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Diphtheria toxin and its ADP-ribosyltransferase-defective homologue CRM197 possess deoxyribonuclease activity

(nuclease)

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ABSTRACT **The cytotoxic mechanism of diphtheria toxin (DTx) is associated with its ability to inhibit protein synthesis by ADP-ribosylation of elongation factor 2. Although DTx intoxication leads to internucleosomal DNA cleavage and cell lysis, these events do not occur when protein synthesis is inhibited by alternative treatments (e.g., cycloheximide). Here we show that endonucleolytic degradation of DNA is an intrinsic activity of DTx and also of the crossreactive mutant protein CRM197. Assays using DNA-impregnated gels as well as linear and supercoiled DNA in solution revealed not only that CRM197 has nuclease activity but also that its specific activity is actually significantly greater than that of the wild-type molecule. Since CRM197 contains a single amino acid substitution that renders it incapable of ADP-ribosylation, we propose that the active sites for ADP-ribosyltransferase and nuclease activities are distinct.**

Diphtheria toxin (DTx) is a well-characterized toxic protein (1, 2). It has two functional domains, A and B, which can be resolved by limited proteolytic digestion and reduction of an interchain disulfide bond. Intoxication of cells by DTx involves endocytosis of receptor-bound toxin. Once the toxin is sequestered in endosomes, acid-triggered conformational changes promote the translocation of fragment A to the cytosol (3–5). Fragment A then proceeds to catalyze the transfer of the ADP-ribosyl group of NAD to the diphthamide residue of translational elongation factor 2 (EF-2). Recently we have discovered that DTx also exhibits a cation-dependent nuclease activity (6). In this report we explore the relationship between the toxin's ADP-ribosyltransferase and nuclease activities. CRM197, a mutant DTx with a $Gly^{52} \rightarrow Glu^{52}$ substitution in its A domain (7), is unable to ADP-ribosylate EF-2 *in vitro* because of its extremely low affinity for NAD (8, 9). We reasoned that if DTx's ability to cleave DNA were in any way related to its ability to bind or cleave the dinucleotide NAD, then CRM197 should show a much lower nuclease activity. For example, the NAD-binding site might be coincident with the nucleic acid-binding site, or NAD might serve as a cofactor for the toxin's nuclease activity. The data presented in this report demonstrate that CRM197 actually exhibits a 20-fold higher level of nuclease activity than DTx. This finding supports a model in which the NAD-binding pocket is distinct from the DNA-cleavage site.

MATERIALS AND METHODS

Protein Sources. "Intact" CRM197 was purchased from List Biological Laboratories (Campbell, CA). As supplied, it was 5–10% cleaved. DTx monomer (5–10% cleaved) was purified (4) from material purchased from Connaught Laboratories. DTx and CRM197 were cleaved into fragments A and B by incubating 105 μg of protein with 0.5 unit of endoproteinase ArgC (Sigma) in 485 μl of 15 mM Tris/150 mM NaCl/1 mM EDTA, pH 7.4 (30 min, 37°C). Pancreatic DNase I (bovine, grade II) was from Boehringer Mannheim.

Nuclease Activity Assay in DNA-Containing Gels. Samples of cleaved DTx monomer, cleaved CRM197, and "intact" CRM197 were solubilized in SDS reducing buffer (containing 0.01% 2-mercaptoethanol) and electrophoresed in a SDS/12.5% polyacrylamide gel (10) prepared with ^{32}P-labeled DNA [0.2 ng/ml; 800,000 cpm/ng of sonicated salmon sperm DNA radiolabeled by the primer-extension method (11, 12), as described (6)]. The stacking gel was omitted. The gels were pre-run at 170 V for 90 min. For nondenaturing gels, SDS was omitted and samples were not boiled. After electrophoresis, the DTx lanes were separated from the CRM197 lanes and each gel piece was washed three times (40 min per wash) with 100 ml of 40 mM Tris/0.04% NaN_3, pH 7.6, at 22°C. The gel pieces were then incubated with the same buffer containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ for 24 hr at 30°C (for SDS gels) or for 12 hr at 37°C (for nondenaturing gels), prior to autoradiography (−85°C, Kodak XAR film, DuPont Cronex screen).

Cleavage of Supercoiled and Linear Plasmid DNA. CRM197, DNase I, and DTx were incubated in 10 μl of 10 mM Tris, pH 7.6/2.5 mM $CaCl_2$/2.5 mM $MgCl_2$ with 0.2 μg of linear (*Eco*RI-cleaved) pBluescript DNA (Stratagene) for 15 min at 22°C. The cleavage was stopped by the addition of 3 μl of tracking dye (13) containing 25 mM EDTA. Samples were analyzed by electrophoresis in a 1% agarose gel using a Tris/borate/EDTA buffer that contained ethidium bromide at 0.5 μg/ml (13).

Determination of Catalytic Rate Constants. Twenty microliters of CRM197 (0.5-mg/ml stock), ArgC-cleaved CRM197 (0.45-mg/ml stock), DNase I (0.25-mg/ml stock), or DTx (1.5-mg/ml stock) in 150 mM NaCl/15 mM Tris/1 mM EDTA, pH 7.8, was added to 280 μl of a mixture containing radiolabeled (12,000 cpm/μg) sonicated salmon sperm DNA (25 ng/μl), 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 40 mM Tris (pH 7.6) at 25°C. Portions (50 μl) were taken at 1-min intervals and mixed with 20 μl of calf thymus DNA (2 mg/ml) in 36 mM Tris (pH 7.6) and 70 μl of ice-cold 10% (wt/vol) trichloroacetic acid. After 15 min on ice, the samples were spun for 15 min at 18,000 × *g* in a microcentrifuge. Two portions (50 μl) from each supernatant were taken for scintillation counting and the data were averaged. Rate constants were calculated by dividing the initial rate of increase in the concentration of acid-soluble DNA by the initial DNA concentration.

RESULTS AND DISCUSSION

When DTx and CRM197 were cleaved with endoproteinase ArgC, reduced, and then tested for nuclease activity in a

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Abbreviations: DTx, diphtheria toxin; EF-2, elongation factor 2; dsDNA, double-stranded DNA.

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[^{32}P]DNA gel assay, we were surprised to find that both DTx and CRM197 displayed fragment A-associated nuclease activity (Fig. 1). Moreover, 2.2 μg of cleaved CRM197 showed the same level of nuclease activity in the gel as did 4 μg of cleaved DTx. "Intact" CRM197 (which contains 5–10% cleaved toxin) also exhibited a high level of fragment A-associated nuclease activity. Thus, 2.2 μg of intact CRM197 had as much fragment A nuclease activity as 2.0 μg of completely nicked DTx. This corresponds to a CRM197/DTx nuclease activity ratio of 10–20:1. It should be noted, however, that the apparent level of activity exhibited by a renatured nuclease analyzed in a DNA-containing SDS/polyacrylamide gel is a function not only of its intrinsic nuclease activity but also of its ability to renature and not leach out of the gel matrix. Correspondence between fragment A bands and nuclease activity bands was demonstrated by the fact that fragment A of CRM197 exhibited a detectably higher molecular weight in SDS/polyacrylamide gels (compared to fragment A of DTx) and the position of nuclease activity shifted accordingly.

When analyzed after electrophoresis under nondenaturing conditions (Fig. 2), the nuclease activity observed for ArgC-cleaved DTx and CRM197 corresponded to the relative positions of the native forms of DTx and CRM197. Since reduction in the absence of detergent or urea does not effect subunit separation, these results establish that fragment A does not need to be separated from fragment B to catalyze DNA degradation. Indeed, identical samples run under nonreducing conditions actually showed higher levels of nuclease activity (data not shown). This is in distinct contrast to the requirements for the activation of the ADP-ribosyltransferase activity of DTx (2). In this nondenaturing gel system, ArgC-cleaved CRM197 appears to have $\approx$10 times more nuclease activity than ArgC-cleaved DTx. Although our CRM197 was obtained from a commercial source and was not purified further, the absence of any nuclease-active bands other than those that comigrated with intact CRM197 or its A fragment makes the possibility of a nuclease contaminant unlikely.

Linear double-stranded (ds) DNA and supercoiled dsDNA were used to assess the substrate specificity and relative activities of intact DTx and CRM197 with respect to pancreatic DNase I. CRM197 and DNase I showed equivalent nuclease activity on a weight-per-weight basis, and both showed $\approx$4-fold higher activity than DTx (Fig. 3*A*). Both CRM197 and DTx were capable of endonucleolytic cleavage of supercoiled dsDNA to unit-sized linear dsDNA (Fig. 3*B*). Again, CRM197 and DNase I showed similar levels of activity on a weight basis, and both were more active than

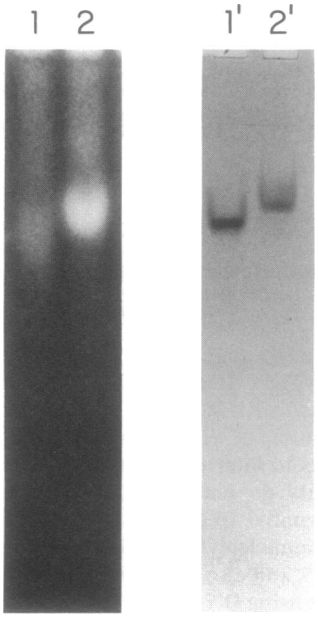

FIG. 2. Nuclease activity of cleaved DTx and CRM197 after electrophoresis under nondenaturing conditions. Lanes 1 and 2 of the autoradiogram show the nuclease activities of ArgC-cleaved, reduced DTx and CRM197 (4 μg each), respectively; lanes 1' and 2' show the locations of the protein bands as revealed by Coomassie blue staining.

DTx. Similar levels of activity were obtained with DTx and CRM197 that were completely cleaved (to fragments A and B) with endoproteinase ArgC (data not shown). Since nuclease activity is frequently expressed as the rate of degradation of [^{32}P]DNA to acid-soluble products, this assay method (6) was used to establish catalytic rate constants for DTx, CRM197, cleaved CRM197, and pancreatic DNase I (see *Materials and Methods*). The rate constants (expressed per μM protein) were as follows: 0.012 $\pm$ 0.001 min^{-1} for DTx, 0.24 $\pm$ 0.03 min^{-1} for CRM197, 0.13 $\pm$ 0.08 min^{-1} for cleaved CRM197, and 0.060 $\pm$ 0.001 min^{-1} for DNase I (averages and standard errors of two determinations). Thus the relative levels of nuclease activity on an equimolar basis are 0.2, 4.0, 2.2, and 1.0, respectively.

NAD, ATP, and various analogues of these compounds bind to the same site on fragment A (9), a site that includes residues His^{21}, Lys^{39}, Trp^{50}, Gly^{52}, and Glu^{148} (7, 14–18). Dinucleotides such as ApUp and ApGp bind in a pocket that includes the NAD-binding site and an adjoining phosphate-

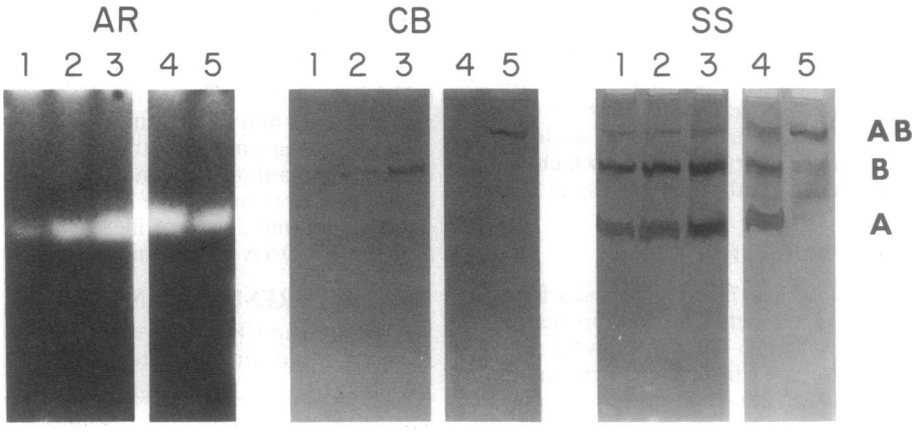

FIG. 1. Nuclease activity of DTx and CRM197 in a [^{32}P]DNA-containing gel. AR, autoradiogram showing the nuclease-active bands; CB, Coomassie blue-stained gel; SS, same gel after silver staining. Lanes 1–3 contained 0.8, 2.0, and 4.0 μg of cleaved DTx, respectively; lane 4 contained 2.2 μg of ArgC-cleaved CRM197; and lane 5 contained 2.2 μg of "intact" CRM197. AB marks the position of intact protein.

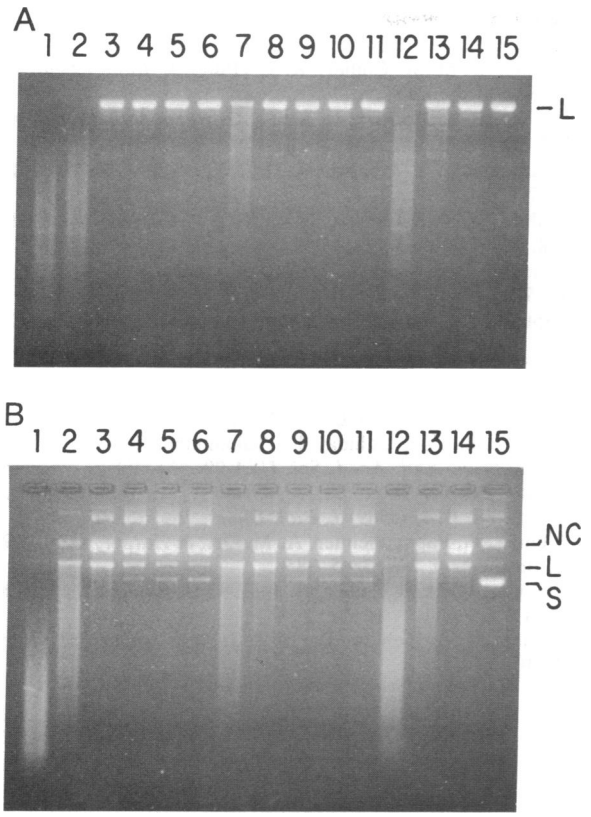

FIG. 3. Nuclease activity of CRM197 relative to pancreatic DNase I and DTx. (*A*) CRM197, DNase I, and DTx were incubated in 10 μl of 10 mM Tris, pH 7.6/2.5 mM $CaCl_2$/2.5 mM $MgCl_2$ with 0.2 μg of linear (*Eco*RI-cleaved) pBluescript DNA (Stratagene) for 15 min at 22°C. Lanes 1–6: 0.1, 0.05, 0.04, 0.03, 0.02, and 0.015 μg of intact CRM197, respectively; lanes 7–11: 0.05, 0.04, 0.03, 0.02, and 0.015 μg of pancreatic DNase I, respectively; lanes 12–14: 0.3, 0.2, and 0.1 μg of intact DTx, respectively; lane 15: 0.2 μg of control DNA. L, linear plasmid. (*B*) Samples were incubated with 0.5 μg of supercoiled pBluescript DNA for 5 min at 22°C. All other conditions were the same as in *A*. NC, nicked circular plasmid; S, supercoiled plasmid.

binding site on the B subunit (19). Indeed, the possibility that DTx evolved from a nucleic acid-binding protein has been suggested (2). While CRM197 fails to bind NAD (9), its reported inability to bind ApUp (19) does not agree with the high A_{260}/A_{280} ratio reported by one research group (20). Our observation that CRM197 is a highly efficient nuclease suggests that the NAD-binding pocket is distinct from the DNA-cleavage site. Further support for this conclusion is that DTx has to be activated by proteolytic nicking and reduction to ADP-ribosylate EF-2 (2). The endonucleolytic activity reported here for DTx and CRM197 does not require such activation. Another way in which the two activities differ is in their ion requirements. Divalent cations reportedly have no effect on the ADP-ribosyltransferase activity of DTx (2) but are absolutely required for nuclease activity (6, 21). That both DTx and CRM197 cleave supercoiled dsDNA to linear dsDNA (Fig. 3*B*) also demonstrates that DNA degradation is not akin to ADP-ribosyltransferase activity, which proceeds through glycohydrolytic removal of the nicotinamide base (2). This represents a strong factor in support of a two-site model because, to our knowledge, no nucleases have been found that use the same active site to achieve glycohydrolytic removal of bases.

Significantly, the single amino acid difference between the sequences of DTx and CRM197 has a profound effect on the conformation of the protein. Not only do the two proteins have different circular dichroism spectra (22, 23) and proteolytic susceptibilities (20), but fluorescence emission measurements (20), membrane binding and insertion experiments (20, 24), and antibody binding studies (25) show that the hydrophobic domains of CRM197 are more exposed to water than those of DTx. Moreover, the studies suggest that the structure of CRM197 at neutral pH is more similar to the structure of DTx at low pH. Such differences in the conformations of the two proteins might explain CRM197's higher level of nuclease activity.

With respect to the biological properties of CRM197, Uchida *et al.* (8) reported that this serologically-related protein gave negative skin reactions in rabbits and was nontoxic when injected into guinea pigs. Although CRM197 binds to the DTx cell surface receptor (8), no cytotoxic effects have been reported. When CRM197 was microinjected into tissue culture cells, the protein was rapidly degraded ($t_{1/2}$ = 2.5 hr; ref. 26). This suggests that the molecule would not have time to mediate significant chromosomal degradation (if it can be assumed to reach the cytoplasm under normal conditions), since observations with DTx-intoxicated cells show that the onset of internucleosomal DNA degradation occurs ≈6 hr after toxin treatment (27). Indeed, sensitivity to proteases might explain the lack of correlation between the *in vitro* and *in vivo* toxic effects observed with mutant forms of DTx (28) and *Pseudomonas aeruginosa* exotoxin A (29).

Because a link has been demonstrated between DTx resistance and possession of non-ADP-ribosylatable forms of EF-2 (30), we propose that the level of internucleosomal DNA degradation might be dependent on protein synthesis inhibition (e.g., in the absence of protein synthesis, expression of DNA-repair enzymes might not be sufficient to counteract DTx-dependent DNA cleavage). Preliminary support for a unique nuclease site came from studies in which 10 mM NAD was shown to have no inhibitory effect on the level of fragment A-associated nuclease activity in a DNA gel assay (6). Recent characterization studies (ref. 21; L. T. Nakamura and B.J.W., unpublished data) designed to establish the optimal conditions for *in vitro* DTx nuclease activity demonstrated that NAD, ATP, adenine, nicotinamide, or ApUp had no competitive effect (at 0.01 to 10 mM) on the DNase activity of DTx. Although we have not established that DTx nuclease activity is the direct cause of chromosome cleavage, what has been shown is that protein synthesis inhibition in general does not lead to the DNA degradation and cell lysis observed with DTx treatment (27).

Detailed studies of the cytolytic pathway of DTx have revealed no simple relationship between the toxin's cytolytic activity *in vivo* and its channel-forming behavior *in vitro* (M. P. Chang, B. Kagan, B.J.W., and J. Bramhall, unpublished data), nor has any correlation been established between its lytic activity and its ability to inactivate EF-2 *in vitro* or *in vivo* (27). In light of the findings described in this report, we propose a model for cell killing in which the toxin acts as a double-edged sword, using apparently nonoverlapping sites to effect translation inhibition and chromosomal cleavage.

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1. Olsnes, S. & Sandvig, K. (1988) in *Immunotoxins*, ed. Frankel, A. E. (Kluwer, Boston), pp. 39–73.
2. Collier, R. J. (1982) in *ADP-ribosylation Reactions*, eds. Hayaishi, O. & Ueda, K. (Academic, New York), pp. 575–592.
3. Neville, D. M. & Hudson, T. H. (1986) *Annu. Rev. Biochem.* **55**, 195–224.

4. Gonzalez, J. E. & Wisnieski, B. J. (1988) *J. Biol. Chem.* **263,** 15257–15259.
5. Zalman, L. S. & Wisnieski, B. J. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3341–3345.
6. Chang, M. P., Baldwin, R. L., Bruce, C. & Wisnieski, B. J. (1989) *Science* **246,** 1165–1168.
7. Giannini, G., Rappuoli, R. & Ratti, G. (1984) *Nucleic Acids Res.* **12,** 4063–4069.
8. Uchida, T., Pappenheimer, A. M., Jr., & Greany, R. (1973) *J. Biol. Chem.* **248,** 3838–3844.
9. Lory, S., Carroll, S. F. & Collier, R. J. (1980) *J. Biol. Chem.* **255,** 12016–12019.
10. Rosenthal, A. L. & Lacks, S. A. (1977) *Anal. Biochem.* **80,** 76–90.
11. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
12. Feinberg, A. P. & Vogelstein, B. (1984) *Anal. Biochem.* **137,** 266–267.
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
14. Zhao, J.-M. & London, E. (1988) *Biochemistry* **27,** 3398–3403.
15. Carroll, S. F. & Collier, R. J. (1974) *Proc. Natl. Acad. Sci. USA* **81,** 3307–3311.
16. Michel, A. & Dirkx, J. (1977) *Biochim. Biophys. Acta* **491,** 286–295.
17. Kandel, J., Collier, R. J. & Chung, D. W. (1974) *J. Biol. Chem.* **249,** 2088–2097.
18. Papini, E., Schiavo, G., Sandona, D., Rappuoli, R. & Montecucco, C. (1989) *J. Biol. Chem.* **264,** 12385–12388.
19. Collins, C. M. & Collier, R. J. (1984) *J. Biol. Chem.* **259,** 15159–15162.
20. Hu, V. W. & Holmes, R. K. (1987) *Biochim. Biophys. Acta* **902,** 24–30.
21. Nakamura, L. T. & Wisnieski, B. J. (1990) *J. Biol. Chem.* **265,** 5237–5241.
22. Collins, C. M. & Collier, R. J. (1985) *Biochim. Biophys. Acta* **828,** 138–143.
23. Mekada, E. & Uchida, T. (1985) *J. Biol. Chem.* **260,** 12148–12153.
24. Papini, E., Colonna, R., Schiavo, G., Cusinato, F., Tomasi, M., Rappuoli, R. & Montecucco, C. (1987) *FEBS Lett.* **215,** 73–78.
25. Bigio, M., Rossi, R., Nucci, D., Antoni, G., Rappuoli, R. & Ratti, G. (1987) *FEBS Lett.* **218,** 271–276.
26. Yamaizumi, M., Uchida, T., Takamatsu, K. & Okada, Y. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 461–465.
27. Chang, M. P., Bramhall, J., Graves, S., Bonavida, B. & Wisnieski, B. J. (1989) *J. Biol. Chem.* **264,** 15261–15267.
28. Uchida, T., Pappenheimer, A. M., Jr., & Harper, A. A. (1973) *J. Biol. Chem.* **248,** 3845–3850.
29. Douglas, C. M. & Collier, R. J. (1987) *J. Bacteriol.* **169,** 4967–4971.
30. Kohno, K. & Uchida, T. (1987) *J. Biol. Chem.* **262,** 12298–12305.