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Involvement of Domain II in Toxicity of Anthrax Lethal Factor*[S]

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Anthrax lethal factor (LF) is a Zn^{2+}-metalloprotease that cleaves and inactivates mitogen-activated protein kinase kinases (MEKs). We have used site-directed mutagenesis to identify a cluster of residues in domain II of LF that lie outside the active site and are required for cellular proteolytic activity toward MEKs. Alanine substituted for Leu^{293}, Lys^{294}, Leu^{514}, Asn^{516}, or Arg^{491} caused a 10–50-fold reduction in LF toxicity. Further, whereas pairwise substitution of alanine for Leu^{514} and either Leu^{293}, Lys^{294}, or Arg^{491} completely abrogated LF toxicity, pairwise mutation of Leu^{514} and Asn^{516} resulted in toxicity comparable with N516A alone. The introduction of these mutations reduced LF-mediated cleavage of MEK2 in cell-based assays but altered neither the ability of LF to bind protective antigen nor its ability to translocate across a membrane. Interestingly, direct *in vitro* measurement of LF activity indicated that decreased toxicity was not always accompanied by reduced proteolytic activity. However, mutations in this region significantly reduced the ability of LF to competitively inhibit B-Raf phosphorylation of MEK. These results provide evidence that elements of domain II are involved in the association of LF into productive complex with MEKs.

Anthrax toxin is derived from an exotoxin produced by the Gram-positive bacterium *Bacillus anthracis.* The toxin is composed of three proteins: protective antigen (PA),[1] edema factor (EF), and lethal factor (LF). PA, by itself, is not toxic; it serves to translocate EF and LF to the cytosol (1–4). Two cell surface receptors for PA (anthrax toxin receptor or ANTXR) have recently been identified (5, 6). Following binding to ANTXR, PA is cleaved by cell surface-associated furin, removing a 20-kDa fragment and leaving a 63-kDa fragment (PA_{63}) attached to the ANTXR. This step is necessary to expose a binding site for EF or LF (7) as well as to remove steric hindrances to the subsequent oligomerization of PA into a heptamer (4, 8, 9). Following EF or LF binding to heptameric PA_{63}, the toxin complex internalizes via the endosomal pathway (10–12). The acidic environment of the endosome triggers a conformational change in the structure of PA, causing it to form a pore through which EF or LF apparently transits to the cytosol (4).

EF is an adenylate cyclase (12). EF plus PA (edema toxin) is not lethal but causes edema when injected subcutaneously (13, 14). LF is a Zn^{2+}-metalloprotease that specifically cleaves the NH_2 termini of mitogen-activated protein kinase kinases (MEKs) 1 and 2 (15, 16), 3 (17) and 4, 6, and 7 (18), but not MEK 5 (18), resulting in their inactivation (15, 19, 20). Combinations of PA plus LF (lethal toxin) do not cause edema, but when injected intravenously rapidly induce hypotensive shock leading to death of the host (13, 14).

LF is a large 776-amino acid (90.2-kDa) protein (21). The crystal structure of LF has been solved to a resolution of 2.2 Å (22) (see Fig. 1). It is composed of four domains. Domain I comprises the NH_2-terminal portion, which binds PA. Domain II (residues 263–297 and 385–550) shows structural similarity with the adenosine diphosphate-ribosylating toxin of *Bacillus cereus* but lacks the residues required for nicotinamide adenine dinucleotide binding and catalysis. Domains III inserts into domain II and contains a series of four tandem imperfect repeats of a helix-turn element present in domain II. A previous report suggests this region is important for LF activity because deletion of the second imperfect repeat (residues 308–326) renders LF nontoxic (23). Acidic residues in domain III form specific contacts with the basic NH_2 termini of MEKs. Domain IV has limited structural homology to thermolysin and contains the catalytic core. Quinn *et al.* (24) have shown that insertional mutagenesis within this domain (*e.g.* insertion of an Arg/Val dipeptide at residue 720) can eliminate the toxicity of LF without blocking its ability to bind PA. Elements of domains II, III, and IV together create a long catalytic groove into which the NH_2 terminus of MEK fits, forming an active site complex.

We have demonstrated the existence of an LF-interacting region located in the COOH-terminal region of MEK1, adjacent to a proline-rich region where other regulatory molecules, including B-Raf, interact with MEK (19). Mutation of conserved residues within this region prevents LF proteolysis of MEKs without altering the kinase activity of MEK (19). The precise function of the LF-interacting region is not certain, although we have hypothesized that it is required for MEK association with LF. If LF and MEK do indeed interact outside the active site complex, then it follows that there exists a corresponding region of LF in which the introduction of mutations at key residues should disrupt toxicity. In this article we have identified, using site-directed mutagenesis, a cluster of residues in domain II of LF that plays a key role in LF-mediated toxicity.

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[1] The abbreviations used are: PA, protective antigen; ANTXR, anthrax toxin receptor; CHO, chinese hamster ovary; *df*, degrees of freedom; EF, edema factor; ERK, extracellular regulated kinase; FPLC, fast pressure liquid chromatography; LF, lethal factor; MEK, mitogen-activated protein kinase kinase; Mops, 3-(*N*-morpholino)propanesulfonic acid.

Interestingly, alanine substitution of the residues in this cluster substantially reduces LF toxicity and blocks MEK cleavage in cells. Functional tests of these mutations indicate that loss of toxicity is not caused by interference with the ability of LF to bind PA, translocate across the membrane, or to cleave MEK *in vitro* but instead by a reduction in the ability of LF to interact with MEK. The region containing this cluster of residues could potentially serve as a therapeutic target for small molecule inhibitors that would disrupt LF-MEK association and block LF-mediated proteolysis of MEK.

MATERIALS AND METHODS

Cell Culture and Reagents—The murine macrophage-derived J774A.1 and the Chinese hamster ovarian (CHO) epithelial K1 cell lines were obtained from the American Tissue Culture Collection (Manassas, VA). J774A.1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. CHO K1 were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Both cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator.

Site-directed Mutagenesis—Alanine substitutions in LF were generated by introducing mutations into a *B. anthracis* LF expression vector pSJ115 (25) with the use of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions except that primer extension was allowed to continue for 18 min, and the deoxynucleotide triphosphate stocks were modified to reflect the high deoxyadenylate and deoxythymidylate content (70%) of the gene encoding LF (21). The primers used for site-directed mutagenesis are listed in the supplementary data. The mutations were confirmed by DNA sequencing of the region containing the mutation. In addition, the genes encoding all LF that demonstrated reduced toxicity were sequenced in their entirety to confirm that only the desired mutations were present.

Protein Expression and Purification—To express the mutagenized proteins, they were first transformed into the *Escherichia coli* dcm^- dam^- strain SCS110 to obtain unmethylated plasmid DNA, which was then transformed into a nontoxigenic, sporulation-defective strain of *B. anthracis* (BH445 (25)) as described elsewhere (26).

To prepare crude preparations of secreted protein, a single colony of transformed cells was used to inoculate 5 ml of FA medium (27). Cultures were allowed to grow at 37 °C for 14–16 h. Culture supernatant (2 ml) was then concentrated using a centrifugal filter (Microcon 100K MWCO; Millipore), and protein was recovered in 40 μl of buffer (20 mM Hepes, pH 7.5, 25 mM NaCl). The concentration of each protein was estimated by direct comparison with Coomassie Blue-stained bovine serum albumin standards (0.5 and 2.0 mg/ml) after separation on 10–20% SDS-PAGE gels.

To make high purity preparations of LF and PA, 50-ml cultures were used to inoculate 5 liters of FA medium in a BioFlo 100 fermentor (New Brunswick Laboratories) at 37 °C, pH 7.4, while sparging with air at 3 liter/min and with agitation set to increase from 100 to 400 rpm as level of dO_2 dropped below 50%. After 17–18 h of growth, the cells were removed by centrifugation (3500 × *g* for 30 min at 4 °C), and the supernatant was sterile-filtered and concentrated by tangential flow filtration using a Millipore prep/scale-TFF cartridge with 1 ft^2 of 30-kDa MWCO polyethersulfone membrane, collecting the filtrate at ~50 ml/min under a 1-bar back pressure. Expressed protein was purified by ammonium sulfate fractionation and fast pressure liquid chromatography (FPLC) using phenyl-Sepharose and Q-Sepharose columns following the procedures of Park and Leppla (25). The concentration of each protein was estimated using the bicinchoninic acid method (28) and by densitometric analyses of Coomassie Blue-stained polyacrylamide gels.

For *in vitro* MEK cleavage assays NH_2-terminal histidine-tagged MEK was prepared as described (15). For all other experiments recombinant human MEK1 protein was expressed in *Spodoptera frugiperda* (Sf9) cells that had been infected with baculovirus containing human MEK1 ligated into the pVL1393 vector backbone (pKM636). Protein was isolated from supernatants of lysed cells and was eluted over 10 column volumes in a linear gradient from 0 to 500 mM NaCl from a 20-ml Q-Sepharose column. The peak fractions containing MEK were pooled and loaded directly onto a 10-ml nickel-nitrilotriacetic acid column. After washing the column with 30 mM imidazole, MEK was eluted with 100 mM imidazole. At this point, the eluate was adjusted to 3 μM EDTA, 3 mM $MnCl_2$, and 2 mM dithiothreitol, and 25 units of protein phosphatase 1 (New England Biolabs, Beverly, MA) were added to the reaction and was incubated at 30 °C for 4 h. The samples were then concentrated and applied to a 320-ml Sephacryl 200 column in 25 mM Hepes, pH 8.0, 100 mM NaCl, 2 mM dithiothreitol, and 10% glycerol.

ERK2 protein was expressed in *E. coli* and purified by FPLC as described earlier (15, 19). Active B-Raf (Δ1–415) was purchased from Upstate Biotechnology, Inc.

Cytotoxicity Assays—The cells were grown in 96-well plates to 70% confluence. To induce lysis, the cells were treated with culture medium containing lethal toxin (PA (0.1 μg/ml) plus LF (0.01–10,000 ng/ml)) and incubated for 3 h at 37 °C. At the end of the experiment, cell viability was determined using the CellTiter 96® aqueous nonradioactive cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions. The concentration of LF required to cause a 50% maximal decrease in absorbance at 570 nm (the EC_{50}) was determined by linear regression.

PA Binding and Translocation Assays—PA binding and translocation assays were performed as described by Lacy *et al.* (29) and quantitated using a Packard Tri-Carb 3100TR liquid scintillation counter.

MEK Proteolysis and B-Raf Kinase Assays—To assay MEK cleavage in cells we made lysates of J774A.1 macrophages that had been incubated for 2 h with 0.1 μg/ml PA and 0.01 μg/ml LF or LF mutants. The lysates were separated by denaturing SDS-PAGE and immunoblotted with antibodies raised against the NH_2 or COOH termini of MEK2 (N-20 and C-16, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). *In vitro* MEK cleavage assays were performed using immunoblotting with antibodies raised against MEK (anti-MEK1/2, 1:1000; Cell Signaling) as described earlier (19). Alternatively, MEK cleavage was assayed indirectly by reacting a constant concentration of MEK with varying the amounts of LF, using MEK activity (*i.e.* ERK phosphorylation) as a readout for LF activity. Briefly, 0.35 μg of MEK1 was added to 3 μl of cleavage buffer (20 mM Mops, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) in the presence of varying amounts of LF or mutant LF (0.002–10 μg) and in a total volume of 10 μl. These cleavage reactions were incubated at 30 °C for 10 min. After cooling on ice for 2 min, 10 μl of kinase buffer (0.5 mM ATP (diluted 9:1 with [γ-^{32}P]ATP (Amersham; 10 mCi/ml, 3000 mCi/mmol), 75 mM $MgCl_2$, and 0.4 μg of ERK2) was added, and samples were incubated for 10 min at 30 °C. After cooling on ice for 2 min one volume of 2× SDS buffer was added, and the samples were incubated in a boiling water bath for 3 min. The proteins were then separated by SDS-polyacrylamide electrophoresis on 10% gels, and ERK2 phosphorylation was quantitated using a Fuji FLA-5000 PhosphorImager.

B-Raf kinase assays were performed as described previously (19) and quantitated using a Fuji FLA-5000 PhosphorImager. The results were normalized to phosphorylation in the absence of LF and compared using an unpaired Students' *t* test.

RESULTS

Site-directed Mutagenesis of Clustered Aliphatic Residues—Because a number of the conserved residues in the LF-interacting region are long chain aliphatic residues, we hypothesized that a complementary region on LF would contain clustered aliphatic residues and would lie close to the groove into which the NH_2 terminus of MEK fits. A surface plot of LF shows three distinct clusters of aliphatic residues meeting this requirement (Fig. 1). The first is composed of aliphatic residues (Ile^{298}, Ile^{300}, Ile^{485}, Leu^{494}, and Leu^{514}) present in domain II and lies at one end of the catalytic groove. The second (residues Ile^{322}, Ile^{343}, Leu^{349}, Leu^{357}, and Val^{362}) is composed of elements of the second, third, and fourth imperfect repeats in domain III and lies at the opposite end of the catalytic groove. A third cluster present in domain IV (Leu^{450}, Ile^{467}, Leu^{677}, Leu^{725}, and Leu^{743}) lies adjacent to the catalytic groove that receives the NH_2 terminus of MEK.

To test our hypothesis we used site-directed mutagenesis to substitute alanine for each of these residues and then evaluated the effects of these mutations upon LF activity using a macrophage toxicity assay (10). The average concentration of crude preparations of wild-type LF required to cause a 50% maximal decrease in absorbance/cell viability (the EC_{50}) was 15.6 ± 16.7 nM. Mutation of most of the aliphatic residues tested caused a less than 5-fold reduction in toxicity (12.9 nM ≤ EC_{50} ≤ 84.3 nM; Fig. 2*a* and supplementary data) and were

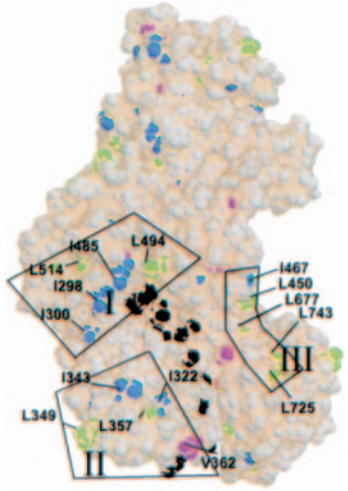

FIG. 1. **A surface plot of anthrax LF highlighting aliphatic residues.** A space-filled surface plot of LF was generated using Protein Explorer® freeware. Aliphatic residues were identified and were found to fall into three clusters (*labeled I*, *II*, and *III*) adjacent to the catalytic groove. Residues are color-coded *green* for leucine, *blue* for isoleucine, and *pink* for valine. The NH_2 terminus of MEK is indicated in *black*.

judged to have a neutral or marginal role in toxicity. By contrast, alanine substitution of Leu^{514} caused a greater than 50-fold reduction in toxicity (EC_{50} = 816 ± 137 nM; Fig. 2*a*).

To determine whether other residues in this region of LF played a role in LF toxicity, we made alanine substitutions at surface-exposed residues that were in proximity to Leu^{514}. Of these, Leu^{285}, Arg^{290}, Gln^{297}, Glu^{515}, and Lys^{518} were judged to have a neutral or marginal role in toxicity (40 nM ≤ EC_{50} ≤ 65 nM; Fig. 2*b* and supplementary data). By contrast, alanine substituted at Leu^{293}, Lys^{294}, Arg^{491}, or Asn^{516} caused a greater than 10-fold reduction in toxicity (141 nM ≤ EC_{50} ≤ 419 nM; Fig. 2*b*). Interestingly, although pairwise mutation of Leu^{514} and either Leu^{293}, Lys^{294}, or Arg^{491} completely abrogated LF toxicity (Fig. 2*c*), pairwise mutation of Leu^{514} and Asn^{516} instead resulted in toxicity comparable with N516A alone (EC_{50} = 200 ± 98 nM for L514A/N516A *versus* 164 ± 13 nM for N516A; Fig. 2*c*). These results indicate that subtle perturbations of the surface composition of domain II caused by alanine substitution of these residues can have a substantial impact upon LF toxicity.

Point Mutations in Domain II Reduce Neither the Affinity of LF for PA nor Its Ability to Translocate across the Plasma Membrane—LF is a Zn^{2+}-metalloprotease that specifically cleaves the NH_2 termini of mitogen-activated protein kinase kinases. To determine whether clustered residues in domain II are required for LF proteolytic activity, we assayed MEK2 cleavage by immunoblotting in J774A.1 macrophages that had been treated for 2 h with PA (0.1 μg/ml) plus wild-type LF or LF containing alanine mutations (0.01 μg/ml) in this region. Of the proteins tested, only wild-type LF and LF containing alanine mutations that had a neutral or marginal effect on toxicity were able to cleave the NH_2 terminus of MEK2 (Fig. 3*a*). By contrast, L293A, K294A, R491A, L514A, and N516A as well as the double mutants L293A/L514A, K294A/L514A, R491A/L514A, and L514A/N516A caused no or reduced MEK2 cleavage. These results are consistent with our observation that only these residues of domain II play key roles in LF toxicity. However, the preceding assay is cell-based and does not distinguish between decreased toxicity caused by a reduced ability of LF to bind PA, to translocate across the endosomal membrane, or to cleave MEKs. Subsequent analyses were performed to elucidate the mechanism by which these mutations interfere with toxicity.

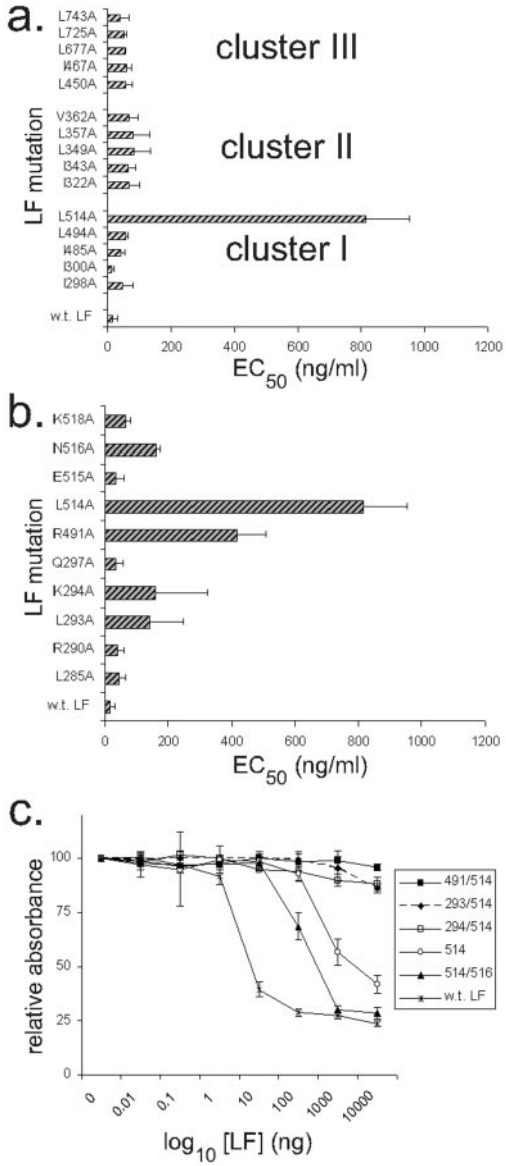

FIG. 2. **The toxicity of mutagenized LF.** The toxicity of mutagenized LF was measured using macrophage lysis assays. The concentrations of LF protein containing alanine mutations of aliphatic residues in clusters I–III (*a*) and alanine mutations of residues located close to Leu^{514} (*b*) that are required to cause a 50% maximal decrease in cell viability (the EC_{50}) were determined by interpolation and are presented as averages of at least three experiments, each of which was performed using independently purified batches of protein ± S.D. between batches. The toxicity of double mutants was also measured using macrophage lysis assays (*c*) and is presented as an average of at least three experiments using independently purified batches of protein ± S.D. between batches.

To test whether our mutant LF were able to bind PA and translocate across the membrane, we performed binding and translocation assays using [^{35}S]Met-labeled LF. In these assays LF and PA_{63} are allowed to bind ANTXR on CHO K1 cells at 4 °C, at which temperature endocytosis does not occur. As a negative control we used LF containing an alanine substitution at LF (Y236A), which has been previously shown to be incapable of binding to PA (29). After unbound protein was washed away, the cells were treated with low or neutral pH buffer. The low pH buffer mimics the endosomal environment and triggers PA_{63} pore formation and the subsequent translocation of LF to the cytosol. After this, cells were exposed to Pronase to remove any surface-bound label, washed, lysed, and assayed for ^{35}S

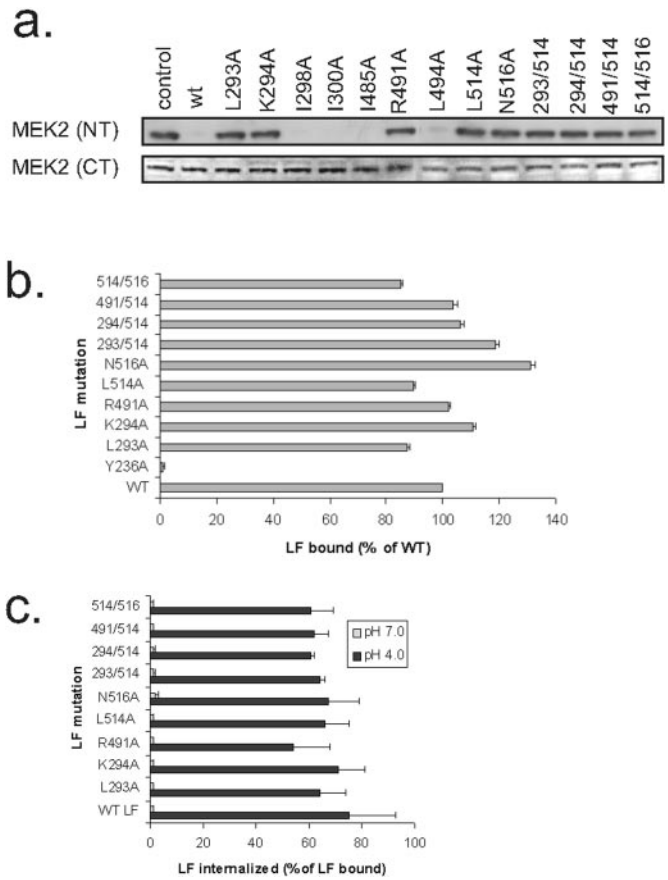

FIG. 3. **The effects of point mutations upon LF functions.** *a*, the effects of point mutations upon proteolytic activity of LF in macrophages was assessed by immunoblotting lysates of toxin-treated (2 h with 0.1 μg/ml PA, 0.01 μg/ml LF) J774A.1 cells with antibodies directed toward the NH_2 termini of MEK2 (MEK2 (NT)). To control for loading and uniform protein expression, these blots were stripped and reprobed with antibodies directed toward the COOH terminus of MEK2 (MEK2 (CT)). Only wild-type (*wt*) LF and LF containing alanine mutations that had a neutral or marginal effect on toxicity were able to cleave MEK2. The results shown are representative of three experiments. *b*, to test whether our mutant LF were able to bind PA [^{35}S]Met-labeled LF and LF mutants were incubated with CHO cells at 4 °C, pH 7.0. After unbound protein was washed away, bound ^{35}S was quantitated using a liquid scintillation counter. LF (Y236A), which has been previously shown to be incapable of binding to PA (29), was used as a negative control. The results shown are an average of at least three experiments and are expressed as percentages of wild-type LF bound to cells ± S.D. *c*, to test whether our mutant LF were able to translocate across a membrane [^{35}S]methionine-labeled LF and LF mutants were incubated with CHO cells at 4 °C, pH 7.0. After unbound protein was washed away, the cells were treated with low or neutral pH buffer. The low pH buffer mimics the endosomal environment and triggers PA_{63} pore formation and the subsequent translocation of LF to the cytosol. After this cells were treated with or without Pronase to remove any surface-bound label, washed, lysed, and assayed for ^{35}S content. The results shown are averages of at least three experiments and are expressed as percentages of label incorporated into cells that had not been treated with Pronase ± S.D.

content. As shown in Fig. 3 (*b* and *c*), wild-type and mutant LF were equally capable of binding PA_{63} and translocating across the plasma membrane. Consistent with published reports (29), LF (Y236A) did not appreciably bind PA_{63} in the same assays. The ability of wild-type and mutant LF to bind PA was confirmed independently by nondenaturing gel shift assays using LF and trypsin-nicked PA (PA_{63}) (data not shown, but see first supplementary figure). These results indicate that loss of toxicity in mutant LF can be explained neither by a loss of the ability to bind PA nor by an inability to translocate across a cell membrane.

Point Mutations in Domain II Do Not Alter LF Proteolytic Activity—Because the preceding assays were performed with relatively crude preparations of protein, it remained a possibility that the results we observed were caused by the effects of contaminants upon mutant LF and not wild-type LF activity. To test this we purified wild-type LF and selected LF double mutants by FPLC and reassessed the toxicity of these preparations using macrophage cytotoxicity assays. The EC_{50} of wild-type LF was 10.9 ± 5.9 nM (Fig. 4*a*), whereas FPLC-purified L514A proved to be less toxic (EC_{50} > 1,000 nM). As noted for crude preparations of protein, the substitution of a second alanine residue for N516 in L514A partially restored the toxicity of this mutant (EC_{50} = 427 ± 74 nM). FPLC-purified K294A/L514A and R491A/L514A were nontoxic ($EC_{50} \gg$ 10,000 nM). Because FPLC-purified LF and LF mutants possess toxicities that are similar to those of crude preparations of the same proteins, it is unlikely that their reduced toxicities may be attributed to the presence of contaminants.

To this point our analyses indicate that point mutations at clustered residues of domain II reduce the proteolytic activity of LF. To directly test this, we assayed the proteolytic activity of FPLC-purified wild-type and mutant LF *in vitro* by immunoblotting. As a control we included FPLC-purified preparations of LF harboring a point mutation in the Zn^{2+}-binding domain (E687C), which has been previously characterized as being nontoxic (30) and proteolytically inactive (15). Incubation of 0.2 μg of wild-type LF, but not E687C, with 0.2 μg of NH_2-terminally His_6-tagged MEK1 increased the electrophoretic mobility of MEK1, consistent with NH_2-terminal proteolysis as described previously (15). Unexpectedly, none of the mutant LF showed reduced proteolytic activity toward MEK (Fig. 4*b*). However, this sort of cleavage assay is qualitative in nature and may not reveal partial reduction of proteolytic activity. To address this we performed cleavage assays in which we varied the concentration of LF in the presence of a constant amount of MEK and used the kinase activity of MEK toward ERK as a indirect, but quantifiable, measure of proteolysis. Wild-type LF caused a robust inhibition of MEK activity and resulted in a 50% suppression of ERK phosphorylation at a molar ratio (LF:MEK) of 0.5 ± 0.3 (Fig. 4*b*). By contrast, E687C had no effect on MEK activity except when present in excess. Consistent with the observed toxicity of these mutants, K294A/L514A and R491A/L514A showed markedly reduced proteolytic activity, causing a 50% suppression of ERK phosphorylation at molar ratios of 1.9 ± 1.1 and 1.6 ± 0.4, respectively. Unexpectedly, L514A and L514A/N516A possessed proteolytic activity that was comparable with wild-type activity, causing a 50% suppression of ERK phosphorylation at a molar ratios of 0.9 ± 0.1 and 0.8 ± 0.3, respectively. These data indicate that although clustered point mutations in domain II decrease LF toxicity, this loss may not be entirely attributed to decreased proteolytic activity.

An alternative explanation for our observations is that decreased LF toxicity may be caused by a loss of substrate affinity that is independent of its proteolytic activity. In lieu of a direct assay of LF binding to MEK, we previously demonstrated that LF could competetively inhibit B-Raf phosphorylation of MEK and that this inhibition was independent of its proteolytic activity (19). Our interpretation of these results was that LF and B-Raf bound adjacent or overlapping epitopes on MEK. To determine whether point mutations at clustered residues of domain II reduce the affinity of LF for MEK, we assayed *in vitro* B-Raf-mediated MEK phosphorylation in the presence of LF or LF mutants. As we reported earlier, LF caused an ~35% inhibition of MEK phosphorylation by B-Raf, and this effect was independent of LF proteolytic activity because E687C also

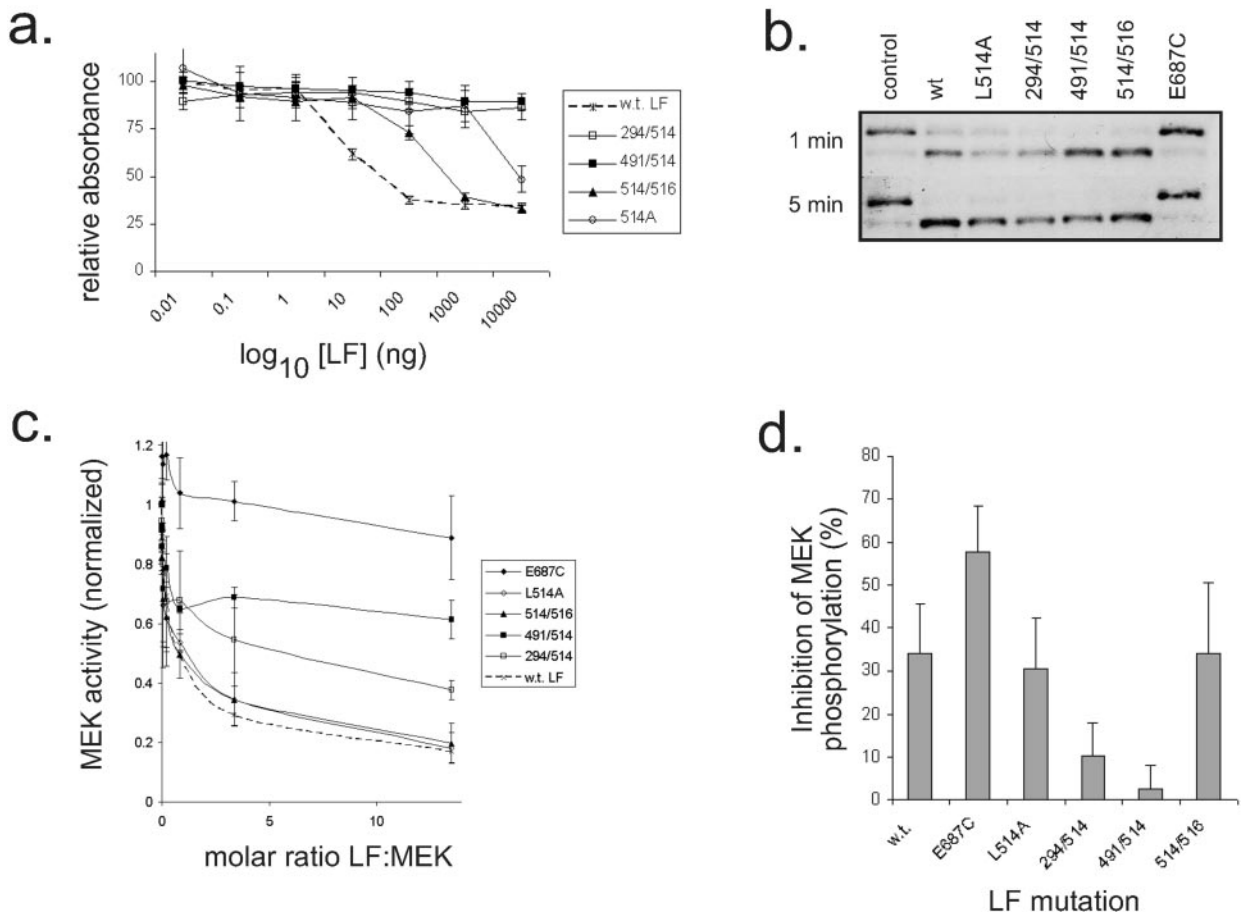

FIG. 4. **Toxicity and proteolytic activity of purified LF and LF double mutants.** *a*, wild-type LF and selected LF double mutants were purified by fast pressure liquid chromatography, and their toxicity was reassessed using macrophage-cytotoxicity assays. J774A.1 cells were treated with PA plus varying concentrations of wild-type LF (x) and LF (L514A) as well as LF containing pairwise alanine mutations of Leu^{514} and Asn^{516}, Leu^{514} and Lys^{294}, or Leu^{514} and Arg^{491} as indicated in the methods section. The cell viability was assessed after 3 h treatment by AQ assay and is presented as an average of five experiments ± S.D. *b*, His_6-tagged wild-type MEK1 (0.2 μg) was incubated with wild-type LF or LF mutants (0.2 μg) at 30 °C for 1 or 5 min. The proteins were separated by SDS-PAGE and immunoblotted with an antibody raised against residues 216–233 of human MEK1. MEK1 not reacted with LF (control) or reacted with inactive LF (E687C) are included as negative controls. MEK1 cleavage is indicated by increased electrophoretic mobility following proteolytic removal of the His_6 tag as well as the NH_2 terminus of MEK1. *c*, *in vitro* MEK proteolysis assays were performed in the presence of a constant concentration of MEK (0.35 μg) while varying the amount of LF (0.002–10 μg), using MEK activity (*i.e.* ERK phosphorylation) as a readout for LF activity. ERK phosphorylation was quantitated using a PhosphorImager. *Ordinate*, ERK phosphorylation normalized to control values obtained in the absence of LF in each experiment. *Abscissa*, the molar ratio of wild-type LF, LF (E687C), and LF (L514A), as well as LF containing pairwise alanine substitutions for Leu^{514} and Asn^{516}, Leu^{514} and Lys^{294}, or Leu^{514} and Arg^{491} to MEK1. The results are expressed as averages of at least three experiments, plus and minus standard deviation. *d*, B-Raf phosphorylation of MEK in the presence of FPLC-purified LF and LF mutants was assayed *in vitro*. MEK phosphorylation was quantitated using a PhosphorImager and normalized to MEK phosphorylation in the absence of LF. The results are expressed as averages of four experiments ± S.D. *wt* and *w.t.*, wild type.

inhibited MEK phosphorylation by B-Raf (Fig. 4*c*). Interestingly, whereas L514A and L514A/N516A had an effect that was similar to that of wild-type LF on MEK phosphorylation, K294A/L514A and R491A/L514A showed significantly reduced inhibition, blocking only 10 ± 7% ($p = 0.014$, $df = 6$) and 2 ± 5% ($p = 0.0026$, $df = 6$) of Raf-mediated phosphorylation, respectively (Fig. 4*c*). These data indicate that decreased LF toxicity resulting from point mutations in clustered residues in domain II may be attributed in part to a decreased ability interact with MEK.

DISCUSSION

LF is the principal virulence factor of anthrax toxin (31–33). To date, its only identified substrates are members of the MEK family of protein kinases. Consequently, the interaction between LF and MEK is an important concern for understanding the pathogenesis of anthrax as well as in the design of targeted therapeutic agents. This study was undertaken to identify regions of LF that are required for interaction with MEK. As a starting point, we reasoned that because a number of the conserved residues in the LF-interacting region are long chain aliphatic residues, any region of LF with which it associated would (i) contain a cluster of surface-exposed aliphatic residues and (ii) lie adjacent to the catalytic groove where the active site complex would form. Regardless of the physiological relevance of these assumptions, tests of this hypothesis led to our identification of a single residue (Leu^{514}) in domain II that, when replaced by an alanine residue, caused a substantial reduction in the toxicity of LF. Further alanine substitution in the vicinity of Leu^{514} identified four additional residues that also play a role in LF toxicity. Although separated in primary sequence, the tertiary structure of LF brings these five residues side by side in a focused region that lies at one end of the groove that forms between domains III and IV and contains the active site (see second supplementary figure).

What role does this region play in LF toxicity? One key observation is that although mutant LF (*i.e.* L514A and L514A/N516A) were incapable of cleaving MEK in cell-based assays, they did so *in vitro*. This indicates that these mutants are

sensitive to the context in which they encounter their substrate MEKs. In cells, the spatial distribution and accessibility of MEKs are influenced by scaffolding proteins such as MP1 (34) and JIP-1 (35). In addition, cellular MEKs may be modified post-translationally (*e.g.* by phosphorylation) and can associate with their cognate mitogen-activated protein kinases as well as other regulatory molecules such as B-Raf. Any of these factors may limit the ability of mutant LF to bind and cleave MEKs in cells. Indeed, although the MEK1 scaffolding protein MP1 can associate with both recombinant MEK1 and MEK2 *in vitro*, it can only bind MEK1 in cells (34). Thus, we propose that the simplest interpretation of our observations is that the region we have identified defines a site that is necessary for LF to associate into a productive complex with MEKs. Several observations support this hypothesis: (i) the effect of the mutations was specific; only mutations in this region, but not in clusters II or III, decreased LF toxicity; (ii) decreased toxicity was accompanied by decreased proteolysis of MEK2 in cells; (iii) mutations in this region altered neither binding to PA nor translocation of LF across the cell membrane; (iv) the LF mutants L514A and L514A/N516A possessed *in vitro* proteolytic activity that was comparable with that of wild-type LF; (v) the LF mutants K294A/L514A and R491A/L514A display reduced ability to competetively inhibit B-Raf phosphorylation of MEK; and (vi) the region identified on domain II is spatially distinct from the active site and thus is not likely to directly participate in substrate proteolysis. An alternative explanation, *i.e.* that the mutations introduce gross structural changes in LF that alter its biochemical activity, is unlikely because mutant LF not only retained the ability to bind PA and internalize into cells but also (in the case of L514A and L514A/N516A) possessed wild-type levels of proteolytic activity.

The precise role this region plays in promoting the association of LF into a productive complex with MEKs is not clear. One possibility is that this region is required to direct LF to MEKs within cells. In this case, mutations in this region of domain II would reduce the ability of LF to associate with proteins which co-localize with MEKs. Alternatively, this region may play a direct role in binding MEKs. The latter possibility is supported by our observations that the LF mutants K294A/L514A and R491A/L514A display reduced ability to competetively inhibit B-Raf phosphorylation of MEK. Moreover, indirect evidence supports the hypothesis that LF and MEK interact at sites outside the active site. Using yeast two-hybrid analysis to identify binding partners of LF, Vitale *et al.* (16) isolated cDNA for MEK2 that lacked the NH_2-terminal cleavage site. In addition, we earlier demonstrated the existence of a conserved region located in the COOH terminus of MEK1, which is required for LF-mediated proteolysis of MEKs (19).

Recent publications have identified lead compounds that may be adapted for use as small molecule inhibitors of LF activity (36–39). These molecules were initially identified by LF cleavage assays that used optimized peptide substrates that mimic the NH_2-terminal cleavage site on MEKs. However, as we have demonstrated, sites outside the active site complex on both LF and MEK are required for efficient proteolysis of MEK. Thus, we propose that alternative therapeutic strategies that employ molecules that are targeted to the region of LF defined by these residues may, alone or in combination with those identified molecules that target the active site of LF, form the basis of a novel and more effective anthrax therapeutic.

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