# Anthrax lethal factor-cleavage products of MAPK (mitogen-activated protein kinase) kinases exhibit reduced binding to their cognate MAPKs

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Anthrax lethal toxin is the major cause of death in systemic anthrax. Lethal toxin consists of two proteins: protective antigen and LF (lethal factor). Protective antigen binds to a cell-surface receptor and transports LF into the cytosol. LF is a metalloprotease that targets MKKs [MAPK (mitogen-activated protein kinase) kinases]/MEKs [MAPK/ERK (extracellular-signal-regulated kinase) kinases], cleaving them to remove a small N-terminal stretch but leaving the bulk of the protein, including the protein kinase domain, intact. LF-mediated cleavage of MEK1 and MKK6 has been shown to inhibit signalling through their cognate MAPK pathways. However, the precise mechanism by which this proteolytic cleavage inhibits signal transmission has been unclear. Here we show that the C-terminal LF-cleavage products of MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7 are impaired in their ability to bind to their MAPK substrates, suggesting a common mechanism for the LF-induced inhibition of signalling.

Key words: anthrax lethal factor protease, docking site, MAPK/ERK kinase (MEK), MAPK kinase (MKK), mitogen-activated protein kinase (MAPK), molecular pathogenesis.

## INTRODUCTION

Anthrax is an extremely rare disease in humans, caused by the gram-positive bacteria *Bacillus anthracis* (reviewed in [1]). However, in recent years anthrax has risen to the forefront of public awareness due to its potential for use as a biological weapon [2]. The most common form of anthrax is cutaneous anthrax caused by entry of bacterial spores through skin abrasions. Cutaneous anthrax is generally curable, but systemic anthrax, usually caused by inhalation of spores, is typically fatal. Anthrax lethal toxin, which consists of two proteins, protective antigen and LF (lethal factor), plays a critical role in the pathogenesis of the disease (reviewed in [1,2]). Intravenous injection of lethal toxin causes rapid death in animals [3]. In addition, lower concentrations of lethal toxin, typical of early infection, disarm the host immune response as part of an evasion strategy of the pathogen [4–8].

Protective antigen binds to a recently described cell-surface receptor and is responsible for the translocation of LF into the cell [9]. LF is a metalloprotease [10–12] that specifically targets the MKKs [MAPK (mitogen-activated protein kinase) kinases] MEK [MAPK/ERK (extracellular-signal-regulated kinase) kinase] 1, MEK2, MKK3, MKK4, MKK6 and MKK7 [4–6,13–15]. MKKs phosphorylate, and thereby activate, specific MAPKs. In so doing, MKKs play a key role in the regulation of signal transduction pathways that orchestrate cellular responses to a diverse array of stimuli, including the response to pathogens [16]. MEK1 and MEK2 target the ERK1 and ERK2 MAPKs, MKK3 and MKK6 target the p38 MAP kinases, and MKK4 and MKK7 target members of the JNK (c-Jun N-terminal kinase) subfamily of MAP kinases [17,18].

LF cleaves its MKK substrates at one or two sites near their N-termini [13]. Cleavage by LF severs a portion the N-terminal domain of the MKK from the bulk of the protein, including the C-terminal protein kinase domain. The crystal structure of LF bound to the first 16 residues of MEK2 has been solved [19].

Anthrax LF-mediated cleavage of MEK1/2 and MKK6 has been shown to inhibit signalling through their cognate MAPK pathways [6,15]; however, the mechanism by which this inhibition occurs has been unclear. One possibility is suggested by the observation that the LF cleavage site in most of the MKK substrates lies adjacent to, or within, a protein interaction site (the MAPK-docking site, or 'D-site') that is required for the binding of the MKKs to their cognate MAPK partners. Functional MAPK-docking sites have been identified and characterized in MEK1 [20,21], MEK2 [21], MKK3 and MKK6 [22], and MKK4 [23]. These docking sites are required for high-affinity MKK–MAPK binding [21,23], and promote efficient MKK-mediated MAPK phosphorylation [20–24]. Cleavage of MEK1, MEK2, MKK3, MKK4 and MKK6 by anthrax LF detaches the MAPK-docking site from the rest of the polypeptide. These observations suggest that disruption of MKK–MAPK docking interactions may contribute to the LF-mediated inhibition of MAPK signalling. Consistent with this, Chopra et al. [25] have recently shown that LF-cleaved MEK1 has a reduced affinity for ERK2.

Knowing the mechanism by which LF-induced MKK cleavage impairs MAPK signalling is important both for understanding the pathogenesis of *B. anthracis*, and because lethal toxin may be useful in anti-tumour and anti-inflammatory therapy [4,26–29]. In the present study, we have examined the hypothesis that the C-terminal anthrax LF-cleavage products of MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7 exhibit reduced binding to their cognate MAPK substrates. We report that, although these MKK cleavage products lack only a part of their N-terminal domains and still contain intact kinase domains, they exhibited much weaker binding to their cognate MAPKs. These findings suggest a common mechanism for the LF-induced inhibition of MKK-mediated signalling.

Abbreviations used: ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; JNKK, JNK kinase; LF, lethal factor, MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEK1-LF (etc.), protein corresponding to the C-terminal product following cleavage of MEK1 by LF (etc.); MKK, MAPK kinase; MKK4-LFA (etc.), protein corresponding to the C-terminal product following cleavage of MKK4 by LF at site A (etc.).

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**Table 1 Oligonucleotides used in the present study**

Where appropriate, restriction sites used for subcloning are underlined, and the translation initiation codon is shown in bold.

| Name | Sequence (5′ → 3′) | Use |
|---|---|---|
| SGMEK1LF | ATAGGATCCACC**ATG**ATCCAGCTGAACCCGGCCCCC | pGEM-MEK1-LF |
| SGMEK2LF | ATAGAATTCACC**ATG**GCGCTCACCATCAACCCTACC | pGEM-MEK2-LF |
| JBMKK3up | GCGAATTCACC**ATG**GAGTCGCCCGCCTCGA | pGEM-MKK3 |
| JBMKK3down | GCGGTACCCTATGAGTCTTCTCCCAGGATCTCC | pGEM-MKK3 and pGEM-MKK3-LF |
| JB850 | GCGAATTCACC**ATG**ATATCCTGCATGTCCAAGCCA | pGEM-MKK3-LF |
| JB851 | GCGGTACCACC**ATG**TTGAATTTTGCAAATCCA | pGEM-MKK4-LFA |
| JB854 | GCGGTACCACC**ATG**TTTACTCTGAATCCCAA | pGEM-MKK4-LFB |
| JB802 | GCGGTACCAAA**ATG**TCTCAGTCGAAAGGCA | pGEM-MKK6 |
| JB803 | GCGTCGACCGATTAAGTCCACTGCTTTTTA | pGEM-MKK6 and pGEM-MKK6-LF |
| JB852 | GCGGTACCACC**ATG**ATTCCAAAAGAAGCATTTGAACAA | pGEM-MKK6-LF |
| JB812A | GCGGATCCACC**ATG**GCGGCGTCCTCCCTG | pGEM-MKK7 |
| JB855 | GCGGATCCACC**ATG**CTCCCGCTGGCCAACGA | pGEM-MKK7-LFA |
| JB853 | GCGGATCCACC**ATG**CTCCCGTCAACCCTGTTCA | pGEM-MKK7-LFB |
| JB818 | GGCGTCGACCTACCTGAAGAAGGGCAGGTG | pGEM-MKK7, pGEM-MKK7-LFA and -LFB |
| p38$\beta$up | GCGAATTCACCATGTCGGGCCCTCGCGC | pGEX-p38$\beta$ |
| p38$\beta$down | CGGTCGACTTATCACTGCTCAATCTCCAGGCTG | pGEX-p38$\beta$ |
| JNK1$\alpha$1up | GCGGGATCCACCATGAGCAGAAGCAAGCGTGA | pGEX-JNK1$\alpha$1 |
| JNK1a1down1 | CGGTCGACTTACTGCTGCACCTGTGCTAA | pGEX-JNK1$\alpha$1 |
| JNK2$\alpha$2up | GCGGATCCACCATGAGCGACAGTAAATGTGACA | pGEX-JNK2$\alpha$2 |
| JNK2$\alpha$2down | CGGTCGACTTATCATCGACAGCCTTCAAGGGG | pGEX-JNK2$\alpha$2 |

## EXPERIMENTAL

### Genes

The following mammalian genes were used in this study: human MEK1 (*MAP2K1*; accession number NM_002755), human MEK2 (*MAP2K2*; NM_030662), human MKK3$\beta$ (*MAP2K3*; D87116), human MKK6 (*MAP2K6*), human MKK4/JNKK1 (JNK kinase 1)/SEK1 [SAPK (stress-activated protein kinase)/ERK kinase 1] (*MAP2K4*; NM_003010), human MKK7/JNKK2 (*MAP2K7*; NM_005043), human ERK1 (*MAPK3*; X60188), rat ERK2 (M64300), human p38$\beta$ (*MAPK11*, NM_002751), human JNK1$\alpha$1 (*MAPK8*; L26318), human JNK2$\alpha$2 (*MAPK9*; L31951) and human JNK3$\alpha$1 (*MAPK10*, NM_002753).

### Plasmids for *in vitro* transcription/translation of mammalian genes

Plasmids pGEM-MEK1, pGEM-MEK2 [21] and pGEM-MKK4 [23] have been described previously. To construct pGEM-MEK1-LF, the corresponding region of the MEK1 coding sequence was amplified by high-fidelity PCR using *Pfu* DNA polymerase (Stratagene), primers SGMEK1LF (see Table 1 for sequences of oligonucleotides used in this study) and LB113 [21] and template plasmid pBS-MEK1 [30]. The resulting PCR product was digested with *Bam*HI and *Sal*I and inserted into the corresponding sites of pGEM4Z (Promega). To construct pGEM-MEK2-LF, the corresponding region of the MEK2 coding sequence was amplified by PCR using primers SGMEK2LF (Table 1) and LB116 [21] and template plasmid pBS-MEK2 [30]. The resulting PCR product was digested with *Eco*RI and *Xho*I and inserted into pGEM4Z that had been digested with *Eco*RI and *Sal*I. To construct pGEM-MKK3 and pGEM-MKK3-LF, the corresponding regions of the MKK3 coding region were amplified by PCR using primers JBMKK3up (for pGEM-MKK3) or JB850 (for pGEM-MKK3-LF) together with JBMKK3down, and, as template, a cDNA clone obtained from Invitrogen Corp. (Genestorm clone ID #RG000058). The resulting PCR products were digested with *Eco*RI and *Kpn*I and inserted into the corresponding sites of pGEM4Z. To construct pGEM-MKK4-LFA and pGEM-MKK4-LFB, the corresponding regions of the MKK4 coding region were amplified by PCR using primers JB851 (for pGEM-MKK4-LFA) or JB854 (for pGEM-MKK4-LFB) together with MKK4endRNEW [23] and template pcDNA3-FLAG-MKK4 (a gift from Roger Davis, University of Massachusetts Medical School, Worcester, MA, U.S.A.). The resulting PCR products were digested with *Kpn*I and *Sal*I and inserted into the corresponding sites of pGEM4Z. To construct pGEM-MKK6 and pGEM-MKK6-LF, the corresponding regions of the MKK6 coding region were amplified by PCR using primers JB802 (for pGEM-MKK6) or JB852 (for pGEM-MKK6-LF) together with JB803, and, as template, a cDNA clone obtained from the I.M.A.G.E. consortium [31] (via Open Biosystems corporation; IMAGE ID #44727). The resulting PCR products were digested with *Kpn*I and *Sal*I and inserted into the corresponding sites of pGEM4Z. To construct pGEM-MKK7, pGEM-MKK7-LFA and pGEM-MKK7-LFB, the corresponding regions of the MKK7 coding region were amplified by PCR using primers JB812A (for pGEM-MKK7), JB855 (for pGEM-MKK7-LFA) or JB853 (for pGEM-MKK7-LFB) together with JB818, and, as a template, I.M.A.G.E. consortium clone ID #5269063. The resulting PCR products were digested with *Bam*HI and *Sal*I and inserted into the corresponding sites of pGEM4Z.

The authenticity of all plasmids constructed for *in vitro* transcription and translation was confirmed by DNA sequencing.

### Plasmids for the production of GST (glutathione S-transferase) fusion proteins

Plasmids pGEXLB and pGEXGERK2 [21] and pGEX-JNK3 [23] have been described previously. Plasmid pGEX-ERK1 was a gift from Steven Pelech (Kinexus Corp., Vancouver, Canada) [32]. To construct pGEX-p38$\beta$, the p38$\beta$ coding region was amplified by PCR using primers p38$\beta$up and p38$\beta$down and, as template, pGEM-p38$\beta$ [23]. The resulting PCR product was digested with *Eco*RI and *Sal*I and inserted into the corresponding sites in pGEXLB. To construct pGEX-JNK1$\alpha$1, the JNK1$\alpha$1 coding region was amplified by PCR using primers JNK1$\alpha$1up and JNK1$\alpha$1down and, as template, Invitrogen Genestorm clone ID #RG000191. The resulting PCR product was digested with *Bam*HI and *Sal*I and inserted into the corresponding sites in pGEXLB. To construct pGEX-JNK2$\alpha$2, the coding region of JNK2$\alpha$2 was amplified by PCR using primers JNK2$\alpha$2up and

JNK2$\alpha$2down and, as template, Invitrogen Genestorm clone ID #RG000037. The resulting PCR product was digested with *Bam*HI and *Sal*I and inserted into the corresponding sites in pGEXLB.

### Protein production and binding assays

MKK polypeptides were generated and $^{35}$S-labelled using an *in vitro* coupled transcription/translation system (SPT3; Novagen). Translation products were partially purified by ammonium sulphate precipitation [33]. Samples of the translation reactions were then analysed by SDS/PAGE and the amount of label incorporated into the complete translation product was quantified using a PhosphorImager™ (Molecular Dynamics). Using this information, equimolar amounts ($\approx$1 pmol) of each complete translation product were used in binding reactions. GST fusion proteins were expressed in bacteria and purified by affinity chromatography using glutathione–Sepharose (Amersham-Pharmacia), and quantified as described elsewhere [21]. Purified GST–p38$\alpha$ protein was purchased from Upstate Biotechnology for use in the experiments shown in Figure 3. Binding assays using GST fusion proteins were performed as described previously [21]. Binding assay results were quantified using a PhosphorImager, so as to determine the amount of each complete translation product bound as a percentage of input. This measurement is advantageous because it is relatively insensitive to variations in the molar amount of input labelled protein. Binding affinities were determined as described elsewhere [23,33].

## RESULTS

### Sites of cleavage by anthrax LF in MKKs lie adjacent to or within MAPK-docking sites

Anthrax LF is a zinc-binding endopeptidase with specificity for the MKKs/MEKs MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7 [13–15]. Cleavage occurs within a stretch of amino acids that approximately fits the consensus sequence ++++X$\varphi$X$\downarrow\varphi$ (basic and hydrophobic residues are indicated by + and $\varphi$ respectively, X indicates any amino acid and the cleavage site is indicated by $\downarrow$) [13]. Figure 1(A) shows all of the known cleavage sites in the human MKKs. Anthrax LF cleaves MEK1, MEK2, MKK3 and MKK6 once, while MKK4 and MKK7 have two cleavage sites, designated hereafter as A for the more N-terminal and B for the more C-terminal. All of the anthrax LF cleavage sites are within the N-terminal region of the MKKs, outside the protein kinase domains.

Figure 1(B) shows the high-affinity MAPK-docking sites, or D-sites, that have been identified (and proven to be functional) in MEK1 [20,21], MEK2 [21], MKK3 and MKK6 [22], and MKK4 [23]. These D-sites all fit the consensus sequence +++X$_{1-5}\varphi$X$\varphi$, which is clearly quite similar to the consensus for cleavage by LF. Each of these D-sites contains within it a site of LF cleavage (the residues between which cleavage by LF occurs are underlined in Figure 1B). For MKK4 it is the more N-terminal LF cleavage site (site A) that corresponds to the MAPK-docking site; site B does not exhibit high-affinity MAPK binding [23]. In all cases, cleavage occurs within the C-terminal end of the MAPK-docking site, resulting in the separation of the basic residues, and in some cases the first hydrophobic residue, of the D-site from the rest of the MKK. The basic [21,23,34] and hydrophobic [20–23,35] residues of the D-sites in MKKs have been shown to be critical for efficient MAPK binding. These considerations suggest that cleavage of MEK1, MEK2, MKK3, MKK4 and MKK6 by anthrax LF may compromise MAPK binding to the catalytic portion of the protein.

**A**

| | | | |
|---|---|---|---|
| **MEK1** | MP | **KKKPTP.I** QLNPAP | 8/9 |
| **MEK2** | MLA | **RRKPVLP.A** LTINPT | 10/11 |
| **MKK3** | GKS | **KRKKDLR.I** SCMSKP | 26/27 |
| **MKK6** | KGK | **KRNPGLK.I** PKEAFE | 14/15 |
| **MKK4** | MQG | **KRKALK.L** NFANPP | 45/46 |
| **MKK4** | ANP | **PFKSTAR.F** TLNPNP | 58/59 |
| **MKK7** | ISP | **RPRPTLQ.L** PLANDG | 44/45 |
| **MKK7** | PPA | **RPRHMLG.L** PSTLFT | 76/77 |
| **consensus** | | **++++X$\varphi$X.$\varphi$** | |

**B**

| | | | |
|---|---|---|---|
| **MEK1** | MP | **KKK--PTPIQL** NPAPDG | 1-17 |
| **MEK2** | MLA | **RRKPVLPALTI** NPTIAE | 1-20 |
| **MKK3** | GKS | **KRKK---DLRI** SCMSKP | 17-33 |
| **MKK6** | SKG | **KKR--NPGLKI** PKEAFE | 4-21 |
| **MKK4** | MQG | **KRK----ALKL** NFANPP | 37-52 |
| **consensus** | | **+++ X$_{1-5}$ $\varphi$X$\varphi$** | |

**Figure 1 Alignment of sites of cleavage by LF and MAPK-docking sites in MKKs**

(**A**) Known LF cleavage sites in mammalian MKKs. Conserved residues are in bold and marked as either basic (+) or hydrophobic ($\varphi$), and the cleavage sites are indicated by full stops (.). Spaces are for visual clarity. The numbers of the residues between which cleavage occurs are indicated to the right of each sequence. (**B**) Known MAPK-docking sites in mammalian MKKs. Conserved residues are in bold and marked as either basic (+) or hydrophobic ($\varphi$). Dashes denote gaps inserted to optimize the alignment; spaces are for visual clarity. The corresponding residue numbers in the full-length proteins are shown to the right of the sequences. The residues that LF cuts between are underlined.

A MAPK-docking site in MKK7 remains to be identified, but both anthrax LF cleavage sites in MKK7 occur within amino acid stretches that have some similarity to the D-site consensus. Furthermore, a third putative D-site lies N-terminal to the first cleavage site [21,34].

### Products of MEK1 and MEK2 cleavage by anthrax LF show reduced binding to ERK1 and ERK2

The MEK1/MEK2 $\rightarrow$ ERK1/ERK2 cascade regulates mitosis, survival, and many other signal-regulated changes in gene expression. Recently, Agrawal et al. [4] demonstrated that anthrax lethal toxin inhibits this cascade in dendritic cells, thereby disrupting the early immune response. Other studies also indicate that cleavage of MEK1 and MEK2 by LF correlates with reduced signalling through the ERK pathway [15,27]. One explanation for this could be that cleavage of MEK1/2 by LF severs the MAPK-docking site from the protein kinase domain, reducing ERK1/2 binding.

To test this hypothesis directly, a plasmid was constructed that contained the coding region for MEK1 from the C-terminal side of the LF cleavage site to the end of the gene: pGEM-MEK1-LF. This plasmid was used to programme an *in vitro* transcription and translation reaction in the presence of [$^{35}$S]methionine, resulting in synthesis of an $^{35}$S-labelled protein corresponding to the C-terminal product of LF cleavage of MEK1 (designated MEK1-LF). For comparison, *in vitro*-transcribed and translated, full-length wild-type MEK1 protein was generated from plasmid pGEM-MEK1. The ability of these two proteins to bind to GST–ERK1 and GST–ERK2 fusion proteins was then compared in a quantitative co-sedimentation (pull-down) assay (Figure 2A).

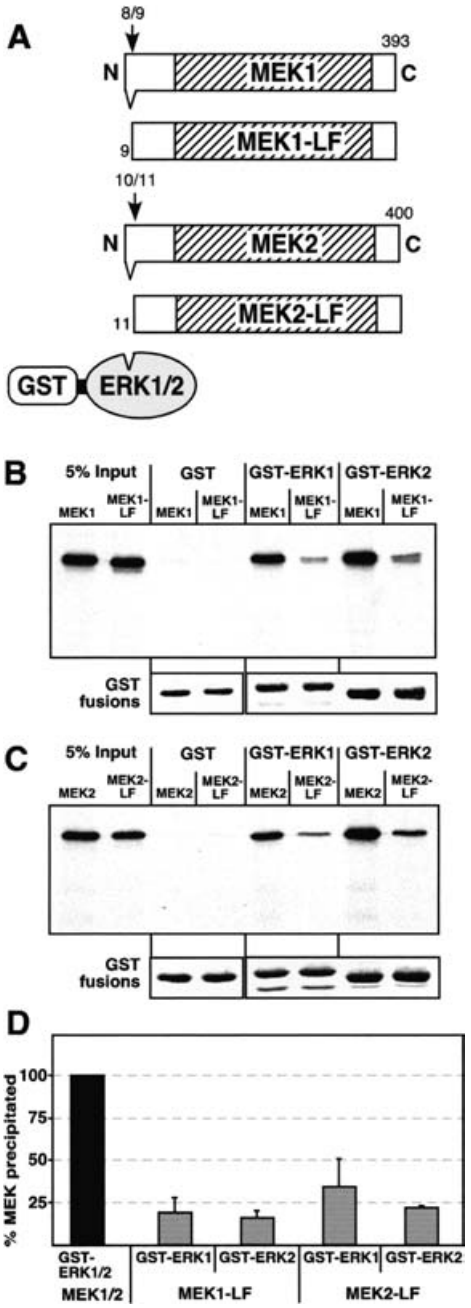


**Figure 2 LF-cleavage products of MEK1 and MEK2 display reduced ERK binding**

(**A**) Schematic representation of the experiment. Full-length wild-type MEK1 and MEK2 containing intact MAPK-docking sites (triangle) and the corresponding LF-cleavage products MEK1-LF and MEK2-LF are shown. Arrows indicate the LF cleavage sites. The N- and C-terminus of each protein is indicated, and the hatched boxes represent the catalytic domains. (**B**–**D**) $^{35}$S-labelled MEK1, MEK1-LF, MEK2 and MEK2-LF proteins (approx.1 pmol) were prepared by *in vitro* transcription and translation, partially purified by ammonium sulphate precipitation, and then incubated with 10 $\mu$g of purified GST, 14 $\mu$g of purified GST–ERK1 or 14 $\mu$g of purified GST–ERK2 pre-bound to glutathione–Sepharose beads. Bead-bound protein complexes were isolated by sedimentation and resolved by SDS/PAGE (10 % gels). Gels were analysed by autoradiography for visualization of the bound radiolabelled proteins (upper panels in **B** and **C**) and by staining with Gelcode Blue (Pierce) for visualization of the bound GST fusion protein in order to verify equal amounts in each reaction (lower panels in **B** and **C**). (**B**) Autoradiogram of a representative experiment for binding of MEK1 and MEK1-LF to GST–ERK1 and GST–ERK2. (**C**) Autoradiogram of a representative experiment for binding of MEK2 and MEK2-LF to GST–ERK1 and GST–ERK2. (**D**) Relative binding of MEK1-LF and MEK2-LF to GST–ERK1 and GST–ERK2 expressed as a percentage of the binding of the corresponding full-length wild-type MEK. Relative binding was determined using a Phosphorimager. Results are means $\pm$ S.D. for two to four experiments.

We chose to use this *in vitro* binding assay because (a) it is quantitative, and (b) it minimizes the possibility of complications resulting from other cellular MKK or MAPK binding proteins (e.g. upstream kinases, downstream substrates and scaffold proteins) that might inhibit or augment MKK–MAPK interactions.

When tested in this *in vitro* binding assay, wild-type MEK1 protein bound to GST–ERK1 and GST–ERK2 with low micromolar affinity ($K_d \approx 15$–$40$ $\mu$M; Figure 2B), consistent with our previous results [21]. This binding was specific, because MEK1 did not bind to GST alone (Figure 2B). Compared with this relatively strong binding, MEK1-LF protein showed minimal binding to ERK1 and ERK2 (Figure 2B). Moreover, as expected, this protein did not bind to GST. These data demonstrate that the LF-cleavage product of MEK1 is defective in high-affinity binding (i.e. docking) to its cognate MAPKs. Using a different experimental approach, Chopra et al. [25] have also demonstrated that LF-cleaved MEK1 has reduced affinity for ERK2.

Using a similar strategy, the coding region of MEK2 from the C-terminal side of the LF cleavage site to the end of the gene was inserted into pGEM4Z to make pGEM-MEK2-LF, and this plasmid was used to produce MEK2-LF protein by *in vitro* transcription and translation. The ability of MEK2-LF, compared with full-length MEK2 protein (generated using pGEM-MEK2), to bind to GST–ERK1 and GST–ERK2 was determined (Figure 2A). Like MEK1, wild-type MEK2 protein bound to ERK1 and ERK2 with low micromolar affinity ($K_d \approx 10$–$20$ $\mu$M; Figure 2C), consistent with our previous results [21]. MEK2-LF protein showed markedly reduced binding to ERK1 and ERK2 compared with full-length MEK2 (Figure 2C).

Figure 2(D) shows the means of several experiments for binding of MEK1-LF and MEK2-LF to ERK1 and ERK2. ERK1 binding to MEK1-LF and MEK2-LF was 19 % and 35 % respectively of that seen with the full-length wild-type MEKs. ERK2 binding to MEK1-LF and MEK2-LF was 16 % and 22 % respectively of the binding seen with the full-length MEKs. Compared with that of the full-length proteins, the binding affinity of the LF-cleavage products was reduced by 4–7-fold. These data indicate that cleavage of MEK1 and MEK2 by anthrax LF severely compromises their ability to bind to their cognate MAPK substrates ERK1 and ERK2. Notably, cleavage of MEK1/2 by LF compromised ERK binding almost to the same degree as did removal of the entire D-site (10–20 % of wild type) [21].

### Products of MKK3 and MKK6 cleavage by anthrax LF show reduced binding to p38

The MKK3/MKK6 signal transduction pathway is responsible for the phosphoregulation of the p38 MAPKs. This pathway is activated in immune cells by inflammatory cytokines and, in turn, regulates cytokine expression and the expression of other genes [17]. A series of complex interactions between *B. anthracis* and host macrophages are central to disease pathogenesis (reviewed in [36,37]). Recently, Park et al. [6] have demonstrated that treatment of lipopolysaccharide-activated macrophages with anthrax lethal toxin inhibits MKK6-mediated p38 activation, and consequently induces macrophage apoptosis. One possible mechanism for this decrease in MKK6 $\rightarrow$ p38 signalling is that LF-catalysed removal of the MAPK-docking site from MKK6 impairs p38 binding (Figure 3A). We have tested this hypothesis, and also examined binding of the MKK3 LF-cleavage product to p38 MAPKs.

Using a strategy similar to that outlined above for MEK1-LF and MEK2-LF, proteins corresponding to the C-terminal portions of MKK3 and MKK6 following cleavage by LF were synthesized *in vitro* using plasmids pGEM-MKK3-LF and pGEM-MKK6-LF respectively. *In vitro*-translated MKK6 and MKK6-LF both

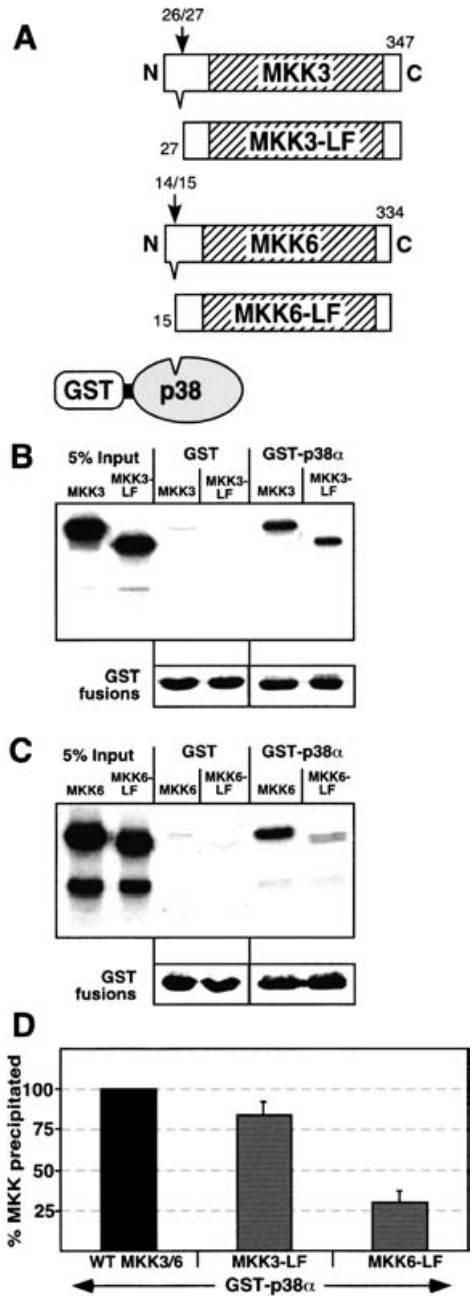


**Figure 3 LF-cleavage products of MKK3 and MKK6 display reduced binding to p38α**

(**A**) Schematic representation of the experiment. Full-length wild-type MKK3 and MKK6 containing intact MAPK-docking sites (triangle) and the corresponding LF-cleavage products MKK3-LF and MKK6-LF are shown. Other details as in Figure 2. (**B**–**D**) As described in the legend to Figure 2, except that 10 $\mu$g of purified GST or 6 $\mu$g of purified GST–p38$\alpha$ was incubated at 30 °C for 15 min with *in vitro* transcribed and translated MKK3, MKK3-LF, MKK6 or MKK6-LF prior to the addition of glutathione–Sepharose beads. Reactions were incubated further at room temperature for 1 h before isolation of bead-bound complexes. (**B**) Autoradiogram of a representative experiment for binding of MKK3 and MKK3-LF to GST–p38$\alpha$. (**C**) Autoradiogram of a representative experiment for binding of MKK6 and MKK6-LF to GST–p38$\alpha$. (**D**) Relative binding of MKK3-LF and MKK6-LF to GST–p38$\alpha$ expressed as a percentage of the binding of the corresponding full-length wild-type (WT) MKK. Results are the means ± S.D. for two experiments.

migrated on SDS/PAGE gels as two forms (Figure 3C): a major, slower migrating form, corresponding to the complete translation product, and a minor, faster migrating form of lower molecular mass. Such faster migrating forms are often seen in cell-free translation reactions, and are typically caused by a low frequency of premature translation termination or internal initiation [38]; such forms were excluded from all calculations in the present study. In the case of MKK6, the faster migrating form was presumably due to an internal initiation downstream of the LF cleavage site, because it was the same size for both MKK6 and MKK6-LF.

We first compared the ability of MKK3, MKK6 and their LF-cleavage products to bind to the best characterized p38 protein, p38$\alpha$, the predominant p38 family member in leucocytes [39]. Wild-type MKK3 and MKK6 proteins bound to GST–p38$\alpha$ with low micromolar affinities ($K_d \approx 50$ and $\approx 35$ $\mu$M respectively; Figures 3B and 3C). MKK3-LF demonstrated a slightly reduced ability to bind to purified p38$\alpha$ as compared with wild-type MKK3 protein (Figure 3B). In contrast, MKK6-LF bound considerably less strongly than wild-type MKK6 protein to GST–p38$\alpha$ (Figure 3C). The results of several experiments are averaged in Figure 3(D). Overall, binding of MKK6-LF to GST–p38$\alpha$ was 32% of that of full-length MKK6, corresponding to a 3.4-fold decrease in binding affinity, while MKK3-LF binding to GST–p38$\alpha$ was 84% of that of full-length MKK3, corresponding to only a 1.4-fold decrease in binding affinity.

Because p38$\beta$ has also been observed to bind to MKK3 and MKK6 [22], we also tested this member of the p38 subfamily of MAPKs. Wild-type MKK3 and MKK6 proteins bound to GST–p38$\beta$ with affinities similar to those that they exhibited for p38$\alpha$. Furthermore, as was seen with p38$\alpha$, MKK6-LF exhibited much weaker binding to p38$\beta$ (16%, corresponding to a 9-fold decrease in affinity) compared with wild-type MKK6 (Figures 4B and 4C). Finally, as shown in Figures 4(A) and 4(C), there was a more marked reduction in the binding of MKK3-LF when binding to p38$\beta$ was examined (57% of wild type, corresponding to a 1.7-fold decrease in affinity).

Thus the anthrax LF-cleavage products of MKK3 and (especially) MKK6 show a decrease in their ability to bind their cognate MAPKs relative to their wild-type counterparts. The finding that binding of MKK3-LF to p38, and especially to p38$\alpha$, was still significant may reflect the existence of an additional MAPK-binding motif(s) between the LF cleavage site and the kinase domain of MKK3, or a relatively high-affinity interaction between the kinase domain and p38.

### Products of MKK4 and MKK7 cleavage by anthrax LF show reduced binding to JNKs

In the stress-activated signal transduction pathway, MKK4 and MKK7 phosphorylate members of the JNK subfamily of MAPKs. This cascade is activated primarily in response to cytokines and environmental stress, and plays an important role in regulating apoptosis [18,40]. Although both MKK4 and MKK7 are cleaved by anthrax LF, it has yet to be clearly demonstrated that this cleavage disrupts signalling to the JNKs, or contributes to anthrax pathogenesis. MKK4 and MKK7 contain two LF cleavage sites each (Figure 1A). In MKK4 the cleavage site closest to the N-terminal end of the protein lies within the MAPK-docking site. Thus cleavage at either of the LF sites in MKK4 would be expected to sever the MAPK-docking site from the bulk of MKK4, yet leave the C-terminal protein kinase domain intact (Figure 5A). To date, no MAPK-docking site in MKK7 has been demonstrated to be functional.

In order to examine the ability of MKK4 LF-cleavage products to bind their cognate JNKs, plasmids pGEM-MKK4-LFA and pGEM-MKK4-LFB were constructed. These plasmids contained the coding region of MKK4 from the C-terminal side of LF cleavage site A or B respectively to the end of the gene. Plasmids

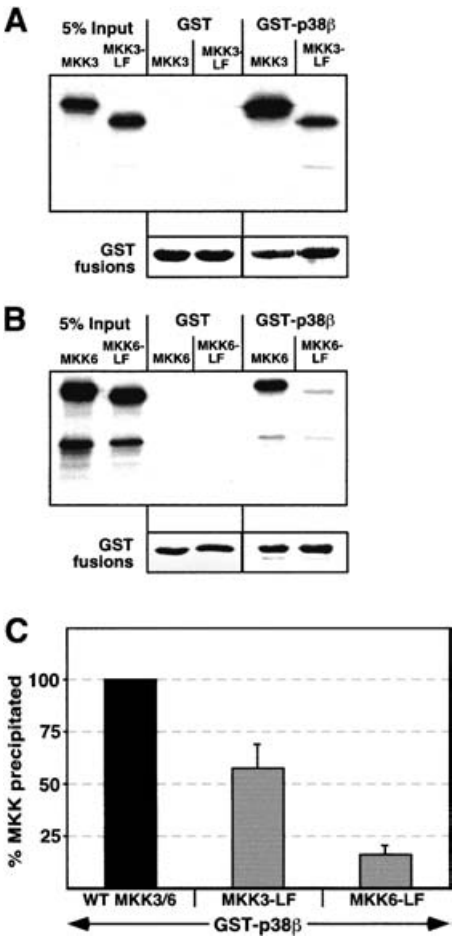


**Figure 4 LF-cleavage products of MKK3 and MKK6 exhibit reduced binding to p38$\beta$**

(**A**–**C**) As described for in Figures 2(B)–2(D), except that 10 $\mu$g of GST or 20 $\mu$g of GST–p38$\beta$ was used with *in vitro* transcribed and translated MKK3, MKK3-LF, MKK6 or MKK6-LF. (**A**) Autoradiogram of a representative experiment for binding of MKK3 and MKK3-LF to GST–p38$\beta$. (**B**) Autoradiogram of a representative experiment for binding of MKK6 and MKK6-LF to GST–p38$\beta$. (**C**) Relative binding of MKK3-LF and MKK6-LF to GST–p38$\beta$ expressed as a percentage of the binding of the corresponding full-length wild-type (WT) MKK. Results are means $\pm$ S.E.M. of four to five experiments.

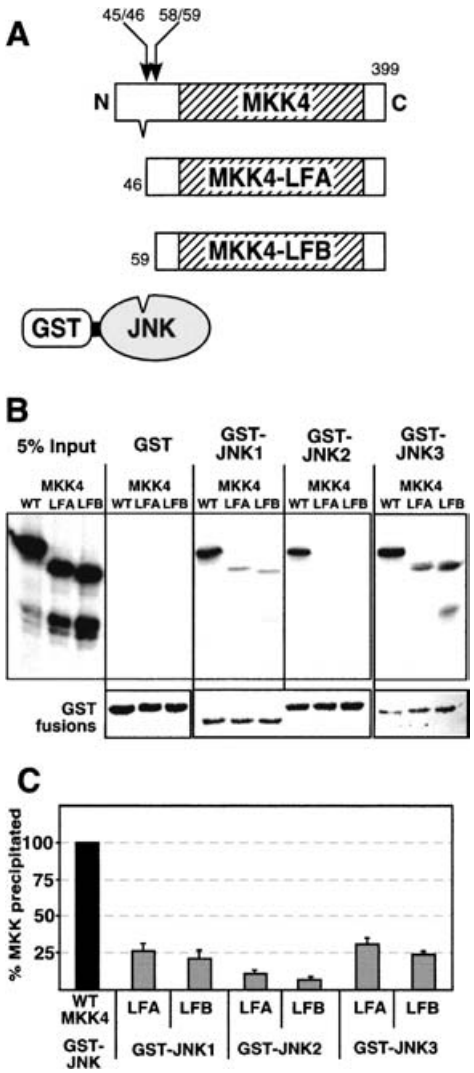


**Figure 5 LF-cleavage products of MKK4 show impaired binding to JNK**

(**A**) Schematic representation of the experiment. Full-length wild-type MKK4 containing an intact MAPK-docking site (triangle) and the corresponding LF-cleavage products MKK4-LFA and MKK4-LFB are shown. Other details are as in Figure 2. (**B**, **C**) As described in the legend to Figure 2, except that 10 $\mu$g of GST, 20 $\mu$g of GST–JNK1$\alpha$1, 20 $\mu$g of GST–JNK2$\alpha$2 or 20 $\mu$g of GST–JNK3 was used with *in vitro* transcribed and translated MKK4, MKK4-LFA or MKK4-LFB. (**B**) Autoradiogram of a representative experiment for binding of MKK4, MKK4-LFA and MKK4-LFB to GST–JNK1$\alpha$1, GST–JNK2$\alpha$2 and GST–JNK3. (**C**) Relative binding of MKK4-LFA and MKK4-LFB to GST–JNK1$\alpha$1, GST–JNK2$\alpha$2 and GST–JNK3 expressed as a percentage of the binding of the corresponding full-length wild-type (WT) MKK. Results are means $\pm$ S.E.M. for four to six experiments.

pGEM-MKK4-LFA and pGEM-MKK4-LFB were used to generate MKK4-LFA and MKK-LFB proteins respectively, using *in vitro* transcription and translation. As was seen with MKK6 (Figures 3C and 4B) and MKK7 (see below), there were some minor, incomplete translation products present in the *in vitro*-translated MKK4 preparation (Figure 5B), most likely caused by a low frequency of internal initiation. The amount of these forms was increased somewhat for the LF-cleavage products, perhaps due to differences in the secondary structure of the respective RNAs. These incomplete products did not bind appreciably to JNK, and were excluded from all calculations.

MKK4 and its LF-cleavage products were tested for their ability to bind GST–JNK1$\alpha$1, GST–JNK2$\alpha$2 and GST–JNK3 in pull-down assays. Full-length, wild-type MKK4 bound to all three JNK proteins with low micromolar affinity ($K_d \approx 25$–$55$ $\mu$M; Figure 5B), consistent with our previous results [23]. For all three JNK proteins tested, the binding of MKK4-LFA and MKK4-LFB was markedly reduced compared with that of wild-type MKK4 (Figure 5B). Binding of MKK4-LFA to GST–JNK1$\alpha$1, GST–JNK2$\alpha$2 and GST–JNK3 was 26%, 11% and 31% respectively of the binding of full-length MKK4 (Figure 5C). Binding of MKK4-LFB was 22%, 7% and 24% respectively of wild-type binding (Figure 5C). Binding affinities for JNK1 and JNK3 were reduced by 4–5-fold, and those for JNK2 by 9–16-fold. The reduced binding of the MKK4 LF-cleavage products was undoubtedly due to the removal of the recently characterized JNK-docking site (residues 38–48 of MKK4) [23]. MKK4-LFB did not exhibit substantially weaker binding to GST–JNKs than MKK4-LFA. This is consistent with there being no second JNK-docking site in MKK4 overlapping the more C-terminal LF cleavage site (site B) [23].

Although no D-site has been functionally demonstrated in MKK7, Tournier et al. [41] found that the N-terminal domain of MKK7 is necessary and sufficient for JNK binding. Both anthrax

LF cleavage sites in MKK7 (44/45 and 76/77) have some amino acid sequence similarity to the D-site consensus $+{+}{+}X_{1-5}\varphi X\varphi$; they contain two of the three usual basic residues followed by the rest of the consensus (hydrophobic, Xaa, hydrophobic). In addition, residues 25–34 of MKK7 (sequence RRRIDLNLDI), which would be removed by cleavage at either LF site, also fit the D-site consensus. Whether or not these regions can act as *bona fide* MAPK-docking sites remains to be determined.

Wild-type MKK7, as well as both MKK7 LF-cleavage products, were tested for binding to GST–JNKs. We utilized the MKK7$\beta$ isoform [41], which appears to be the predominant isoform in most human cells, and is also the only isoform for which LF-cleavage sites have been mapped [13]. Plasmid pGEM-MKK7 was constructed and used to generate full-length MKK7; plasmids pGEM-MKK7-LFA and pGEM-MKK7-LFB, containing the coding region of MKK7 from the C-terminal side of cleavage site A or B respectively to the end of the gene, were constructed and used to generate MKK7-LFA and MKK7-LFB proteins (Figure 6A). Full-length MKK7 bound to JNK1$\alpha$1 and JNK3 with low micromolar affinity ($K_d \approx 15$–$40$ $\mu$M; Figure 6B), whereas binding to JNK2$\alpha$2 was considerably weaker ($K_d \approx 300$ $\mu$M; Figure 6B). MKK7-LFA and MKK7-LFB exhibited reduced binding to all three JNK proteins relative to binding of wild-type MKK7 protein (Figures 6B and 6C). In all cases binding of the LF-cleavage products was less than 50 % that of wild-type MKK7 (Figure 6C), corresponding to a decrease in binding affinity of approx. 4-fold. The finding that the MKK7-LFA protein exhibited such impaired JNK binding implies that there is a JNK-binding site within the first 44 N-terminal amino acid residues of MKK7. MKK7-LFB binding to GST–JNKs was no weaker than MKK7-LFA binding, suggesting that the residual binding to GST–JNKs seen with MKK7-LFA is not dependent upon the sequence between residues 45 and 77.

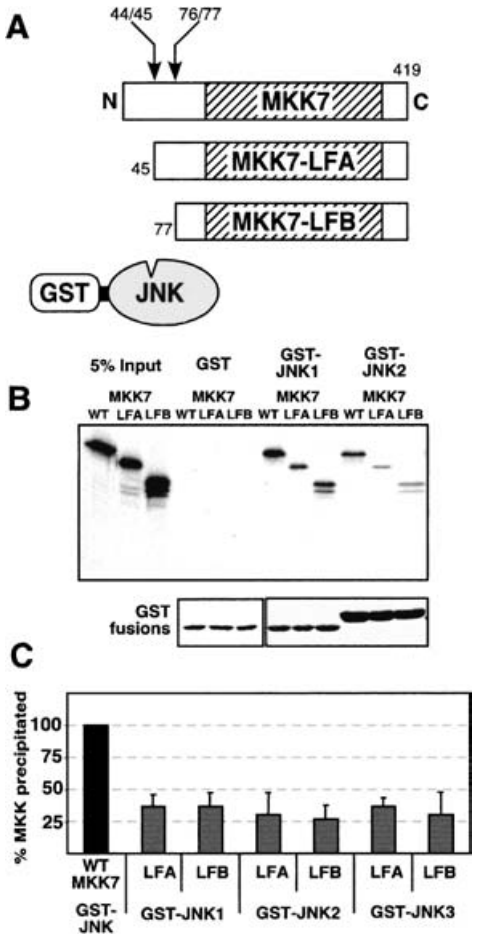


**Figure 6 LF-cleavage products of MKK7 display reduced binding to JNK**

(**A**) Schematic representation of the experiment. Full-length wild-type MKK7 containing an intact MAPK-docking site (triangle) and the corresponding LF-cleavage products MKK7-LFA and MKK7-LFB are shown. Other details as in Figure 2. (**B**, **C**) As described in the legend to Figure 2, except that 10 $\mu$g of GST, 20 $\mu$g of GST–JNK1$\alpha$1, 40 $\mu$g of GST–JNK2$\alpha$2 or 20 $\mu$g of GST–JNK3 was used with *in vitro* transcribed and translated MKK7, MKK7-LFA or MKK7-LFB. (**B**) Autoradiogram of a representative experiment for binding of MKK7, MKK7-LFA and MKK7-LFB to GST–JNK1$\alpha$1 and GST–JNK2$\alpha$2. (**C**) Relative binding of MKK7-LFA and MKK7-LFB to GST–JNK1$\alpha$1, GST–JNK2$\alpha$2 and GST–JNK3 expressed as a percentage of the binding of the corresponding full-length wild-type (WT) MKK. Results are means $\pm$ S.E.M. for four to six experiments.

## DISCUSSION

MKKs/MEKs are the only known proteolytic targets of anthrax LF. Lethal toxin treatment has been shown to inhibit signalling through the ERK and p38 pathways [4,6,15,27]. Moreover, the product of MEK1 cleavage by LF has been shown to have reduced protein kinase activity [15,25]. However, the mechanism by which removal of a relatively short N-terminal segment could inhibit signal transmission and kinase activity has been unclear. In the present study we have tested proteins that correspond to the C-terminal LF-cleavage products of MEK1, MEK2, MKK3, MKK4, MKK6 and MKK7 for binding to their cognate MAPKs. We found that even though all of the MKK LF-cleavage products still contained the bulk of the protein, including the entire kinase domain, they were compromised with regard to MAPK binding. We suggest that this is a direct result of the removal or disruption of the MAPK-docking site (or D-site) in MKKs that is required for efficient MAPK binding [21–23]. Thus we propose that the disruption of MKK–MAPK docking represents a common molecular mechanism of pathogenesis due to anthrax LF.

### Products of MEK/MKK cleavage by LF are defective in MAPK binding

Recent studies clearly demonstrate a role for LF-catalysed MKK cleavage in dismantling the host immune response [4–8]. In contrast, the role that MKK cleavage plays in the end stages of *B. anthracis* infection, characterized by bacteraemia, necrosis and toxic shock-like symptoms, remains to be determined. MEK1, MEK2 and MKK6 appear to play crucial roles in the early inflammatory signalling events in host macrophages and dendritic cells that are suppressed by lethal toxin [4,6]. We found that the LF-cleavage products of these three MKKs displayed a substantial and significant decrease in their MAPK-binding ability (Figures 2–4). In contrast, the LF-cleavage product of MKK3 displayed considerable residual binding to p38 (Figures 3 and 4), suggesting that LF-catalysed cleavage may have a less severe effect on the activity of this enzyme. In accordance with this suggestion, MKK3 isoforms lacking their docking site can still activate p38$\alpha$ [22]. Nevertheless, in the absence of MKK6, MKK3 does not seem to be able to protect macrophages from lethal toxin-induced apoptosis [6]. Thus it appears that MKK6 (but not MKK3) transmits a critical pro-survival signal in activated macrophages that is disrupted by anthrax lethal toxin.

No role for the LF-induced cleavage of MKK4 and MKK7 in immune suppression or other aspects of anthrax pathogenesis has yet been demonstrated, although these kinases and their JNK substrates are important for aspects of both innate and adaptive immunity [16]. We found that the anthrax LF-cleavage products of MKK4 (in particular) and MKK7 displayed reduced binding

to JNK1, JNK2 and JNK3 (Figures 5 and 6). JNK1 and JNK2 are expressed in most cell types, and some evidence suggest that JNK1 phosphorylation in activated macrophages is inhibited by lethal toxin treatment [6]. JNK3 is expressed predominantly in the nervous system [40], and thus is probably not relevant to anthrax pathogenesis. Based upon the marked decrease in binding exhibited by the MKK4 LF-cleavage products (Figure 5), combined with the known importance of JNK docking in MKK4 signalling [23,42] and the functions of MKK4 in immune cells [16], we suggest that the LF-catalysed cleavage of MKK4 may indeed play a role in suppression of the host immune system by anthrax. The same may also be true of MKK7.

### Correspondence of MAPK-docking sites and LF cleavage sites

The correspondence between the D-site class of MAPK-docking sites and LF cleavage sites can now be summarized as follows. There are seven MKKs in the human genome [43]; six of these are cleaved once or twice in their N-terminal regulatory domains by anthrax LF protease [13]. LF cleaves MEK1, MEK2, MKK3 and MKK6 once, within a *bona fide* D-site that has been shown to be necessary and sufficient for relatively high-affinity MAPK binding [21,22]. As shown in the present study, in the case of MEK1, MEK2 and MKK6, this cleavage essentially abolishes D-site function, resulting in a substantial decrease in MAPK binding (even though, in MEK1 and MEK2, the hydrophobic submotif of the D-site is not removed). For MKK3, there is a more modest decrease in MAPK binding.

MKK4 is cleaved twice by LF [13]. The first cleavage site is within a *bona fide* D-site; the sequence flanking the second cleavage site, although it resembles the D-site consensus, does not bind with high affinity to the ERK, JNK or p38 MAPKs [23]. As shown in the present study, cleavage at either the first site (which splits the D-site in two) or the second site (which clips off the D-site plus 10 more residues) greatly reduces MAPK binding. LF-mediated cleavage of MKK7 also occurs at two sites [13], both of which share some similarity to identified MKK D-sites. In addition, there is a third putative D-site in MKK7 that lies N-terminal to the two LF-cleavage sites. However, none of these three D-sites have yet been proven to be functional. We showed in the present work that both MKK7 cleavage products were similarly compromised in JNK binding. This suggests that JNK binding was impaired because of the loss of the most N-terminal potential D-site, and/or that which overlaps the first LF cleavage site. The region flanking the second LF-cleavage site in MKK7 does not appear to be sufficient for MAPK binding.

Of the seven human MKKs, only MEK5 is not a substrate of LF [13]; consistent with this, the N-terminal domain of MEK5 does contain a clear match to the D-site consensus. To summarize, all D-sites in MKKs are sites of cleavage by LF, and most (but not all) LF-cleavage sites in MKKs are D-sites.

Functional D-sites have also been found in MAPK substrates, phosphatases and scaffolds [44,45], but those that have been tested are not substrates for LF [25]. LF appears to be specifically targeted to MKKs, because it makes additional contacts with their catalytic domains [25].

### Conclusions

Is disruption of MKK–MAPK docking sufficient to explain the observed defects in MAPK cascade signalling, or might there be additional mechanisms by which LF-mediated cleavage inhibits MKK-dependent signalling in affected cells? It is certainly possible that there are additional mechanisms by which LF hampers MKKs. For example, in the case of MKK4, the region clipped off by LF is also required for binding to the MKK4 activator MEKK1 (MEK kinase 1) [42]. In addition, it has been suggested that LF-cleaved MEK1 may have a reduced 'intrinsic' kinase activity [25]. On the other hand, peptide inhibition studies suggest that hindering MKK–MAPK docking is sufficient to partially inhibit signal transmission [20,21,24]. In these *in vitro* biochemical studies, D-site peptides derived from MEK1, MEK2 or yeast Ste7 were able to inhibit MEK1 or MEK2 activity by 75–80 % [21,24]. These peptides almost certainly acted by binding to ERK2 and obstructing MEK binding. Hence the observed inhibition was independent of any structural effects on the MKK, and did not require any other proteins.

In conclusion, our results demonstrate that disruption of MKK–MAPK docking is a common molecular mechanism by which anthrax lethal toxin can impede MAPK signal transduction pathways in affected cells; this may play a critical role in the immune suppression and toxicity seen in anthrax.

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