Anthracis 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), inactivates members of the mitogen-activated protein kinase kinase or MEK family through proteolysis of their NH$_2$ termini. However, neither the substrate requirements for LF cleavage nor the mechanism by which proteolysis inactivates MEK have been demonstrated. By means of deletion mutant analysis and site-directed mutagenesis, we have identified an LFIR (LF interacting region) in the COOH-terminal kinase domain of MEK1 adjacent to the proline-rich region, which is essential for LF-mediated proteolysis of MEK. Point mutations in this region block proteolysis but do not alter the kinase activity of MEK. Similar mutations in MEK6 also prevent proteolysis, indicating that this region is functionally conserved among MEKs. In addition, NH$_2$-terminal proteolysis of MEK1 by LF was found to reduce not only the affinity of MEK1 for its substrate MAPK but also for catalytic activity.

The lethal effects of Bacillus anthracis have been attributed to an exotoxin, which it produces (1). This exotoxin is composed of three proteins: protective antigen (PA), edema factor, and to an exotoxin, which it produces (1). This exotoxin is composed of three proteins: protective antigen (PA), edema factor, and lethal factor (LF), inactivates members of the mitogen-activated protein kinase kinase or MEK family through proteolysis of their NH$_2$ termini. However, neither the substrate requirements for LF cleavage nor the mechanism by which proteolysis inactivates MEK have been demonstrated. By means of deletion mutant analysis and site-directed mutagenesis, we have identified an LFIR (LF interacting region) in the COOH-terminal kinase domain of MEK1 adjacent to the proline-rich region, which is essential for LF-mediated proteolysis of MEK. Point mutations in this region block proteolysis but do not alter the kinase activity of MEK. Similar mutations in MEK6 also prevent proteolysis, indicating that this region is functionally conserved among MEKs. In addition, NH$_2$-terminal proteolysis of MEK1 by LF was found to reduce not only the affinity of MEK1 for its substrate MAPK but also for catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Constitutively activated MEK (x7, an3/S222D) was a kind gift of N. Ahn (University of Colorado, Boulder, CO). Expression vectors encoding His$_x$-tagged wild-type rat ERK2 as well as a chimeric protein consisting of the first two subdomains of p38 MAPK fused to subdomains 2–10 of ERK2 (PIE) were a kind gift from M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX). Mouse MEK6 cDNA was a kind gift from J. Han (Scripps Institute, La Jolla, CA). CL100, c-Jun, activating factor-2, and B-Raf were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies raised against the NH$_2$ terminus of MEK1 (MEK1/NT) were obtained from Upstate Biotechnology. Polyclonal antibodies raised against the COOH terminus of MEK1 (C-18) as well as the NH$_2$-terminal His-tag (H-15) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Construction of Deletion and Point Mutants**—COOH-terminal deletion mutants of MEK1 were made by digesting a plasmid encoding His$_x$-tagged human wild-type MEK1 (pMKK1) with the indicated restriction enzymes and ligating the resulting fragment into the appropriate pRSET vector as follows: Δc1-(160–318) EcoRI (Δc2-(218–318) NheI/NcoI, and Δc3-(229–318) NheI/BI). Additional COOH-terminal deletion mutants of MEK1 were made by PCR amplification of pMKK1 using PCR primers 5’-GGACAGCAAATGGGTCGGG-3’ (corresponding to the multiple cloning site on pRSET) and 5’-CCGTATAAGCCTTAGG-GGCC-3’ to introduce a novel HindIII site at position 924. The resulting PCR product was digested with BamHI and HindIII, gel-purified, and ligated into BamHI/HindIII-digested pRSET yielding a construct encoding Δc5-(292–392) as well as one encoding Δc4-(261–392) because of a fortuitous error. Internal deletion mutants of MEK1 were made by digesting pMKK1 with the indicated restriction enzyme and re-ligating the gel-purified plasmid as follows: Δ1i-(20–318) HindI and Δ12-94 (219–318) Msal. To construct Δ5-(292–318), we used the Stratagene QuickChange site-directed Mutagenesis kit to introduce novel Xhol sites into pMKK1 at position 910 using the primer 5’-GGCCAAAGGACCTTCG-5’ and its complementary sequence and then at position 988 using the primer 5’-GTGGGATTACATGCTTCCCAG-GCGTC-3’ and its complementary sequence. The resulting construct
Anthrax LF Proteolysis and Inactivation of MEK

Fig. 1. Locating the LF-interacting region on MEK1. A, a graphic representation of the MEK1 deletion mutants (identified at the left of the boxed regions) distinguishes those portions of wild type (w.t.) MEK1 that has been deleted (in white) from those that remain intact (in gray). The line at the left of the boxed region indicates the approximate site of cleavage, whereas the two open circles above the boxed region are intended to represent activating phosphorylation sites at residues 218 and 222. Because in vitro cleavage assays might generate false-positive results attributed to the presence of high concentrations of protease and substrate and/or the presence of unstable translation products, we elected instead to assay cleavage in a cell-like background. To do this, we incubated recombinant MEK protein and LF in Xenopus oocyte lysates and then immunoprecipitated with an antibody raised against the NH2 terminus of MEK. This antibody does not recognize MEK1 that has been cleaved by LF (10). The immunoprecipitates were then analyzed by SDS-PAGE and immunoblotting with antibodies to the NH2 terminus of MEK1 (MEK1-NT) (1:1000). Selected Western blots of Δi1 (B), Δi2 (C), and Δi5 (D) are shown. Lysates were mixed with nothing (lane 1), active LF (lane 2), active LF (3) plus a MEK deletion mutant (lane 3), inactive LF(E687C) (lane 4), or inactive LF plus a MEK deletion mutant (lane 5). E, His6-tagged wild-type and Δi3 MEK (0.2 μg) were incubated with LF (0.02 μg) in assay buffer (4) at 30 °C for 5–10 min, and proteins were separated by SDS-PAGE and immunoblotting as described above. Lanes 1 and 2, wild-type MEK in the absence or presence of LF, respectively; lanes 3 and 4, Δi3 MER in the absence or presence of LF, respectively.

was digested with XhoI and re-ligated following gel purification to generate Δi3 (292–318). All of the sequences were confirmed by direct DNA sequencing.

Constructs containing MEK1 mutations in the LF-interacting region (LFIR) were generated by introducing the mutations into pM KK1 with the use of the QuikChange site-directed mutagenesis kit. The primers used were 5′-CCCAACACTGCAATTTGCTGGG-3′ for F310A, 5′-CCCAACACTGCAATTTGCTGGG-3′ for E311H, 5′-GGATTACAAGCTTTCTCTCC-3′ for E319H, 5′-CCAGTTTAACTGGTCTCAGTTG-3′ for Phe310-Glu311-Glu319 (FEE), 5′-ATTGTGTCGGCCGATATAAGCT-3′ for I15D, 5′-CTCAAACAGGTGGCAGAGGAGCCATCGCC-3′ for V136A, 5′-GGACACAAGGACATCGCCAACTCCCACGAG-3′ for P276A, and 5′-CCTCCTCCTCAGAGAAGCCGGATGGAGT-3′ for L234A and their respective complementary sequences. MEK6 was PCR-cloned from a plasmid containing mouse MEK6 cDNA and ligated into a pRSET (NH2-terminal His6-tagged) bacterial expression vector. Constructs containing MEK6 point mutations were generated by introducing the mutations with the use of the QuikChange site-directed mutagenesis kit. The primers used were 5′-ggaaacctgtgctgtaagaccaaaaagaagcctt-3′ for H15D, 5′-CTTTACAGTGCCGACAGAGGACCATGGCC-3′ for V271A, 5′-GGAGGAGGCATGGCAACACCTCCCACGAGACG-3′ for P276A, and 5′-CCATCGCCACAGGCCCAGAGACGACAG-3′ for L278A and their respective complementary sequences. ERK(CD) was made by site-directed mutagenesis of the His6-tagged wild-type ERK2 expression vector to introduce asparagine to asparagine substitutions at residues 321 and 324 with the primers 5′-gctgaggtatattactgcgacacgc-3′ and its complementary sequence. All of the sequences were confirmed by direct DNA sequencing.

Protein Expression and Purification—Anthrax lethal factor was produced in a non-toxicogenic, sporulation-defective strain of B. anthracis 9430 (3). Recombinant MEK protein was expressed in Escherichia coli and purified by fast pressure liquid chromatography essentially as described earlier (10). Wild-type ERK2 and ERK(CD) were expressed similarly with the exception that cultures were grown and induced overnight at 30 °C. MEK Cleavage Analysis—To measure MEK cleavage in a cell-based assay, lysates of Xenopus laevis oocytes were prepared as described previously (16). Recombinant MEK proteins (0.5 μg) were added as indicated in the text to 50 μl of oocyte lysate and diluted to a final volume of 0.5 ml with oocyte extraction buffer (0.25 M sucrose, 0.1 M NaCl, 0.02 M Hepes (pH 7.5), 2.5 mM MgCl2). MEK1(NT) antibody (5 μl) was added, and lysates were incubated overnight on a rotorator shaker at 4 °C. Immune complexes were precipitated with protein A-agarose, washed, separated by SDS-PAGE, and immunoblotted using polyclonal antibodies raised against MEK1(NT).

Cleavage of MEK proteins in vitro was measured by adding 0.2 μg of MEK and 0.2 μg of LF to 2.5 μl of 4× assay buffer (20 mM NaCl, 20 mM EGTA, 320 mM potassium phosphate buffer, pH 7.2), and distilled water was added to a final volume of 10 μl. After incubation at 30 °C for 5–10 min, proteins were separated by SDS-PAGE, blotted onto polyvinylidenefluoride diffusion membrane, and probed with antibodies to the NH2 terminus of MEK1 (1:1000).

Kinase Assays—To measure B-Raf phosphorylation of MEK deletion mutants, 2 μl (0.4 units) of recombinant B-Raf, 0.2 μg of MEK protein, 3 μl of AB (20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol), and 3 μl of ATP mixture (γ32P]ATP (10 μCi/ml, 3000 mCi/mmol, Amersham Biosciences) diluted 1:3 in 0.5 mM ATP, 75 mM MgCl2) were mixed with distilled water to a final volume of 10 μl and incubated for 15 min at 30 °C. When assaying the ability of B-Raf to phosphorylate MEK1 in the absence or presence of LF, B-Raf was added last and the reaction was incubated for 5 min at 30 °C. Proteins were then separated by SDS-PAGE upon 10 μg gels and processed for autoradiography. To measure MEK activity in the presence of LF or inactive LF(E687C) (0.2 μg), samples were prepared in a similar manner with the exception that ERK (wild type, PHG, or ERK(CD)) was added. Proteins were then separated by SDS-PAGE on 14% gels and processed for autoradiography.

RESULTS

1. Identification of an LFIR—To identify regions of MEK that are distal to the NH2-terminal cleavage site and are required for interaction with LF, we undertook an analysis of internal and carboxyl-terminal deletion mutants of MEK1. Two internal and five COOH-terminal deletion mutants (Fig. 1A) were generated and analyzed each for their ability to be recognized and cleaved by LF. Of the deletion mutants analyzed, only Δi2 was noticeably cleaved (Fig. 1, B–D, data not shown). This finding was surprising considering that Δi2 lacks residues 94–219, corresponding to the amino-terminal kinase domain and suggests that the NH2 terminus may form a stable association with the COOH-terminal portion of MEK1. Because neither Δi5 (292–329) nor Δi1 (20–318) was cleaved by LF but Δi2 was, we hypothesized that those regions absent from Δi5
and Δ1 but present in Δ2 must be necessary for binding and/or cleavage. To test this hypothesis, we used site-directed mutagenesis to introduce novel restriction sites in MEK1 to produce a new deletion mutant, Δ3, lacking residues 292–318. Analyses with Δ3 showed that it was resistant to cleavage by LF (Fig. 1E). These results indicate that a region contained either in whole or in part within residues 292–318 is necessary for binding and/or cleavage by LF. We have called this region the LF-interacting region or LFIR.

**2. The LFIR Is Functionally Conserved**—Because not only MEK1 but also MEK2 and MEK6 and MEK7 are cleaved by LF, we reasoned that critical elements of the LFIR must be conserved among the MEKs. Residues 292–306 are present at the COOH-terminal end of a proline-rich insert, which is unique to MEKs 1 and 2, and thus are not likely to play an important role in LF substrate recognition. However, an analysis of a sequence alignment of MEKs in the region following the proline-rich insert revealed the presence of conserved elements (Fig. 2A). We used site-directed mutagenesis to determine the importance of these residues in LFIR function. Neither the single mutations in Phe310, Glu311, Glu319, and Leu313 nor a triple mutation in FEE interfered with the ability of LF to cleave MEK1 (Fig. 2B). However, the substitution of alanine for Ile316, Val317, Pro322, or Leu324 inhibited the ability of LF to cleave MEK1, indicating that these residues are critical for binding and/or cleavage by LF. Moreover, the LFIR appears to be functionally conserved because the introduction of similar mutations at Val271, Pro276, or Leu278 in MEK6 also blocked proteolysis by LF (Fig. 2C). These results indicate that a conserved COOH-terminal region of MEKs is required for LF-mediated proteolysis.

**3. The LFIR Is Proximal to COOH-terminal Regulatory Epitopes**—The COOH end of the proline-rich region of MEK1 is necessary for the association of MEK with the MEK1 activator Raf (17, 18), suggesting the possibility that the LFIR and Raf-binding regions in MEK1 may overlap. To further evaluate this possibility, we assayed the ability of B-Raf to phosphorylate and activate Δ3 or Δc5 MEK. Consistent with the hypothesis that LF interacts with MEK in a region that contains a Raf-interacting site, in vitro kinase assays demonstrated that B-Raf could phosphorylate neither Δ3 nor Δc5. Subsequent analyses of the ability of B-Raf to phosphorylate MEK1 with point mutations in the LFIR indicated that with the exception of Phe310, alanine substitutions interfered with the ability of B-Raf to phosphorylate (Fig. 2D) but not activate MEK1. These results indicate that although the LFIR and the B-Raf-interacting region of MEK1 are not identical, they are adjacent or overlapping. If the LFIR and the B-Raf-interacting regions of MEK1 do indeed overlap, LF should antagonize B-Raf-induced activation of MEK. We tested this hypothesis by assaying the ability of B-Raf to phosphorylate MEK1 in vitro in the presence of increasing amounts of LF. The presence of an equimolar amount of LF but not bovine serum albumin was sufficient to reduce the ability of B-Raf to phosphorylate MEK1 by half (Fig. 3A). Collectively, these results indicate that adjacent or overlapping epitopes of MEK1 are required not only for cleavage by LF but also for B-Raf-mediated phosphorylation.

**4. Mechanism of LF Inhibition of MEK Activity**—The preceding observation raises the intriguing possibility that LF may inhibit the activity of MEK by blocking its activation by Raf. However, LF(E687C), a non-toxic inactive LF containing a single amino acid substitution in the putative zinc binding site (20), was as able as wild-type LF to inhibit B-Raf-mediated MEK phosphorylation (Fig. 3A). Moreover, we have found that the ability of constitutively activated MEK1 (Δn3/S222D) to phosphorylate and activate ERK2 is decreased in the presence of LF (Fig. 3B). Because the kinase activity of Δn3/S222D MEK1 is not dependent upon B-Raf-mediated activation, the decrease in phosphorylation and activation of ERK associated with exposure to LF must have resulted from a decrease in the intrinsic kinase activity of MEK1 and/or a decrease in the ability of MEK1 to interact with ERK. To test this hypothesis, we assayed the effects of LF upon the ability of MEK1 to phosphorylate ERK2 protein containing mutations at the common docking (CD) domain (D321N/D324N) through which the NH₂-terminus of MEK1 binds ERK2 (21). We reasoned that if LF reduced the ability of MEK1 to interact with ERK through its CD domain, LF should not inhibit MEK1-mediated phosphorylation of ERK(CD). However, we found that although LF reduced ERK2 phosphorylation by approximately two-thirds, it still reduced ERK(CD) phosphorylation by half as much (Fig. 3C). Similar results were obtained when we assayed phosphorylation of PIIE, a chimeric protein consisting of the first two subdomains of p38 MAPK fused to subdomains 2–10 of ERK2 and lacking a putative docking site on ERK2 (Fig. 3C) (22). These results indicate that the loss of MEK activity following proteolysis by LF cannot be attributed entirely to a decreased ability to bind ERK.

In the preceding experiments, phosphorylation was measured with MEK and ERK present in approximately equimolar amounts. Thus, the extent of phosphorylation reflected not only the affinity of MEK for ERK but also the intrinsic kinase activity of MEK. However, the relative contribution of each of these factors may be altered by varying the ratio of substrate to kinase so that at relatively low concentrations of ERK the rate...
of phosphorylation is limited by the affinity of MEK for ERK, whereas at relatively high concentrations of ERK, the reaction becomes saturated and the rate of phosphorylation largely reflects the intrinsic kinase activity of MEK. Therefore, in the following experiments, the level of MEK1 protein was kept constant at 1.5 μg while the amount of substrate was varied from 0.25 to 12 μg. Rather than conforming to classic Michaelis-Menten enzyme kinetics, a plot of the extent of wild-type MEK1-mediated ERK phosphorylation versus substrate concentration revealed a sigmoidal activation of MEK, more closely resembling that of an allosteric enzyme (Fig. 3D). Similar observations have been made in Xenopus oocytes and were attributed to a combination of protein synthesis-dependent positive feedback and the intrinsic ultrasensitivity of the MEK-ERK kinase cascade (23, 24). Because our results were obtained in vitro in the presence of recombinant proteins, they indicate that ERK itself is capable of positively regulating MEK activity. Consistent with the accepted view that the NH2-terminal docking (D) domain of MEK1 binds ERK2, treatment with LF lowered the affinity of MEK for ERK (Fig. 3C). However, LF treatment also decreased the extent of ERK phosphorylation at saturating concentrations, indicating that the NH2-terminal residues 1–7 of MEK1 play a role in maintaining intrinsic kinase activity. To verify this result, we repeated this assay, substituting ERK(CD) as the substrate (Fig. 3E). Under these conditions, LF did not alter the affinity of MEK for its substrate but did decrease the extent of ERK(CD) phosphorylation under saturating conditions. Thus, LF inhibits MEK activity not only by lowering its affinity for ERK but also by decreasing its intrinsic kinase activity.

**DISCUSSION**

To date, MEKs are the only identified physiological substrates of LF. A comparison of the LF cleavage sites on MEKs 1–4, MEK6, and MEK7 reveals elements of homology. In all cases, the cleavage site is preceded by a series of basic residues and followed immediately by an aliphatic residue. Also, with the exception of MEKs 3 and 4, the cleavage site is preceded by one or more proline residues. Synthesizing these results, the consensus site for LF cleavage fits the pattern B(B/P/PX)X1–3-Al, where B represents a basic residue, P is proline, X is the variable, and Al represents aliphatic residues. This motif is similar to a described generic MAPK binding site or D domain, consisting of a basic amino acid center, which is flanked by hydrophobic residues on one or both sides (21). This raises the possibility that LF may be a D domain protease, which targets both activators and substrates of MAPKs. However, using in vitro cleavage assays, we have been unable to detect LF-induced cleavage of CL-100, c-Jun, or activating transcription factor-2, known substrates of MAPKs that contain a D domain (data not shown). In addition, yeast two-hybrid analyses for binding partners of LF have isolated cDNA for MEK2, which lacks the NH2-terminal cleavage site (13). Thus, other regions of MEKs, in addition to the NH2-terminal cleavage site, must be required for LF substrate recognition.

Using mutational analysis, we have identified a functionally conserved COOH-terminal region of MEKs that is essential for LF-mediated proteolysis of MEK. The presence of a conserved region distal to the cleavage site, which is necessary for binding and/or cleavage by LF, may explain in part the failure to identify physiological LF substrates other than MEKs. The fact that other MEK1-regulatory proteins, such as B-Raf (17, 18), p21-activated kinase (25), and the scaffolding protein MEK partner-1 (26, 27), also interact with MEK in this region suggests that this region constitutes a key regulatory domain of MEK1 and perhaps other MEKs.

The presence of a shared regulatory domain may have func-
tional implications for LF toxicity because the presence of MEK-binding proteins may alter the access of LF to its substrates. Conversely, LF might decrease MEK activity by competitively displacing positive regulators of MEKs. However, by itself, this mechanism seems insufficient to explain how LF inactivates MEKs, because as noted in the preceding section, LF can inhibit the activity of constitutively activated MEK1. Because phosphorylation under these conditions is dependent not only upon kinase activity but also substrate affinity, we reasoned that LF might inhibit MEK by either reducing its intrinsic kinase activity or decreasing its affinity for ERK. The latter seemed more probable because MEK1 deletion mutants lacking the 32 NH2-terminal residues are deficient in their ability to bind ERK (22, 28) and because mutations in the docking domain decrease the efficiency with which MEK1 activates ERK (21). However, we found that as well as decreasing the affinity of MEK for its substrate, LF also decreased the intrinsic kinase activity of MEK. The latter result was not expected but is without precedence. Based upon homology to the A-helix of cAMP-dependent protein kinase and its observation that NH2-terminal deletions and activation loop substitutions synergize to activate MEK1, Ahn and colleagues (29) have hypothesized that regions of the NH2 terminus form long range interactions with the activation loop and that perturbation of the structure within this region promotes conformational changes within the activation loop that favor activation. In addition, the structural analyses of the α-subunit of the serine/threonine protein kinase CKII, which is constitutively active, indicate that a cluster of basic residues at its NH2 terminus stably associates with the activation loop, keeping it in an open conformation (30, 31). By analogy, we predict that the NH2 terminus of MEK1 associates with its activation loop to promote its activity. NH2-terminal structure may also promote protein stability, because we previously noted that the long term stability of MEKs is decreased in cells treated with PA and LF (10). The proximity of the D domain to the activation loop may coordinate MEK-ERK interaction and facilitate ERK phosphorylation and activation.

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