REFERENCES AND NOTES

1. E. D. Cope, *The Origin of the Fittest* (Appleton, New York, 1887).
2. N. D. Newell, *Evolution* **3**, 103 (1949); B. Kurtén, *Ann. Zool. Fenn.* **76**, 1 (1953).
3. P. D. Gingerich, *Annu. Rev. Earth Planet. Sci.* **8**, 407 (1980).
4. K. J. McNamara, in *Evolutionary Trends*, K. J. McNamara, Ed. (Univ. of Arizona Press, Tucson, AZ, 1990), pp. 205–231.
5. D. Jablonski, in *Evolutionary Paleobiology*, D. Jablonski, D. H. Erwin, J. H. Lipps, Eds. (Univ. of Chicago Press, Chicago, 1996), pp. 256–289; *Nature* **385**, 250 (1997).
6. B. J. MacFadden, *Paleobiology* **12**, 355 (1987).
7. S. M. Stanley, *Evolution* **27**, 1 (1973).
8. M. L. McKinney, *Paleobiology* **12**, 282 (1986).
9. A. J. Arnold, D. C. Kelly, W. C. Parker, *J. Paleontol.* **69**, 203 (1995).
10. R. D. Norris, *Paleobiology* **17**, 388 (1991); J. Trammer and A. Kaim, *Acta Palaeontol. Pol.* **42**, 1 (1997).
11. D. W. McShea, *Evolution* **48**, 1747 (1994)
12. S. J. Gould, *J. Paleontol.* **62**, 319 (1988); M. L. McKinney, in *Evolutionary Trends*, K. J. McNamara, Ed. (Univ. of Arizona Press, Tucson, AZ, 1990), pp. 75–118.
13. S. Legendre, *Palaeovertebrata* **16**, 191 (1986); S. Legendre and C. Roth, *Hist. Biol.* **1**, 85 (1988).
14. J. Damuth, in *Body Size in Mammalian Paleobiology: Estimation and Biological Implications*, J. Damuth and B. J. MacFadden, Eds. (Cambridge Univ. Press, Cambridge, 1990), pp. 229–253.
15. G. F. Gunnell, *Univ. Mich. Pap. Palaeontol.* **27**, 1 (1989).
16. ——— and P. D. Gingerich, *Contrib. Mus. Paleontol. Univ. Mich.* **29**, 413 (1996).
17. B. J. MacFadden and R. C. Hulbert Jr., in *Body Size in Mammalian Paleobiology: Estimation and Biological Implications*, J. Damuth and B. J. MacFadden, Eds. (Cambridge Univ. Press, Cambridge, 1990), pp. 337–363.
18. J. Alroy, *Paleobiology* **18**, 326 (1992).
19. ———, *ibid.* **20**, 191 (1994).
20. S. L. Wing, J. Alroy, L. J. Hickey, *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **115**, 117 (1995).
21. J. Alroy, *ibid.* **127**, 285 (1996).
22. P. D. Gingerich, in *Phylogenetic Analysis and Paleontology*, J. Cracraft and N. Eldredge, Eds. (Columbia Univ. Press, New York, 1979), pp. 41–77; D. C. Fisher, in *Interpreting the Hierarchy of Nature—From Systematic Patterns to Evolutionary Theories*, L. Grande and O. Rieppel, Eds. (Academic Press, Orlando, FL, 1994), pp. 133–171; J. P. Huelsenbeck, *Paleobiology* **20**, 470 (1994); P. J. Wagner, *ibid.* **21**, 153 (1995); J. P. Huelsenbeck and B. Rannala, *ibid.* **23**, 174 (1997).
23. J. Felsenstein, *Am. Nat.* **125**, 1 (1985); P. H. Harvey and M. D. Pagel, *The Comparative Method in Evolutionary Biology* (Oxford Univ. Press, Oxford, 1991); E. P. Martins, Ed., *Phylogenies and the Comparative Method in Animal Behavior* (Oxford Univ. Press, Oxford, 1996).
24. M. Foote and D. M. Raup, *Paleobiology* **22**, 121 (1996); M. Foote, *ibid.*, p. 141.
25. J. Gauthier, A. G. Kluge, T. Rowe, *Cladistics* **4**, 105 (1988); R. Hitchin and M. J. Benton, *Paleobiology* **23**, 20 (1997).
26. M. V. Lomolino, *Am. Nat.* **125**, 310 (1985); J. H. Brown, P. A. Marquet, M. L. Taper, *ibid.* **142**, 574 (1993); J. Damuth, *Nature* **365**, 748 (1993); J. H. Brown, *Macroecology* (Univ. of Chicago Press, Chicago, 1995).
27. J. H. Brown and B. A. Maurer, *Nature* **324**, 248 (1986); J. H. Brown and P. F. Nicoletto, *Am. Nat.* **138**, 1478 (1991); B. A. Maurer, J. H. Brown, R. D. Rusler, *Evolution* **46**, 939 (1992).
28. E. O. Wilson, *Evolution* **13**, 122 (1959); J. D. Rummel and J. Roughgarden, *ibid.* **39**, 1009 (1985); D. B. Miles and A. E. Dunham, *ibid.* **50**, 594 (1996).

29. I thank A. DeQueiroz, W. DiMichele, C. Marshall, D. Raup, P. Wagner, P. Wilf, and the anonymous reviewers for comments on the paper. This work was supported by the Evolution of Terrestrial Ecosystems (ETE) program and the Smithsonian Institution.

18 December 1997; accepted 6 March 1998

Proteolytic Inactivation of MAP-Kinase-Kinase by Anthrax Lethal Factor

Nicholas S. Duesbery, Craig P. Webb, Stephen H. Leppla, Valery M. Gordon, Kurt R. Klimpel,* Terry D. Copeland, Natalie G. Ahn, Marianne K. Oskarsson, Kenji Fukasawa, Ken D. Paull, George F. Vande Woude†

Anthrax lethal toxin, produced by the bacterium *Bacillus anthracis,* is the major cause of death in animals infected with anthrax. One component of this toxin, lethal factor (LF), is suspected to be a metalloprotease, but no physiological substrates have been identified. Here it is shown that LF is a protease that cleaves the amino terminus of mitogen-activated protein kinase kinases 1 and 2 (MAPKK1 and MAPKK2) and that this cleavage inactivates MAPKK1 and inhibits the MAPK signal transduction pathway. The identification of a cleavage site for LF may facilitate the development of LF inhibitors.

Anthrax toxin, produced by the bacterium *Bacillus anthracis*, is composed of three proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF) (*1*). PA binds to specific cell surface receptors and, upon proteolytic activation to a 63-kD fragment (PA63), forms a membrane channel that mediates entry of EF and LF into the cell (*2*). EF is an adenylate cyclase and together with PA forms a toxin referred to as edema toxin (*3*). LF and PA together form a toxin referred to as lethal toxin. Lethal toxin is the dominant virulence factor produced by *B. anthracis* and is the major cause of death of infected animals (*4*). Intravenous injection of lethal toxin into rats causes death in as little as 38 min (*5*), and addition of the toxin to mouse macrophages in culture causes lysis within 2 hours (*6*). LF is a 776–amino acid protein that contains a putative zinc-binding site [HEFGF (*7*)] at residues 686 through 690, which is characteristic of metalloproteases. Mutation of the H or E residues inactivates LF (*8*) and reduces its zinc-binding activity (*9*). However, no physiological substrate has been identified.

The National Cancer Institute maintains a database of antineoplastic drugs that have been tested against a panel of 60 human cancer cell lines [NCI's ADS (*10*)]. A screen of this database aimed at identifying novel inhibitors of the mitogen-activated protein kinase (MAPK) signal transduction pathway, an evolutionarily conserved pathway that controls cell proliferation and differentiation, revealed that anthrax LF had an activity profile similar to that of PD09859, a compound that selectively inhibits the MAPK pathway (*11*). We therefore examined the effect of LF on the MAPK pathway.

In response to extracellular signals, MAPK is phosphorylated and activated by MAPK kinases 1 and 2 (MAPKK1 and MAPKK2). In oocytes of the frog *Xenopus laevis*, progesterone-stimulated synthesis of Mos, a serine/threonine kinase, leads to activation of the MAPK pathway, which is essential for the activation of maturation-promoting factor (that is, cyclin B/p34^{cdc2} kinase) and the resumption of meiosis (maturation) (*12*). Addition of PA and LF to oocyte culture medium had no effect on progesterone-induced oocyte maturation (*13*). In contrast, injection of 1 ng of LF into oocytes inhibited maturation by 50% as judged by an assay of germinal vesicle (nuclear envelope) breakdown (GVBD), and GVBD was completely inhibited by 10 ng of LF (Table 1). Injection of LF Glu^{687} → Cys^{687} (E687C), an inactive LF containing a single amino acid substitution in the putative zinc-binding site (*8*), had no effect on GVBD (Table 1). Because a decrease in adenosine 3′,5′-monophosphate–dependent protein kinase A activity is also required for oocyte maturation (*12*), there was concern that low levels of EF may have been present as a contaminant. However, preparations of LF from strains of *B. anthracis* deficient in the production of EF also

N. S. Duesbery, C. P. Webb, T. D. Copeland, M. K. Oskarsson, G. F. Vande Woude, Advanced BioScience Laboratories–Basic Research Program, National Cancer Institute–Frederick Cancer Research and Development Center, Post Office Box B, Frederick, MD 21702, USA.
S. H. Leppla, V. M. Gordon, K. R. Klimpel, National Institute of Dental Research–National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA.
N. G. Ahn, Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado, Campus Box 215, Boulder, CO 80309, USA.
K. Fukasawa, Department of Cell Biology, University of Cincinnati, College of Medicine, P.O. Box 670521, Cincinnati, OH 45267, USA.
K. D. Paull, Division of Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

*Present address: Biopraxis, Post Office Box 9100–78, San Diego, CA 92191, USA.
†To whom correspondence should be addressed.

blocked oocyte maturation (Table 1). In contrast, LF did not inhibit GVBD induced by injection of Δ90 cyclin B, a truncated nondegradable form of cyclin B and a potent activator of $p34^{cdc2}$ kinase (*14*) (Table 1).

The addition of LF, but not of LF E687C, inhibited Mos-induced activation of MAPK in oocyte lysates (Fig. 1A), which suggests that oocytes cannot mature in the presence of LF because of a failure in MAPK activation. When immunoblots of these lysates were probed with antibodies to the COOH-terminus of MAPKK1, we detected antigen throughout the incubation with a slightly increased mobility (albeit at reduced levels in comparison to control lysates). In contrast, antibodies to the NH_2-terminus of MAPKK1 did not detect the antigen after the addition of LF. These results suggest that LF may have proteolytically modified MAPKK1, rendering it undetectable by antibodies against its NH_2-terminus.

We tested the effects of LF on tumor-derived NIH 3T3 (490) cells that express a form of the human *V12HRas* oncogene (*V12-S35 Hras*) that has a mutant effector domain. This mutant of Ras constitutively activates the MAPK pathway but is defective in other effector functions (*15*). The addition of PA and LF, but not LF E687C, to these cells inhibited MAPK activation (Fig. 1B). This inhibition was accompanied by an increase in the electrophoretic mobility of MAPKK1 observed with the COOH-terminal antibody, as well as a loss of MAPKK1 epitopes observed with the NH_2-terminal antibody, observations that are consistent with the notion that LF proteolytically modifies MAPKK1.

We next ascertained the effects of LF on MAPK activation in vitro by assaying myelin basic protein (MBP) phosphorylation in the presence of MAPKK1 and MAPK. The addition of LF, but not LF E687C, prevented MBP phosphorylation (Fig. 2A). To exclude the possibility that contaminants in the LF preparation inhibited MBP phosphorylation, we adsorbed LF to the proteolytically activated PA

Table 1. Effects of anthrax LF on *Xenopus* oocyte maturation. Oocytes were isolated, defolliculated, injected, and induced to mature with progesterone as described (*22*). After progesterone-treated control oocytes had completed GVBD, oocytes were fixed and dissected to score GVBD. In experiment 1, LF was purified from culture supernatants of *B. anthracis* Sterne, a strain that produces PA, LF, and EF, with the use of methods described previously (*23*), and GVBD was scored by dissection 20 hours after progesterone treatment. In experiment 2, LF was produced as a recombinant fusion protein, PA20-LF, and cleaved with Factor X to release the PA20 domain (*8*). The expression host was *B. anthracis* lacking the EF gene. GVBD was scored 4 hours after progesterone treatment, when GVBD was complete in control oocytes. The injection buffer was 0.1 M KCl and 10 mM Hepes (pH 7.5).

Material injected	Progesterone treatment	GVBD*	Frogs used (*n*)
	Experiment 1		
None	−	0/128	7
None	+	143/158	7
Buffer	+	61/75	4
LF (1 ng)	+	24/52	3
LF (10 ng)	+	0/50	3
LF (40 ng)	+	0/75	4
LF E687C (40 ng)	+	57/73	4
	Experiment 2		
None	−	0/99	3
None	+	108/113	3
LF (40 ng)	+	0/75	3
Δ90 cyclin (24 ng)	−	43/43	2
LF (40 ng), Δ90 cyclin (24 ng)	−	75/75	3

*Proportion of oocytes injected that had undergone GVBD.

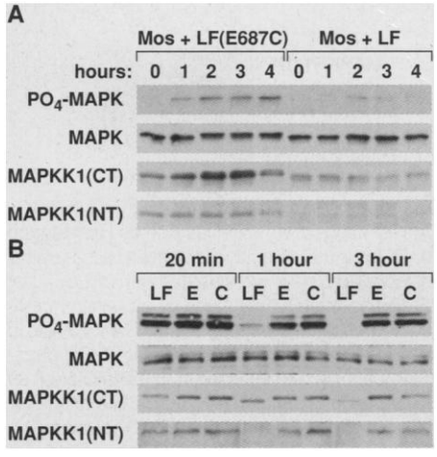

Fig. 1. LF inhibition of MAPK phosphorylation. (**A**) LF or LF E687C (4 μg from a stock containing 1 mg/ml) was added to 40 μl of oocyte lysate (*24*), which was activated 0.5 hour later by the addition of 2.6 μg of maltose-binding protein–Mos fusion protein [stock (0.75 mg/ml) purified from bacteria (*25*)]. Samples were taken at 1-hour intervals; analyzed by SDS-PAGE (*22*) with antibodies against phosphorylated MAPK (PO_4-MAPK, New England Biolaboratories, 1:1000), MAPK (Zymed clone ERK-7D8, 1:1000), or the COOH- or NH_2-terminus of MAPPK1 [MAPKK1 (CT) or (NT); Upstate Biotechnology; 1:500]; and visualized by chemiluminescence. (**B**) NIH 3T3 cells expressing the *V12-S35 Hras* oncogene were grown to approximately 70% confluence in Dulbecco's minimal essential medium (DMEM) + 10% fetal bovine serum (FBS) and then incubated in DMEM + 10% FBS containing PA (1 μg/ml) for 10 min. Control medium (C), LF, or LF E687C (E) (0.1 μg/ml) was then added directly to the cells. Cells were lysed in lysis buffer [20 mM Pipes (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% SDS, aprotonin and leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate] at the times indicated and clarified by centrifugation (15,000*g* for 15 min at 4°C). Samples (10 μg) were analyzed as described above, except that antibodies against PO_4-MAPK were obtained from Promega (1:15,000).

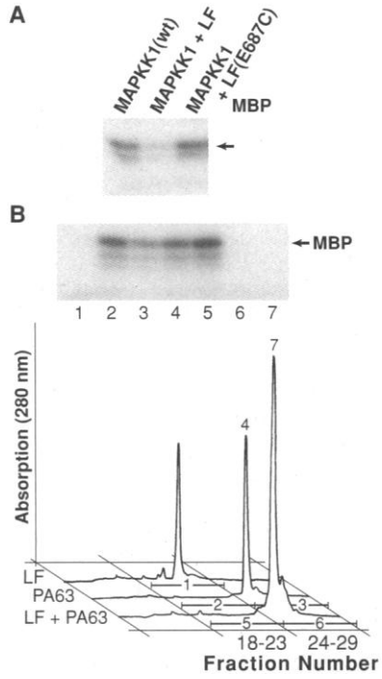

Fig. 2. LF inhibition of in vitro MAPK activation. (**A**) His^6-MAPKK1 prepared from bacterial lysates (*17*) (0.25 μg from a stock containing 0.1 mg/ml) was incubated for 15 min at 30°C in the presence or absence of 0.25 μg of LF or LF E687C and then assayed for MAPK activity (MBP phosphorylation) (*26*). (**B**) Comigration of MAPKK1 inhibitory activity with LF repurified by adsorption to PA63. LF was chromatographed on a MonoQ HR5/5 column in the presence or absence of the PA63 heptamer. The MonoQ column was eluted with a gradient of NaCl in 10 mM 2-(*N*-cyclohexylamino)ethane sulfonic acid (CHES) and 0.06% aminoethanol (pH 9.0). The samples applied to the columns were 250 μg of LF, 250 μg of PA63, and 250 μg of LF + 350 μg of PA63. Fractions were pooled and assayed for inhibition of MAPKK1 activity (*26*). The numbered peaks correspond to the lane position in the autoradiograph.

heptamer (PA63), to which it tightly binds (*16*), and re-purified it by column chromatography. In all cases, the activity that inhibited MBP phosphorylation coeluted with LF (Fig. 2B).

The increase in electrophoretic mobility of MAPKK1 and the disappearance of NH_2-terminal epitopes in *Xenopus* oocyte lysates and *ras*-transformed cells (Fig. 1) suggested that LF might proteolytically cleave MAPKK1. We tested this hypothesis by adding LF to a His^6-tagged MAPKK1 fusion protein produced in bacteria. Within seconds of LF addition, the apparent molecular mass of MAPKK1 decreased by ~6 to 8 kD (Fig. 3A). Cleavage by LF was enzymatic, because proteolysis of MAPKK1 was observed within 15 min with as little as 2 ng of LF per 200 ng of MAPKK1 (≅1 mol of LF: 400 mol of MAPKK1)(Fig. 3B). LF also increased the electrophoretic mobility of His^6-tagged MAPKK2 (Fig. 3B).

Because the MAPKK1 used in these analyses was His^6-tagged at the NH_2-terminus, the actual decrease in its mass was 5 kD less (*17*), which suggests that LF cleaves MAPKK1 in the first 30 amino acids. To test this directly, we assayed NH_2-terminal deletion mutants of MAPKK1 (*17*) for their ability to serve as substrates for LF. These analyses showed that Δn3 (32–51), Δn4 (44–51), Δn5 (38–43), and Δn6 (32–37) were susceptible to LF proteolysis, whereas Δn1 (1–32) and Δn2 (1–52) were resistant (Fig. 3C). Thus, the NH_2-terminal 32 amino acids are essential for cleavage or binding of MAPKK1 by LF. To determine the exact site of cleavage, we performed NH_2-terminal sequence analysis of the larger MAPKK1 proteolytic fragment (*18*) and identified the amino acid sequence IQLNPAPDG (*7*), which corresponds to amino acids 8 through 16 of MAPKK1. Thus, LF cleaves MAPKK1 between amino acids 7 and 8, resulting in the loss of the NH_2-terminal seven residues [PKKKPTP (*7*)]. These results also suggest that these NH_2-terminal residues are essential for MAPKK1 activity. Consistent with this, bacterially produced MAPKK1 lacking the NH_2-terminal seven residues was resistant to proteolysis by LF and possessed no activity toward MAPK (Fig. 4, A and B). These results agree with previous findings that MAPKK1 mutants with deletions of the NH_2-terminal 32 amino acids are less active than wild-type kinase (*19*). In addition, the NH_2-terminal 32 amino acids of MAPKK1 contain a MAPK binding site (*20*), which suggests that LF may prevent the association of MAPKK1 with its substrate.

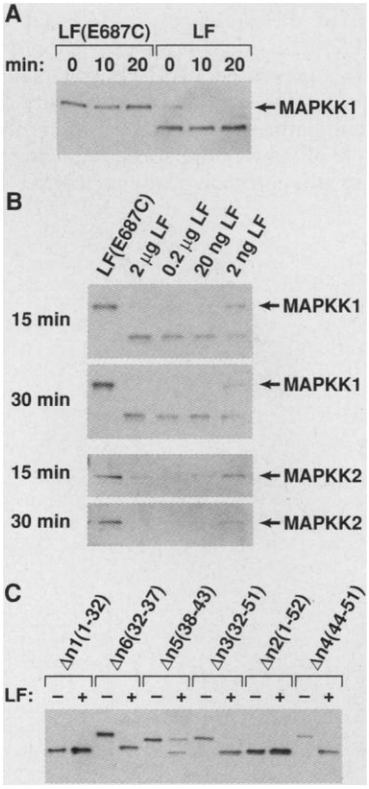

Fig. 3. Proteolysis of MAPKK1 by LF. (**A**) His^6-MAPKK1 (0.1 μg) was incubated in 16 μl of assay buffer (*26*) in the presence of 1 μg of LF E687C or 1 μg of LF. Samples were withdrawn at 0, 10, or 20 min and analyzed by SDS-PAGE and protein immunoblotting with antibodies against the COOH-terminus of MAPKK1. (**B**) His^6-MAPKK1 or His^6-MAPKK2 (0.2 μg) was incubated in the presence of 2 μg of LF E687C or LF that had been serially diluted in ADB. Aliquots were withdrawn at 15 and 30 min and analyzed by SDS-PAGE and immunoblotting with antibodies against the COOH-terminus of MAPKK1 or the NH_2- and COOH-terminus of MAPKK2 (Upstate Biotechnology; 1:1000). (**C**) His-tagged MAPKK1 deletion mutants (0.1 μg) isolated from bacterial lysates (*17*) were incubated in assay buffer (*26*) in the presence or absence of LF (1 μg) for 15 min at 30°C and analyzed by protein immunoblotting. Δn1 and Δn2 were completely resistant to proteolysis, whereas Δn3, Δn4, and Δn6 were cleaved. Δn5 showed partial resistance to proteolysis, which suggests that structural modifications in this construct may partially hinder LF activity.

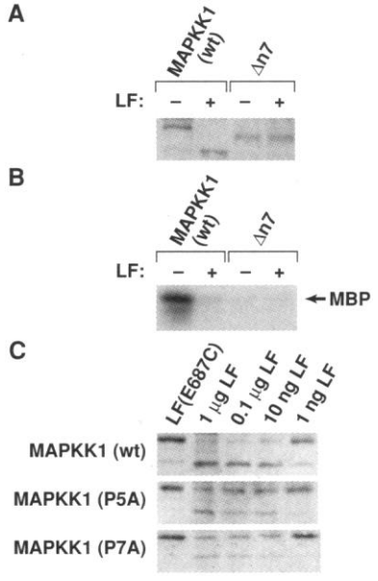

Fig. 4. Analysis of the cleavage site in MAPKK1. (**A**) His^6-tagged MAPKK1 as well as His^6-tagged MAPKK1 mutants lacking the seven NH_2-terminal residues (ΔN7) (*27*) were treated with LF E687C or LF and assayed for cleavage by protein immunoblotting as described (Fig. 3). (**B**) MAPKK1 and Δn7 were assayed for MAPK activity (MBP phosphorylation) in the presence of LF E687C or LF as described (Fig. 2A). (**C**) His^6-tagged MAPKK1 (0.1 μg) as well as His^6-tagged MAPKK1 mutants (0.1 μg) containing proline-to-alanine mutations at residues 5 (P5A) or 7 (P7A) were incubated for 15 min in the presence of 1 μg of LF E687C or LF that had been serially diluted in ADB. Samples were analyzed by SDS-PAGE and immunoblotting with antibodies against the COOH-terminus of MAPKK1.

Our results indicate that frog, mouse, and human MAPKK1 are all substrates of LF. In addition, NH_2-terminal sequence analysis of the larger MAPKK2 proteolytic fragment (*18*) revealed that LF cleaved MAPKK2 between residues 9 and 10, resulting in the loss of NH_2-terminal residues LARRKPVLP (*7*). MAPKK1 and MAPKK2 share a similar NH_2-terminal sequence consisting of three positively charged residues followed by two prolines that are separated by one or two amino acids. Because other residues near the cleavage site are not conserved, the basic residues and prolines may constitute the sequence recognized by LF. This is consistent with a recent report that LF cleaves synthetic peptides after the sequence RRP (*7*, *21*). We generated MAPKK1 mutants in which either Pro^5 or Pro^7 was changed to Ala and assayed each mutant for its ability to serve as a substrate for LF. These analyses showed that compared to wild-type MAPKK1, both mutants were resistant to LF cleavage (Fig. 4C), indicating that the proline residues constitute an important component of the cleavage site.

Further characterization of the cleavage site for LF will be an important step in the development of LF inhibitors. Although LF activity correlates with the activity of the MAPKK inhibitor PD09859 in the NCI's ADS, we cannot exclude the possibility that LF has other cellular substrates. Indeed, MAPKK3 (but not MAPKK4) contains a similar sequence at its NH_2-terminus, and sequences resembling the putative consensus cleavage site occur frequently in protein databases. However, because the MAPK signaling pathway plays such a fundamental role in signal transduction, it is likely that inhibition of MAPKK activities is important in the pathogenesis of anthrax.

REFERENCES AND NOTES

1. S. H. Leppla, in *Handbook of Natural Toxins*, J. Moss, B. Iglewski, M. Vaughan, A. T. Tu, Eds. (Dekker, New York, 1995), vol. 8, pp. 543–572.
2. V. M. Gordon, S. H. Leppla, E. L. Hewlett, *Infect. Immun.* **56**, 1066 (1988); J. C. Milne, D. Furlong, P. C. Hanna, J. S. Wall, R. J. Collier, *J. Biol. Chem.* **269**, 20607 (1994).
3. S. H. Leppla, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3162 (1982).
4. C. Pezard, P. Berche, M. Mock, *Infect. Immun.* **59**, 3472 (1991).
5. J. W. Ezzell, B. E. Ivins, S. H. Leppla, *ibid.* **45**, 761 (1984).
6. A. M. Friedlander, *J. Biol. Chem.* **261**, 7123 (1986).
7. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
8. K. R. Klimpel, N. Arora, S. H. Leppla, *Mol. Microbiol.* **13**, 1093 (1994).
9. S. K. Kochi, G. Schiavo, M. Mock, C. Montecucco, *FEMS Microbiol. Lett.* **124**, 343 (1994); A. Menard, E. Papini, M. Mock, C. Montecucco, *Biochem. J.* **320**, 687 (1996).
10. J. N. Weinstein *et al.*, *Science* **275**, 343 (1997); H.-M. Koo *et al.*, *Cancer Res.* **56**, 5211 (1996).
11. D. T. Dudley, L. Pang, S. J. Decker, A. J. Bridges, A. R. Saltiel, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7686 (1995).
12. For reviews, see T. Moriguchi, Y. Gotoh, E. Nishida, *Adv. Pharmacol.* **36**, 121 (1996); M. S. Murakami and G. F. Vande Woude, *Methods Enzymol.* **283**, 584 (1997); W. T. Matten and G. F. Vande Woude, *Sem. Dev. Biol.* **5**, 173 (1994).
13. N. S. Duesbery and G. F. Vande Woude, unpublished data.
14. M. Glotzer, A. W. Murray, M. W. Kirschner, *Nature* **349**, 132 (1991).
15. M. A. White *et al.*, *Cell* **80**, 533 (1995).
16. Y. Singh, K. R. Klimpel, N. Arora, M. Sharma, S. H. Leppla, *J. Biol. Chem.* **269**, 29039 (1994).
17. S. Mansour, J. M. Candia, K. K. Gloor, N. G. Ahn, *Cell Growth Differ.* **7**, 243 (1996); S. J. Mansour *et al.*, *Science* **265**, 966 (1994).
18. Wild-type MAPKK1 (0.5 μg) was incubated with LF (0.2 μg) as described in Fig. 2A for 30 min at 30°C. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride membrane in CAPS transfer buffer {3-[cyclohexylamino]-1 propanesulfonic acid (10 mM, pH 11) and 10% methanol} at 300 mA constant current for 30 min. After transfer, membranes were quickly stained with Ponceau S solution (0.1% Ponceau S and 5% acetic acid) and rinsed with distilled deionized water. The appropriate band was cut from the membrane and subjected to automated Edman degradation in an Applied Biosystems 477A gas-phase sequencer, and phenylthiohydantoin derivatives were identified online with a 120 phenylthiohydantoin analyzer.
19. S. J. Mansour, J. M. Candia, J. E. Matsuura, M. C. Manning, N. G. Ahn, *Biochemistry* **35**, 15529 (1996).
20. M. Fukuda, Y. Gotoh, E. Nishida, *EMBO J.* **16**, 1901 (1997).
21. S. E. Hammond and P. C. Hanna, abstract presented at the 97th General Meeting of the American Society of Microbiology, Miami Beach, FL (1997).
22. N. S. Duesbery *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9165 (1997).
23. S. H. Leppla, *Methods Enzymol.* **165**, 103 (1988).
24. E. K. Shibuya, T. G. Boulton, M. H. Cobb, J. V. Ruderman, *EMBO J.* **11**, 3963 (1992).
25. N. Yew, M. L. Mellini, G. F. Vande Woude, *Nature* **355**, 649 (1992).
26. MAPKK and LF were diluted in 16 μl of assay buffer {assay dilution buffer [ADB, Upstate Biotechnology; consisting of 20 mM Mops (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol] containing 10 mM protein kinase C inhibitor peptide, 1 mM protein kinase A inhibitor peptide, and 10 mM compound R24571}. After incubation at 30°C for 0.5 hours, 11 μl of kinase buffer {[γ-^{32}P]ATP (Amersham; 10 mCi/ml, 3000 mCi/mmol) diluted 1:9 in ADB containing 0.5 mM ATP, 75 mM $MgCl_2$, 0.35 μg of MAPK [ERK2; D. J. Robbins *et al.*, *J. Biol. Chem.* **268**, 5097 (1993)], and 10 μg of MBP (Upstate Biotechnology)} was added, and samples were incubated for a further 15 min at 30°C. Proteins were separated by SDS-PAGE on 14% gels and processed for autoradiography.
27. A MAPKK1 construct containing a seven–amino acid NH_2-terminal deletion was generated by polymerase chain reaction using primer pairs A and B and pKH-1, encoding His^6-tagged human wild-type MAPKK1 [S. J. Mansour *et al.*, *J. Biochem.* **116**, 304 (1994)], as a template. The amplified fragment was digested with Xho I and Hind III and introduced into pRSETA (Invitrogen). The primers used were 5′-AAGAAGCGG**CTCGAG**ATCCAGCTGAACCCG-3′ (A) and 5′-CTTTGTTAGCAGCCGGATCAAGCTTCGAAT-3′ (B). Constructs containing MAPKK1 mutations $Pro^{5\ or\ 7}$ to Ala were generated by introducing the mutations into pKH-1 with the use of the Quickchange Site-Directed Mutagenesis Kit (Stratagene). The primers used were 5′-ATGCCCAAGAAGAAG**G**CGACGCCCATCCAGCTGAACCCG-3′ for P5A, 5′-CCCAAGAAGAAGCCGACG**G**CCATCCAGCTGAACCCGGCC-3′ for P7A, and their respective complementary sequences. The sequences of these constructs were confirmed by direct DNA sequencing. To produce recombinant protein, *Escherichia coli* strain TOP10F′ was transformed with these constructs. Cultures were grown at 37°C to an optical density at 600 nm of 0.3 in 750 ml of SOB and ampicillin (100 μg/ml) and then incubated for 1 hour in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside, at which point M13/T7 helper phage was added to a concentration of three plaque-forming units per cell. Cells were harvested by centrifugation 3 hours later, resuspended in 36 ml of extraction buffer (EB) [50 mM potassium phosphate (pH 8.0); 10% (v/v) glycerol; 0.25% (v/v) Tween-20; and 1 mM dithiothreitol (DTT), containing protease inhibitors (EDTA-free Complete tablets; Boehringer Mannheim)], and lysed by four sonication/freeze/thaw cycles. Soluble protein was separated by centrifugation (at 14,000 rpm for 30 min with an SS-34 rotor), and the supernatant was sterile-filtered and adjusted to 1% Tween-20, 1 M KCl, and 20% glycerol. The supernatant was then loaded on a Ni^{2+}-charged HiTrap Chelating column (Pharmacia), washed with EB containing 10 mM imidazole, and eluted with EB containing 200 mM imidazole. Fractions containing recombinant protein were then pooled and desalted and concentrated in buffer containing 50 mM tris (pH 8.0), 0.1 M NaCl, 10% glycerol, 0.01% Triton X-100, and 1 mM DTT by centrifugation with a Centriprep-30 centrifugal concentrator (Amicon, Beverely, MA, XX).

28. This paper is dedicated to the memory of Ken Paull, whose untimely death is a significant loss to NCI and the cancer research community. The authors thank A. Cline and R. Frederickson for preparing the manuscript, L. Miller for technical assistance, H.-M. Koo for helpful discussions, M. Jeffers and S. Koochekpour for critical comments on the manuscript, and A. Murray for providing Δ90 cyclin. This research was sponsored in part by NCI, Department of Health and Human Services, under contract with Advanced BioScience Laboratories. By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. government and its contractors and agents to retain a nonexclusive royalty-free license in and to any copyright covering the article.

8 December 1997; accepted 10 March 1998

Disruption of Splicing Regulated by a CUG-Binding Protein in Myotonic Dystrophy

Anne V. Philips, Lubov T. Timchenko, Thomas A. Cooper*

Myotonic dystrophy (DM) is caused by a CTG expansion in the 3′ untranslated region of the *DM* gene. One model of DM pathogenesis suggests that RNAs from the expanded allele create a gain-of-function mutation by the inappropriate binding of proteins to the CUG repeats. Data presented here indicate that the conserved heterogeneous nuclear ribonucleoprotein, CUG-binding protein (CUG-BP), may mediate the trans-dominant effect of the RNA. CUG-BP was found to bind to the human cardiac troponin T (cTNT) pre–messenger RNA and regulate its alternative splicing. Splicing of cTNT was disrupted in DM striated muscle and in normal cells expressing transcripts that contain CUG repeats. Altered expression of genes regulated posttranscriptionally by CUG-BP therefore may contribute to DM pathogenesis.

Myotonic dystrophy (DM) is a dominantly inherited neuromuscular disorder caused by a trinucleotide (CTG) expansion in the 3′ untranslated region (UTR) of *DM*, the gene that encodes DM protein kinase (*1*). The severity of symptoms correlates with the length of the expansion (*2*). It has been proposed that the CUG repeats in the *DM* RNA create a gain-of-function mutation that disrupts processing or transport of heterologous RNAs in a trans-dominant manner (*3–5*). This hypothesis is supported by the observation that transcripts of the expanded *DM* allele accumulate in the nucleus (*6*). One possible mediator of a trans-dominant effect is CUG-BP (or hNab50), a conserved heterogeneous nuclear ribonucleoprotein (hnRNP) that binds specifically to RNA that contains CUG repeats (*7, 8*). CUG-BP is ubiquitously expressed, distributes to both the nucleus and cytoplasm, and exists in two isoforms that differ by the extent of phosphorylation (*7, 9*). The hypophosphorylated isoform accumulates in the nucleus in tissues and cultured cells

A. Philips and T. Cooper, Department of Pathology, Baylor College of Medicine, Houston, TX 77030, USA.
L. Timchenko, Department of Medicine, Section of Cardiology, Baylor College of Medicine, Houston, TX 77030, USA.

*To whom correspondence should be addressed. E-mail: tcooper@bcm.tmc.edu