or absence of metaloprotease inhibitors and EDTA. The processed ColE7 products were separated using SDS-PAGE, and detected by Western blot hybridization using an antibody raised against the ColE7 C-domain, revealing a processed fragment with a size similar to the constructed nuclease domain (see Figure 1C). Addition of EDTA but not serine or cysteine protease inhibitors inhibited the processing, implying that the processing might involve metaloproteases.

The processed ColE7 product was then extracted from the SDS-PAGE and analyzed by N-terminal sequencing, revealing the first five residues of the processed ColE7 as R-N-K-P-G, corresponding to residues R447 to G451. Therefore the ColE7 cleavage site lay between K446-R447, located in a region between its R-domain and C-domain. By incubating the free form of ColE7 (without Im7) with periplasmic extracts, we found the processing pattern (see Figure 1D) similar to that of the ColE7/Im7 complex. This result shows that periplasmic extracts cleave ColE7 between K446-R447 in the presence or absence of bound Im7.

**R447 ColE7 mutants lose in vivo cell killing activity**

Several single- or double-point ColE7 mutants were constructed to determine which of the amino acids flanking the K446-R447 cleavage site, including K446E, R447E, N448A and K446E/R447E, would affect cleavage. In the cell death assay, wild-type ColE7/Im7, spotted on a disk filter paper placed in an agar plate seeded with *E. coli* M15 strains, killed cells ad gave a clear region around the filter paper. However, two of the mutants with mutations at the 447 site, R447E and K446E/R447E, failed to kill target bacteria, as evidenced by the lack of a clear region surrounding the disk (see Figure 2A). In contrast, K446E and N448A mutants retained significant cell killing activities. R447 was then further mutated to a similar basic residue (Lys) or to a non-charged residue (Ala). However, neither R447K nor R447A killed any cells even upon application at high concentrations (see Figure 2B). This result demonstrates the critical importance of R447 in mediating the cell killing activity of ColE7 in vivo. Circular dichroism and tryptophan fluorescence spectroscopy were used to detect any conformational changes in R447 mutants (see Figure 3). No obvious differences were detected in secondary and tertiary structures between wild-type ColE7 and R447 mutants, indicating that the failure to kill cells, as exhibited by R447 mutants, was likely not to be due to any change in protein conformation.

**R447 ColE7 mutants retain DNase activity**

Since it was possible that the R447 mutants lacked cell lethality because they had lost their endonuclease activity, full-length wild-type and mutated ColE7 proteins were prepared and purified for nuclease activity assays. Plasmids pUC18 or pQE70 were used as substrates to monitor ColE7 endonuclease activity from any DNA topological changes seen in agarose gel electrophoresis. Figure 4A shows that the supercoiled plasmid was cleaved into linear/open-circular forms by either wild-type or mutated ColE7 (K446E, R447E and
The wild-type nuclease domain of ColE7 (residues 444-576) and the corresponding R447A mutant were further constructed and purified. These two C-domain proteins also cleaved the plasmid substrate efficiently (see Figure 4B). Nevertheless, R447A C-domain had about a 10-fold lower endonuclease activity than the wild-type protein.

The catalytic activities of wild-type and mutant C-domains were further measured by a fluorescent method using a fluorophore and quencher-labeled oligonucleotide as the substrate. For a control to compare with R447 mutants, a mutant, N560D, was selected for the measurement of enzyme and cell killing activity. N560 is a conserved residue in the HNH motif located in the C-domain of ColE7 therefore the mutant N560D was expected to have reduced endonuclease activity. Cleavage of the fluorophore-labeled oligonucleotides by the C-domain proteins gave increasing fluorescence emission intensities. The measured intensities showed that R447A C-domain had 15.0 % and N560D C-domain had 0.7 % of wild-type enzyme activity (Table 1). The reduced catalytic activity of R447A was not due to any decrease in enzyme activity, but mostly resulted from an increased $K_m$, indicating that the affinity between R447A and DNA was lower than that of the wild-type enzyme. N560D mutant, with a much lower overall endonuclease activity of only 0.7 %, was still able to kill target cells (Figure 4C), indicating that any failure to kill cells by R447 mutants did not result from any reduction in endonuclease activity.

ColE7 R447E mutants are not imported into cytoplasm nor processed at the 447 site

In order to further elucidate the underlying cause for the failure of R447 mutants to kill cells, the imported ColE7 in the cytoplasm of target cells was characterized. Escherichia coli cells containing an endogenously expressed His-tagged Im7 were treated with full-length wild-type ColE7/Im7 and R447E/Im7 complexes. The translocation product in complex with the endogenous His-tagged Im7 was then purified by Ni-NTA resin and detected by Western blot hybridization using an antibody against nuclease-ColE7. Fragments of nuclease domains were detected in target cells treated with wild-type ColE7/Im7 complex. However, no nuclease domain fragments were detected in the cells treated with R447E/Im7 complex (Figure 5A). This result indicates that the ColE7 R447E mutant was not imported into the cytoplasm of target cells in the same manner as that of wild-type ColE7.

It was intriguing why ColE7 R447 mutant was not imported into the cytoplasm of target cells. It is thus necessary to determine if R447E could be processed correctly in periplasm since the incorrect processing may lead to the failure in import. Full-length ColE7 R447E mutant was incubated with periplasmic extracts and the digested protein products were separated in SDS-PAGE and detected by Western blot hybridization (see Figure 5B). The R447E mutant was not cleaved between K446-E447. However, a larger fragment was found, resulting from a cut between K438-A439 as analyzed by the N-terminal sequencing. This result suggests that cleavage at R447 is necessary for ColE7 import into target cells and that is why the mutant R447E, not processed at 447, was not imported into the cytoplasm.

**DISCUSSION**

**Specific cleavage at R447 in ColE7 is required for its cell killing activity**

The nuclease colicins acting in the cytoplasm of sensitive bacterial cells have to traverse the second inner membrane, as a result they require additional steps in translocation as compared to pore-forming colicins. Here we
present several lines of evidence demonstrating that cleavage between K446-R447 in ColE7 is essential for its import into the cytoplasm of sensitive cells. The arginine at the P1’ site (cleavage between P1-P1’) is of critical importance since replacing it with other residues abolished the specific cleavage and protein translocation, resulting in a non-lethal colicin. This cleavage is independent of immunity protein, i.e., Im7 cannot protect ColE7 from this cleavage. This cleavage process occurs extracellularly, most likely in the periplasm of sensitive cells. However, since outer membrane associated proteins may have also been extracted under the preparation conditions used in the isolation of periplasmic extracts, it cannot be excluded that cleavage may take place during ColE7 translocation across the outer membrane.

If one compares R447 cleavage in ColE7 to the LepB-mediated cleavage in ColD during translocation (19), similar features can be found. Firstly, the cleavages of ColD and ColE7 are required for their import into cytoplasm and for their cell killing activity. Secondly, several single-, double- and triple-mutants have been constructed for ColD, but only the mutants in which the basic residue K603 was replaced (R602L/K603E and R602L/K603E/L596P) were not cleaved, similar to the R447 mutants in ColE7. However, cleavage of ColD by whole cell extracts was inhibited by the immunity protein and mediated by an inner-membrane associated peptidase (19), which was not present in the periplasmic extract used in this analysis. Moreover, the signal peptidase LepB is a serine protease but not a metalloprotease. But the processing of ColE7 by periplasmic extracts is inhibited by EDTA, indicating that a metalloprotease may be involved. Therefore it is likely that the observed cleavages in ColE7 and ColD occur at different stages or are processed by different proteins during translocation. The difference observed in processing may also be a consequence of two different types of colicins using two different translocation pathways for import: the nuclease ColE7 depends on Tol proteins and tRNase ColD depends on TonB.

**Cleavage site in ColE7 is located in a linker region between R-domain and C-domain**

The crystal structure of an E-group rRNase colicin, ColE3, has been determined. ColE3 shares high sequence identity with ColE7 in the T- and R-domains (75.4 % identity). We therefore constructed a ColE7 structure model by fusing the crystal structure of ColE3 T- and R-domain (PDB: 1JCH) to the crystal structure of a ColE7 C-domain (the nuclease domain) in complex with Im7 (PDB: 7CEI) (11,22). The overall structure and a closer view of the endonuclease active site in ColE7 are shown in Figure 6. In the crystal structure of ColE7 C-domain (PDB: 1MZ8), R447 hydrogen bonds to the phosphate ion mimicking the scissile phosphate that is directly bound to the metal ion in the active site (23). This explains why R447A mutant has a lower affinity for DNA substrates even though it retains endonuclease activity, since R447 indeed is involved in DNA binding but not directly involved in catalysis.

The location of R447 in the exposed linker region seems ideal for protease processing. This site is located distant from Im7, in agreement with the fact that Im7 cannot protect ColE7 from cleavage. From comparisons of ColE7 sequence with those of other nuclease colicins, conserved basic residues, either arginine or lysine, are seen to align at the same position as R447 for all the Tol-dependent colicins. The E-group rRNase (E3, E4, E6) and tRNase (E5) colicins, all have a lysine whilst the non-specific nuclease colicins (E2, E7, E8 and E9) all have an arginine at this position (marked by the red box in Figure 6C). The Tol-dependent DF13 which binds to the receptor IutA (24), also shares high sequence homology with the E-group colicins in this linker
region and DF13 also has a lysine aligned with R447 in ColE7. This implies that the cleavage observed in ColE7 at R447 may be common to other Tol-dependent enzymatic colicins. The Ton-dependent nuclease colicin ColD shares low sequence homology with ColE7. However, it will be interesting to find out if ColD requires cleavage at K603 for translocation. Additional experiments are needed to find out if the processing seen for ColE7 at the R447 site is general for the import of all enzymatic colicins and the protein components involved in this process.

REFERENCES

1. Sandvig, K., and Falnes, P. O. (2000) Curr. Opin. Cell Biol. 12, 407-413
2. Riley, M. A., and Wertz, J. E. (2002) Annu. Rev. Microbiol. 56, 117-137
3. Cao, Z., and Klebba, P. E. (2002) Biochimie 84, 399-412
4. Zakharov, S. D., and Cramer, W. A. (2002) Biochem. Biophys. Acta 1565, 333-346
5. Zakharov, S. D., and Cramer, W. A. (2004) Frontiers Biosci. 9, 1311-1317
6. Gillor, O., Kirkup, B. C., and Riley, M. A. (2004) Adv. Appl. Microbiol. 54, 129-146
7. Barnard, T. J., Watson, M. E. J., and McIntosh, M. A. (2001) Mol. Microbiol. 41, 527-536
8. Lazzaroni, J.-C., Dubuisson, J.-F., and Vianney, A. (2002) Biochimie 84, 391-397
9. Riley, M. A., and Wertz, J. E. (2002) Biochimie 84, 365-380
10. Masaki, H., and Ogawa, T. (2002) Biochimie 84, 433-438
11. Soelaiman, S., Jakes, K., Wu, N., Li, C., and Shoham, M. (2001) Mol. Cell 8, 1053-1062
12. Hsia, K.-C., Li, C.-L., and Yuan, H. S. (2005) Curr. Opin. Struct. Biol. 15, in press
13. James, R., Penfold, C. N., Moore, G. R., and Kleanthous, C. (2002) Biochimie 84, 381-389
14. Wiener, M., Freymann, D., Ghosh, P., and Stroud, R. M. (1997) Nature 385, 461-465
15. Kurisu, G., Zakharov, S. D., Zhalnina, M. V., Bano, S., Eroukova, V. Y., Rokitskaya, T. I., Antonenko, Y. N., Wiener, M. C., and Cramer, W. A. (2003) Nature Struct. Biol. 10, 948-954
16. Vetter, I. R., Parker, M. W., Tucker, A. D., Lakey, J. H., Pattus, F., and Tsernoglou, D. (1998) Structure 6, 863-874
17. Hilsenbeck, J. L., Park, H., Chen, G., Youn, B., Postle, K., and Kang, C. (2004) Mol. Microbiol. 51, 711-720
18. Liao, C.-C., Hsia, K.-C., Liu, Y.-W., Liang, P. H., Yuan, H. S., and Chak, K.-F. (2001) Biochem. Biophys. Res. Commun. 284, 556-562
19. de Zamaroczy, M., Mora, L., Lecuyer, A., Geli, V., and Buckingham, R. H. (2001) Mol. Cell 8, 159-168
20. Chak, K.-F., Kuo, W.-S., Lu, F.-M., and James, R. (1991) J. Gen. Microbiol. 137, 91-100
21. Witholt, B., Boekhout, M., Brock, M., Kingma, J., Heerikhuizen, H. V., and Leij, L. D. (1976) Anal. Biochem. 74, 160-170
22. Ko, T.-P., Liao, C.-C., Ku, W.-Y., Chak, K.-F., and Yuan, H. S. (1999) Structure 7, 91-102
23. Sui, M.-J., Tsai, L.-C., Hsia, K.-C., Doudeva, L.-G., Chak, K.-F., and Yuan, H. S. (2002) Protein Sci. 11, 2947-2957
24. Thomas, J. A., and Valvano, M. A. (1993) J. Bacteriol. 175, 548-552
FOOTNOTES

* This work was supported by research grants from the Academia Sinica and National Science Council (NSC92-2321-B001-012) of the Republic of China to H. S. Yuan.

FIGURE LEGENDS

Fig. 1. Processing of ColE7 by E. coli periplasmic extracts. A, The periplasmic extract was prepared by osmotic shock using Escherichia coli M15 cells containing a plasmid encoding His-tagged Im7. The quality of the periplasmic extract was confirmed by immunoblotting the cell fractions (3 µg each) with antibodies raised against the cytoplasmic markers GroEL (EMD Biosciences) and His-tagged Im7. GroEL and His-tagged Im7 were only identified in total cell and cytoplasmic fractions, but not in periplasmic fractions. B, The ColE7/Im7 complex (3 µg) was incubated with cytoplasmic or periplasmic extracts at 37°C for 12 hours. ColE7 cleaved products were resolved by 12% SDS-PAGE followed by Western blot hybridization with anti-serum raised against the C-domain of ColE7. The full-length ColE7 was mostly cleaved by the periplasmic fraction, but was left almost intact by the cytoplasmic fraction. C, The ColE7/Im7 complex (3 µg) was further incubated with E. coli periplasmic extract (1 µg) for 2 to 12 hours in the presence or absence of protease inhibitors and EDTA at 37°C. A small fragment of ColE7 was generated by cleavage between K446-R447 as determined by N-terminal sequencing. Addition of EDTA inhibited the processing but addition of serine or cysteine protease inhibitors did not inhibit the processing. D, ColE7/Im7 complex and the free-form of ColE7 were respectively incubated with the periplasmic extract for 12 hours. ColE7 was cleaved in the presence or absence of bound Im7.

Fig. 2. R447 mutants fail to kill sensitive E. coli. A, A drop of 20 µl of wild-type or mutated ColE7/Im7 (2 mg/ml) was spotted onto a disk filter paper in a LB agar plate seeded with E. coli M15 strain. Plates were then incubated overnight at 37°C. Wild-type ColE7 killed bacteria and generated a clear circle surrounding the spotted site. N448A and K446E mutants gave reduced clear zones as compared to that of the wild-type ColE7. R447E and K446E/R447E failed to kill cells and gave no clear zone. B, Wild-type and R447 mutated ColE7/Im7 were spotted onto agar plates in different amounts from 0.02 to 20 µg. All of the R447 mutants, R447E, R447A and R447K, failed to kill ColE7 sensitive E. coli. C, The N560D ColE7/Im7 mutant was spotted onto the agar plates and it induced cell death from 0.2 to 20 µg.

Fig. 3. Circular dichroism and tryptophan fluorescence spectra of wild-type and R447 mutated ColE7/Im7. A, The CD spectra of the wild-type ColE7/Im7 (—), K446E/Im7 (---), R447E/Im7 (·) and K446E/R447E/Im7 (----) were recorded from 200 to 250 nm with protein concentrations of 0.04 mg/ml in 20 mM sodium phosphate (pH 7.0) at 25°C. B, The fluorescence spectra of wild-type and mutated ColE7/Im7 were measured using an excitation wavelength of 295 nm at 25°C. Protein concentrations were 0.04 mg/ml in 20 mM sodium phosphate (pH 7.0).
Fig. 4. In vitro endonuclease activity assay for wild-type and mutated ColE7. A, Plasmid pUC18 (300 ng) was incubated with 15 ng of full-length ColE7 proteins for two or three hours at 37°C. The digested DNA was resolved by agarose gel (1 %) electrophoresis. Supercoiled plasmid DNA was cleaved into linear or open-circular forms by the wild-type and ColE7 mutants (K446E, R447E and K446E/R447E). B, Various amounts (0.12 to 120 ng) of wild-type ColE7 C-domain (residues 444-576) and the corresponding R447A mutant were incubated with 300 ng of plasmid pQE70 for 5 minutes at room temperature. The R447A C-domain had about a 10-fold lower endonuclease activity in comparison to the wild-type C-domain.

Fig. 5. R447E ColE7 mutant is not imported into the cytoplasm of E. coli cells and is cleaved at different sites by a periplasmic extract. A, A cell culture of 200 ml of E. coli M15 containing a plasmid encoding His-tagged Im7 (pQE30-cei) was treated respectively with wild-type ColE7/Im7 (5 mg) and R447E/Im7 (5 mg) for 4 hours. The proteinase K treated cells were then harvested and the processed ColE7 bound to cytoplasmic His-tagged Im7 was purified by the Ni-NTA chromatography. Western blots with polyclonal antibodies against the C-domain of ColE7 identified processed ColE7 fragments in cells treated with wild-type ColE7/Im7. However no ColE7 fragments were identified in cells treated with R447E/Im7. Full-length and C-domain ColE7 proteins were used as markers. A control of M15 cells were treated with only sodium phosphate buffer. B, ColE7/Im7 and R447E/Im7 were treated with the periplasmic extract and the ColE7 processed fragments were resolved by 12 % SDS-PAGE followed by Western blot. The ColE7 R447E mutant was not processed at the 447 position, but a larger fragment with a cleavage between K438-A439 was observed.

Fig. 6. A structural model of ColE7 and sequence alignments of nuclease colicins. A, The structure of the H-N-H motif in the C-domain of ColE7 (PDB: 1MZ8) demonstrates that R447 directly binds to the phosphate. Only the phosphate ion and the side chains of R447, H545, H544, H569 and H573 are displayed in the ball-and-stick model. B, Structural model of full-length ColE7/Im7 is shown in a ribbon model. The structure was constructed by fusing ColE3 T- and R-domains (PDB: 1JCH, in blue) with a ColE7 C-domain/Im7 (PDB: 7CEI). The C-domain of ColE7 is displayed in red with only the H-N-H motif in green. The ColE7 inhibitor Im7 is displayed as a magenta coil structure. R447 is located at the linker region between R- and C-domains. C, Sequence alignments of several nuclease colicins near their linker regions. All the Tol-dependent nuclease colicins share high sequence homology with ColE7 at the linker region and have a basic residue, arginine or lysine, that aligns with R447 in ColE7.
Table 1. The endonuclease activity of the wild-type and mutated nuclease domain of ColE7 as measured by the FRET method.

| Protein  | $k_{cat}$ ($10^3$ sec$^{-1}$) | $K_m$ (nM) | $k_{cat}/K_m$ ($10^4$ M$^{-1}$ sec$^{-1}$) | Relative activity toward a 16-bp dsDNA (%) |
|----------|-------------------------------|------------|------------------------------------------|-------------------------------------------|
| Wild-type* | 1.81                          | 27.25      | 1.95                                     | 100                                       |
| R447A    | 1.09                          | 105.50     | 0.30                                     | 15.4                                      |
| N560A    | 0.02                          | 144.00     | 0.014                                    | 0.7                                       |

*The wild-type nuclease domain of ColE7 contains residues from 444-576.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Identification of an essential cleavage site in ColE7 required for import and killing cells
Zhonghao Shi, Kin-Fu Chak and Hanna S. Yuan

J. Biol. Chem. published online April 27, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501216200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts