

# Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor

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The lethal factor (LF) produced by toxigenic strains of *Bacillus anthracis* is a Zn<sup>2+</sup>-endopeptidase that cleaves the mitogen-activated protein kinase kinases (MAPKKs) MEK1, MEK2 and MKK3. Using genetic and biochemical approaches, we have extended the study of LF proteolytic specificity to all known MAPKK family members and found that LF also cleaves MKK4, MKK6 and MKK7, but not MEK5. The peptide bonds hydrolysed by LF within all MAPKKs were identified. Cleavage invariably occurs within the N-terminal proline-rich region preceding the kinase domain, thus disrupting a sequence involved

in directing specific protein–protein interactions necessary for the assembly of signalling complexes. Alignment of the sequences flanking the site of cleavage reveals the occurrence of some consensus motifs: position P2 and P1' are occupied by hydrophobic residues and at least one basic residue is present between P4 and P7. The implications of these findings for the biochemical activity and functional specificity of LF are discussed.

**Key words:** bacterial toxin, metallopeptidase, signal transduction.

## INTRODUCTION

Anthrax lethal factor (LF) is the active component of a protein exotoxin (the lethal toxin, LeTx) secreted by toxigenic strains of *Bacillus anthracis*. LeTx is an A-B type toxin consisting of two non-covalently bound factors, LF and protective antigen (PA) [1]. PA binds to a ubiquitous receptor on the plasma membrane and is processed proteolytically by membrane peptidases, including furin, to a 63 kDa form that is able to oligomerize and bind LF [2,3]. On endocytic uptake, PA undergoes an acid-triggered conformational rearrangement [4] that mediates the transfer of LF from the lumen of a late endocytic compartment to the cytoplasm [5]. In the cytosol LF displays its activity, causing alterations in still poorly understood cellular functions in a manner dependent on cell type and dose. LeTx has specific cytopathic effects on macrophages [6] after the production of reactive oxygen intermediates, which induce cellular lysis beginning approx. 90 min after toxin challenge [7]. Low non-cytolytic doses of the toxin have been reported to mediate the release of cytokines from cultured macrophages [8]. LeTx-induced macrophage cytotoxicity has a primary role in the development of systemic anthrax, a rapid and often fatal disease of several vertebrate species including humans (reviewed in [9]).

LF is a 90 kDa protein containing a C-terminal HEXXH (single-letter amino acid codes) Zn<sup>2+</sup>-binding motif (residues 686–690 of mature LF) present in the active site of metallopeptidases [10]. LeTx cytotoxicity depends on the proteolytic activity of LF, because membrane-permeable metalloprotease inhibitors prevent the cytotoxicity of LeTx in macrophages [10,11]. Moreover, mutations of active-site residues His<sup>686</sup>, Glu<sup>687</sup> and His<sup>690</sup> partly or totally impair LF activity [10,12]. LF has been proposed to cleave specific intracellular substrates in a manner analogous to the metallopeptidase activity of clostridial neurotoxins [12,13]. The specific nature of this activity is high-

lighted by the fact that LF does not cleave general protease model substrates such as casein or haemoglobin [13]. Moreover, specific synthetic peptide substrates are cleaved with very low efficiency [12]. These results suggest that, as with clostridial neurotoxins [14,15], LF interacts with specific structural motifs to recognize its substrate(s) correctly and to achieve full enzymic activity.

The mitogen-activated protein kinase kinases (MAPKKs) MEK1 and MEK2 have been found to be specific substrates of LF endopeptidase activity. This identification was the result of two different experimental approaches. Comparing the activity of several drugs on a panel of different cell lines, LeTx had an effect on cultured cells similar to that of the MEK1 and MEK2 inhibitor PD098059 [16]. A genetic screening in yeast, with the use of the two-hybrid system with a catalytic inactive mutant of LF (LF<sup>E687A</sup>) as a bait, led to the identification of MEK2 as specific prey [17]. MEK1 and MEK2 are cleaved by LF *in vivo* and *in vitro* within the proline-rich N-terminal tails that precede their kinase domains. We have observed recently that MKK3 (an additional member of the MAPKK family) is N-terminally cleaved in macrophages challenged with LeTx [18].

Dual-specificity MAPKKs are central components of three-kinase modular cascades that control cell responses to mitogenic and stress signals (reviewed in [19]). MAPKKs are activated by upstream MAPKK kinases (MAPKKKs); they in turn phosphorylate downstream MAP kinases (MAPKs). The family of MAPKKs is composed of seven members that share the unique ability to phosphorylate the Thr-Xaa-Tyr motif in the activation loop of MAPKs and stress/cytokines-activated protein kinases [20]. All MAPKKs are structurally characterized by a conserved kinase domain and a divergent N-terminal proline-rich extension that contains localization signals [21] and mediates protein–protein interactions that are important in the assembly of specific signalling complexes [22,23]. Through the formation of these

Abbreviations used: GST, glutathione S-transferase; LF, lethal factor; LeTx, lethal toxin; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; PA, protective antigen.

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complexes, the MAPKKs' N-terminal extension has been proposed to co-operate in the specificity and functionality of the signalling pathways [23,24].

MAPK/stress-activated protein kinase signalling has a primary role in the regulation of host's response to bacterial infections [25]; pathogens have developed virulence factors able to interfere with these pathways [26]. For example, the virulence factor YopJ produced by *Yersinia enterocolitica* binds to several MAPKKs and specifically blocks MAP kinase signalling, thus promoting bacterial survival and multiplication [27].

Although the exact role of MAPKKs' cleavage in anthrax pathogenesis is still under investigation, the fundamental cellular role of these signalling molecules and the identification of some members of the family as specific substrates of LF proteolytic activity calls for the definition of the spectrum and the nature of LF specificity with regard to all known MAPKKs. Here we report the cleavage of MAPKKs MKK4, MKK6 and MKK7, but not MEK5 or other unrelated kinases, within their N-termini, and the determination of the cleavage sites.

## EXPERIMENTAL

### Plasmids

All two-hybrid constructs have been described previously [17]. pGADGH H2-2 was obtained in the two-hybrid screen described here and harboured a 2 kbp *EcoRI/XhoI* insert encoding full-length MKK4 (EMBL/GenBank<sup>®</sup> accession no. L36870).

pGEX2TK LF and pGEX2TK LF<sup>E687A</sup> have been described previously [17]. pGEX-3X MKK6 [28] (encoding isoform b of MKK6 according to Han et al. [29]) and pGEX MKK7 (encoding a  $\beta$  isoform of MKK7 [30]) were a gift from Dr Roger Davis. pGEX-KG MEK5 [31] was a gift from Dr Jack Dixon.

pGEX-4T3 MKK4 was constructed by subcloning the *EcoRI/XhoI* fragment of pGADGH H2-2 into pGEX-4T3 (Amersham Pharmacia). The cDNA for MKK3b (EMBL/GenBank<sup>®</sup> accession no. D87115 [32]) was a gift from Dr Kenji Irie; an *XbaI*-filled-in/*XhoI* 1.1 kbp fragment encoding the full-length murine MKK3b isoform was subcloned in the *SmaI/XhoI* sites of pGEX-4T3.

### Yeast two-hybrid system

All yeast media were purchased from Bio101. Two hybrid methodologies have previously been described [17]. Screening of  $7.5 \times 10^6$  independent transformants yielded 173 colonies growing on histidine-lacking medium; these were picked  $4\frac{1}{2}$  days after plating and tested for  $\beta$ -galactosidase activity with a replica filter assay [33]. Only ten clones showed strong  $\beta$ -galactosidase reporter activity and were further analysed. Library plasmids from these clones were rescued into *Escherichia coli* and subsequently analysed by re-transformation tests, restriction analysis and DNA sequencing.

### Protein expression and purification

Fusion proteins of glutathione S-transferase (GST) with LF and LF<sup>E687A</sup> (GST::LF and GST::LF<sup>E687A</sup> respectively) were expressed in *E. coli* strain BL21 DE3 pUBS 520 [34] and purified by affinity chromatography on GSH-Sepharose beads (Pharmacia), and by anion-exchange and size-exclusion chromatography as described [35]. LF was more than 90% pure as judged by SDS/PAGE and Coomassie Blue staining. PA was obtained from the culture supernatant fluids of *B. anthracis* strain RP42 (PA<sup>+</sup>, EF<sup>-</sup>, LF<sup>-</sup>) [36] and purified as described [13].

GST fusions of different MAPKKs were expressed in *E. coli* strain BL21 DE3 pUBS 520 and affinity-purified on GSH-

Sepharose beads. Bacteria were grown in  $2 \times$  YT medium [1.6% (w/v) Bacto tryptone/1% (w/v) yeast extract/0.5% NaCl] supplemented with 200 mg/l ampicillin and 34 mg/l kanamycin to a  $D_{600}$  of 1 and induced for 12 h at 22 °C with 0.1 mM isopropyl  $\beta$ -D-thiogalactoside. Bacterial pellets from 1 litre cultures were resuspended in 15 ml of 50 mM Tris/HCl (pH 7.5)/10 mM EDTA/300 mM NaCl/1 mM dithiothreitol/5 mM ADP/2.5 mM MgCl<sub>2</sub>/10% (v/v) glycerol, supplemented with two protease inhibitors tablets (Roche) and were lysed by passing the cell suspension twice through a French pressure cell at 1200 lb/in<sup>2</sup> (8.3 MPa). The supernatant obtained after high-speed centrifugation was incubated with 1 ml of GSH-Sepharose 4B beads (Amersham Pharmacia). After several washes with 10 mM Tris/HCl (pH 7.5)/0.1 mM EDTA/1 mM dithiothreitol/0.1 mM ADP/10 mM MgCl<sub>2</sub>/10% (v/v) glycerol, decreasing the NaCl concentration stepwise to reach 20 mM, the GSH-Sepharose beads were equilibrated in cleavage buffer [25 mM potassium phosphate buffer (pH 7.5)/20 mM NaCl/0.1 mM CaCl<sub>2</sub>/0.1 mM ZnCl<sub>2</sub>/1 mM dithiothreitol/0.1 mM ADP/10 mM MgCl<sub>2</sub>/10% (v/v) glycerol] and incubated with LF.

### Proteolysis of MAPKKs

HeLa cytosol was prepared as described [37]. The cytosol was treated with the indicated quantities of LF or LF<sup>E687A</sup> for 30 min at 37 °C. Proteolysis was stopped by the addition of Laemmli sample buffer containing 10 mM *o*-phenanthroline.

GSH-Sepharose bead-immobilized MAPKKs were resuspended at approx. 10  $\mu$ M (50  $\mu$ l bed volume of beads in a final volume of 100  $\mu$ l) in cleavage buffer with 1  $\mu$ M LF or LF preincubated for 30 min with 10 mM *o*-phenanthroline, then incubated for 1.5 h at 37 °C. The reactions were separated by SDS/PAGE [13.5% (w/v) gel] and the gel was stained with Coomassie Blue.

### Antibodies

Antibodies generated against the C-terminal epitopes of MKK3, MKK4 and MEK5 were purchased from Santa Cruz Biotechnology (catalogue nos SC-961, SC-837 and SC-1287 respectively). Antibodies against the C-terminal epitopes of MKK6 and MEK5 were from Stressgen (catalogue nos KAP-MA014C and KAP-MA003C respectively). Antibodies against MEK1, ERK2 and MST1 were from Transduction Laboratories (catalogue nos M17020, E16220 and M43720 respectively).

Blots were probed with antibodies diluted in blocking buffer as indicated in figure legends. The blots were washed and processed with the enhanced chemiluminescence (ECL<sup>®</sup>) detection kit (Amersham Pharmacia) in accordance with the protocol suggested by the manufacturer.

### Protein microsequencing

For N-terminal sequence determination, LF cleavage products were obtained by SDS/PAGE [12% (w/v) gel] separation and subsequent electrotransfer to Immobilon-P<sup>®</sup> PVDF sequencing membrane (Millipore) at 4 °C for 1 h at 200 mA with Caps buffer [10 mM Caps/10% (v/v) methanol (pH 11)]. After staining with 0.1% Coomassie Brilliant Blue R250 in 50% methanol and destaining in 50% (v/v) methanol, the proteins were subjected to Edman degradation and sequenced in a pulsed-liquid Applied Biosystems model 477A protein sequencer.

## RESULTS

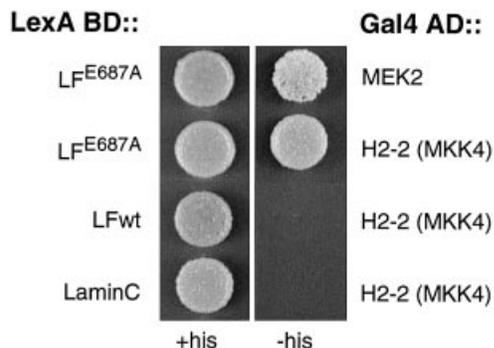
### LF<sup>E687A</sup> interacts with MKK4 in the yeast two-hybrid system

Using the yeast two-hybrid system, we previously identified the MAPKKs MEK1 and MEK2 as proteins capable of interacting with a catalytic inactive mutant of LF (LF<sup>E687A</sup>) [17]. In our original screen, approx.  $3 \times 10^5$  clones were analysed, corresponding to 5% of total library complexity. Having searched for other potential LF substrates, we present here the results of a complete screen of  $7.5 \times 10^6$  yeast colonies transformed with a HeLa cell cDNA library. This screen yielded ten clones positive for both interaction reporters, i.e. growth on histidine-lacking medium and 'strong'  $\beta$ -galactosidase activity on filter assays.

Restriction analysis of library plasmids rescued from nine of these clones revealed patterns similar to the MEK2 clone previously isolated (results not shown); one clone, H2-2, had a 2 kbp insert with a unique restriction pattern and was further characterized: an analysis of its sequence revealed a complete open reading frame encoding full-length MKK4 (also referred as SEK1 and JNKK1 [38]; EMBL/GenBank® accession no. L36870). As previously observed with MEK2, MKK4 specifically interacts with LF<sup>E687A</sup> but not with wild-type LF or with the unrelated bait laminC in the two-hybrid system (Figure 1).

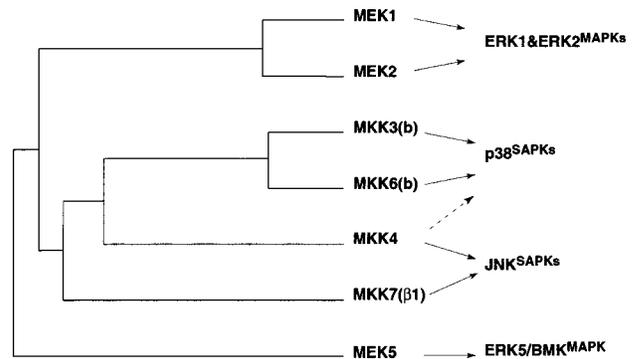
### Several MAPKK family members are substrates of LF peptidase activity *in vitro*

MKK4 belongs to the family of dual-specificity MAPKKs, which are cytosolic enzymes structurally characterized by a conserved kinase domain and a non-conserved N-terminal extension. The seven members of this family are grouped into four subfamilies (Figure 2) [20] on the basis of their functional specificity and mostly reflecting the sequence divergence of the N-terminal tail. The MAPKKs MEK1 and MEK2 specifically phosphorylate and activate ERK1 and ERK2 MAPKs. MKK3 and MKK6 are specific for the MAP kinase p38<sup>hog</sup>, whereas MKK4 and MKK7 phosphoactivate the stress-activated protein kinase JNK, although MKK4 has been reported to phosphorylate p38<sup>hog</sup> as well [38]. MEK5 is the most distantly related



**Figure 1** MKK4 interacts with the catalytic inactive mutant LF<sup>E687A</sup> in the yeast two-hybrid system

*HIS3* reporter gene activation caused by specific interactions between the indicated bait and prey revealed by the ability of the L40 test yeast strain to grow on histidine-lacking medium. Yeast cells co-transformed with the *2 $\mu$ /LEU2* pGAD prey plasmid [encoding the indicated fusions with the Gal4 RNA polymerase II activation domain (Gal4 AD::)] and the *2 $\mu$ /TRP1* pLexA bait plasmid [encoding the indicated fusions with the LexA DNA-binding domain (LexA BD::)], were spotted on synthetic complete medium lacking tryptophan and leucine (+ his) and in medium lacking tryptophan, leucine and histidine (– his). Abbreviation: LFwt, wild-type LF.



**Figure 2** Dendrogram representing the structural relationships and functional specificity of known MAPKK family members

The phylogenetic tree was obtained by aligning the indicated human MAPKK sequences with ClustalW and the distances were calculated by using the neighbour-joining method of Saitou and Nei [41]. Abbreviation: SAPK, stress-activated protein kinase.

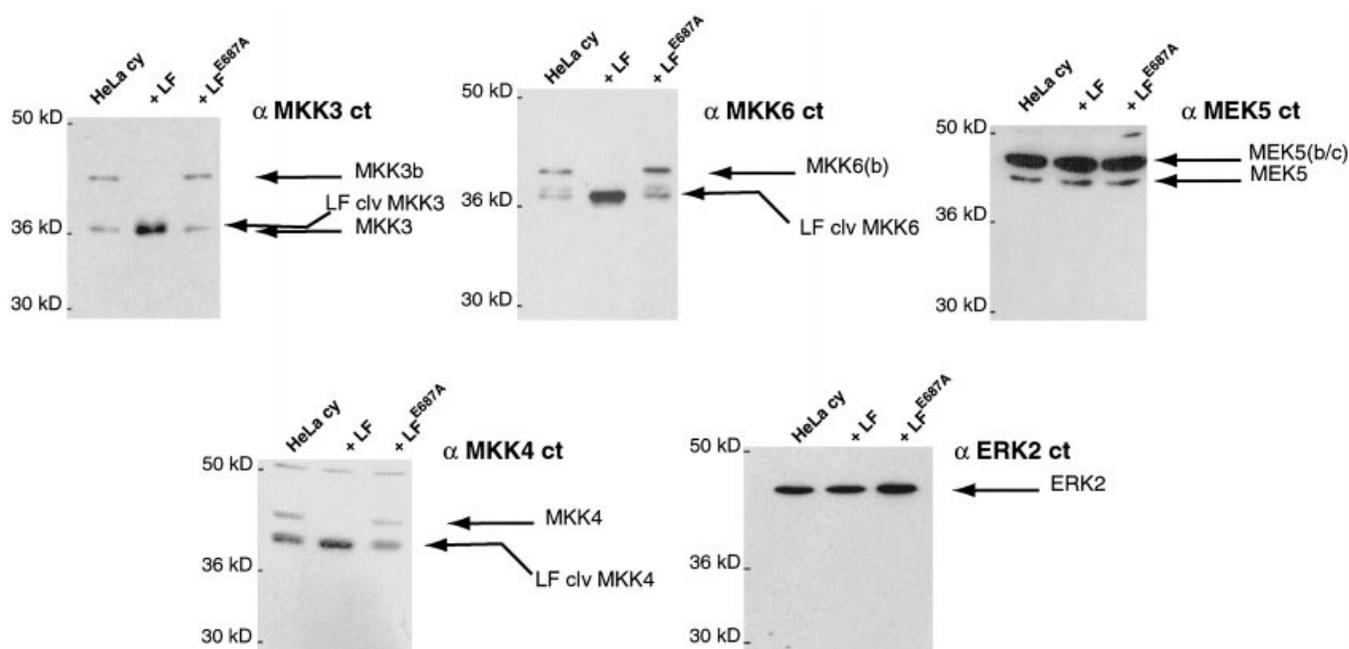
member of the family and is known to phosphoactivate the MAPK Bmk1/ERK5<sup>MAPK</sup>.

The finding that LF recognizes members of different subfamilies in the yeast two-hybrid system prompted us to determine the whole spectrum of LF peptidase activity against known MAPKK family members. To test LF proteolytic activity against potential substrates under native conditions, we assayed changes in the electrophoretic mobility of MAPKK family members after the incubation of HeLa cytosol with purified recombinant LF or with the catalytic inactive mutant LF<sup>E687A</sup>. After separation by SDS/PAGE followed by transfer to nitrocellulose, the reaction products were probed with antibodies directed against C-terminal epitopes specific for different MAPKKs. As shown in Figure 3, we detected an increased electrophoretic mobility of bands recognized by antibodies specific for the C-termini of MKK4 and MKK6 but not of MEK5, indicating that these proteins were cleaved at their N-terminus. To confirm the specificity of the bands recognized by the anti-MEK5 antibody, two commercial polyclonal MEK5 antisera were tested with identical results (see the Experimental section; results not shown). Because commercially available antibodies against MKK7 did not give satisfactory results with HeLa cytosol as well as other cell lysates (results not shown), MKK7 was directly tested as a LF proteolytic substrate with the recombinant molecule (see below).

Changes in electrophoretic mobility were not detected in samples incubated with the metal chelator and metallopeptidase inhibitor *o*-phenanthroline (results not shown), further indicating that MAPKK cleavage is specific and metal dependent. We tested whether LF could cleave two unrelated kinases, the 42 kDa MAPK ERK2 and the 55 kDa serine-threonine kinase MST1, in HeLa cytosol. However, the electrophoretic mobilities of ERK2 (Figure 3) and MST1 (results not shown) were not increased by the incubation with LF.

Figure 3 shows that the anti-MKK3 antibody detected two bands: one of lower electrophoretic mobility probably corresponding to isoform MKK3b and a second band of higher mobility probably corresponding to MKK3a [32], which lacks the N-terminal tail. On cleavage, isoform MKK3b appeared downshifted and migrated in close proximity to isoform MKK3a.

This preliminary analysis indicated that LF cleavage specificity is extended to several but not all members of the MAPKK family and isoforms, and that other structurally and functionally unrelated signalling kinases are not cleaved by LF.



**Figure 3** LF peptidase activity against MAPKKs in HeLa cytosol

LF peptidase activity was tested by incubating LF and LF<sup>E687A</sup> (1 ng) with HeLa cytosol (HeLa cy; 5  $\mu$ g) for 30 min at 37 °C; the reaction products were separated by SDS/PAGE [13.5% (w/v) gel], blotted and probed with the following: a polyclonal antibody specific for the C-terminus of MKK3 ( $\alpha$ MKK3 ct) diluted 1:3000; a polyclonal antibody specific for the C-terminus of MKK6 ( $\alpha$ MKK6 ct) diluted 1:1000; a polyclonal antibody specific for the C-terminus of MEK5 ( $\alpha$ MEK5 ct) diluted 1:500; a polyclonal antibody specific for the C-terminus of MKK4 ( $\alpha$ MKK4 ct) diluted 1:500; and a monoclonal antibody specific for the C-terminus of ERK2 ( $\alpha$ ERK2 ct) diluted 1:1000. A downwards shift of the proteins (indicated as LF clv MKK) reveals proteolysis by LF. Abbreviation: kD, kDa.

### Identification of the peptide bonds cleaved by LF within MAPKK family members

To identify the peptide bonds cleaved by LF, recombinant MAPKKs were expressed in *E. coli* as N-terminal fusion proteins with the GST of *Schistosoma japonicum*. GST::MAPKKs were affinity-purified on GSH-Sepharose; bead-immobilized proteins were assayed for LF peptidase activity. As a control, LF was preincubated with *o*-phenanthroline to remove the active-site Zn<sup>2+</sup> ion essential for catalysis.

As shown in Figure 4, LF was able to proteolyse recombinant GST::MAPKK fusions, generating distinct cleavage products. The products containing the GST moiety and the MAPKK sequence at the N-terminus of the cleavage site were retained on beads, whereas the C-terminal fragments were released into solution. In addition, under these experimental conditions proteolysis of GST::MEK5 was not observed (results not shown), confirming that this MAPKK was not sensitive to cleavage by LF. GST::MKK3b and GST::MKK6b were cleaved at a single peptide bond with the generation of a single fragment, whereas GST::MKK4 and GST::MKK7 generated two distinct soluble products corresponding to cleavage at two different sites.

The soluble proteolytic products were subjected to N-terminal amino acid sequencing by the Edman method. Table 1 summarizes the results obtained and aligns the sequences containing the MAPKKs peptide bonds hydrolysed by LF. The analysis of the sequences flanking the cleavage sites, together with the fact that LF can cleave MKK4 and MKK7 at two different sites, indicates that the LF active site can accommodate peptides of different primary structures. Given the overall sequence diversity around the cleavage site, these findings suggest that the major determinant in the recognition by LF of its substrates is the

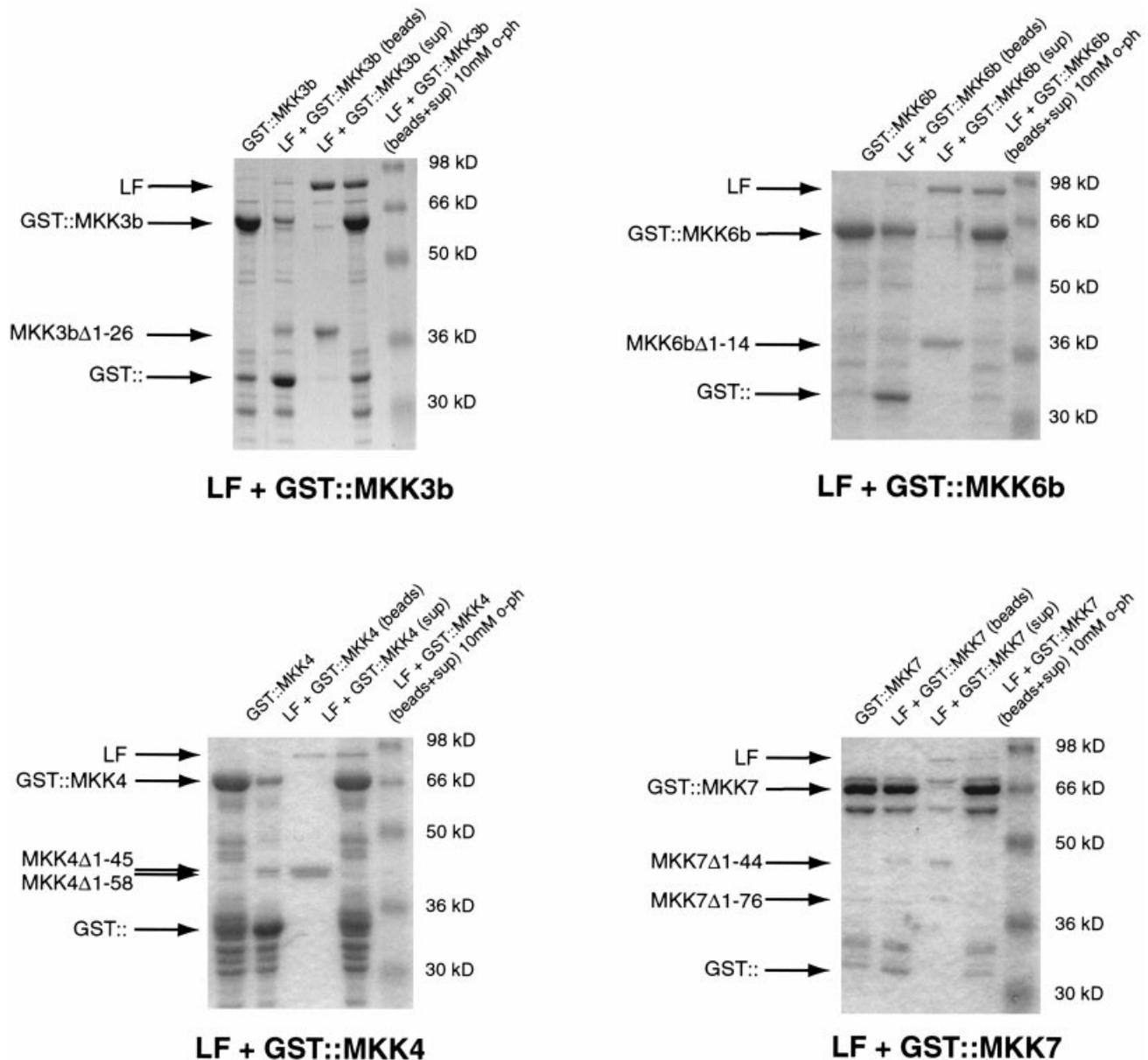
interaction with specific structural elements external to the cleavage site, with a smaller contribution from the sequence containing the cleaved peptide bond. However, a closer inspection of the sequence alignment of Table 1 reveals that position P2 is predominantly hydrophobic, whereas a hydrophobic residue is invariably present at position P1'. Moreover, at least one (but more often two or three) basic residues are present within the P4-P7 segment.

The results document clearly that LF is not an aminopeptidase because it can cleave its substrates even when they are covalently linked via their N-termini to the bulky GST domain, suggesting that LF can cleave MAPKKs complexed with other proteins provided that a limited portion of their N-terminus is accessible.

### DISCUSSION

The present study has analysed the range of metalloproteolytic specificity of anthrax LF and presents evidence that LF is active against all the members of the MAPKK family, except MEK5. The identification of the cleavage site in different MAPKKs provides insight into LF biochemical specificity. The peptide bonds hydrolysed are always located within the N-terminal tail of MAPKKs. The alignment of these regions centred on the cleavage sites shows that position P1' is always occupied by a hydrophobic residue, as is position P2, except in MEK1. Moreover, one or more positively charged residues are present between positions P4 and P8, suggesting that electrostatic interactions are also necessary for a correct positioning of the substrate within the active site of LF.

The present findings do not exclude the possibility that LF might also act on other cellular substrates. Here we have performed an extensive screening of potential LF-interacting



**Figure 4** LF endopeptidase activity against GST::MAPKK fusions

Endopeptidase activity of LF was tested *in vitro* on the basis of its ability to cleave the bulk of the GST sequence from the N-terminus of bead-immobilized recombinant GST::MAPKKs as described in the Experimental section. The reaction products were separated by SDS/PAGE [13.5% (w/v) gel]; gels were stained with Coomassie Blue. In the samples incubated with LF in the absence of *o*-phenanthroline (*o*-ph), the beads were isolated from the supernatants (sup) and washed, then equal amounts of the two fractions were separated in different lanes. Abbreviation: kD, kDa.

proteins with the yeast two-hybrid system employing the catalytic mutant of LF (LF<sup>E687A</sup>) [17] as bait. Glu<sup>687</sup> has previously been shown to be essential for LF activity [10]. This conserved residue of the zinc-binding motif of metalloproteases co-ordinates a water molecule essential for the catalysis; mutagenesis of this essential residue in other Zn<sup>2+</sup>-dependent peptidases produces properly folded but inactive enzymes [39]. The use of this mutant as two-hybrid bait prevents the potential cleavage of preys in the yeast cell with the subsequent loss of the ability to reveal interaction that could occur with active LF. This approach was successful previously in the identification of the MAPKKs MEK1 and MEK2 as targets of LF peptidase activity [17]. The result of the larger screening performed in the present

study was that in nine cases MEK2 and in one case MKK4 were found as interacting partners of LF. However, it is widely accepted that the yeast two-hybrid system cannot be regarded as a method for saturation screening and the possibility that other natural substrates of LF exist cannot be excluded.

The ability of LF to cleave MAPKK as fusions with the N-terminal bulky GST sequence, documented here, indicates that LF acts as an endopeptidase and suggests that enzymic activity requires access to a limited portion of the substrate cleavage site. This observation might have important implications *in vivo*, where the N-terminal extension is engaged in protein-protein interactions that are essential for signal transduction along the several pathways in which members of the MAPKK family are

**Table 1** Alignment of MAPKKs residues flanking the LF cleavage site

Substrate amino acid residues (single-letter codes) are numbered in accordance with the Schechter and Berger nomenclature [42]. The consensus row indicates chemical properties common to several or all (bold) residues at a given substrate site position: + indicates a basic residue; h stands for hydrophobic.

Substrate site	P ...	8	7	6	5	4	3	2	1	1'	2'	3'	4'	5'	6'	7'	8'
MEK1 (Pro <sup>8</sup> -Ile <sup>9</sup> cleavage site)		M	P	K	K	K	P	T	P	I	Q	L	N	P	A	P	D
MEK2 (Pro <sup>10</sup> -Ala <sup>11</sup> cleavage site)		A	R	R	K	P	V	L	P	A	L	T	I	N	P	T	I
MKK3b (Arg <sup>26</sup> -Ile <sup>27</sup> cleavage site)		S	K	R	K	K	D	L	R	I	S	C	M	S	K	P	P
MKK6b (Arg <sup>14</sup> -Ile <sup>15</sup> cleavage site)		K	K	R	N	P	G	L	K	I	P	K	E	A	F	E	Q
MKK4 (Lys <sup>45</sup> -Leu <sup>46</sup> cleavage site)		Q	G	K	R	K	A	L	K	L	N	F	A	N	P	P	F
MKK4 (Arg <sup>58</sup> -Phe <sup>59</sup> cleavage site)		P	P	F	K	S	T	A	R	F	T	L	N	P	N	P	T
MKK7β (Gln <sup>44</sup> -Leu <sup>45</sup> cleavage site)		Q	R	P	R	P	T	L	Q	L	P	L	A	N	D	G	G
MKK7β (Glu <sup>76</sup> -Leu <sup>77</sup> cleavage site)		A	R	P	R	H	M	L	G	L	P	S	T	L	F	T	P
Consensus...			+	+	+	+			h								

implicated. In other words, the fact that MAPKKs are cleaved by LF *in vitro*, notwithstanding the presence of the bulky GST sequence, is compatible with a situation *in vivo* where LF might interfere with several signal transduction pathways that control cellular responses to both stress and mitogenic stimuli, suggesting that the toxin might have a more pleiotropic role than previously thought.

LF invariably cleaves within the MAPKK N-terminal extension, a proline-rich region divergent in length and primary sequence that precedes the kinase domain. Several studies have shown that this region harbours important protein-protein interaction determinants. For example the recently described 'MAPK docking site' present within the N-terminal region of all MAPKK family members [23,24] is implicated in the formation of stoichiometric, non-catalytic complexes with target downstream MAPKs. It is noteworthy that the LF cleavage site almost invariably coincides with the MAPK docking site as defined by Takuji et al. [24].

Although the data presented here on the biochemical specificity of LF do not provide a direct insight into the mechanisms of its cytotoxicity in macrophages, the cleavage of a MAPKK sequence involved in directing specific protein-protein interactions suggests that LF intracellular activity significantly interferes with several MAP kinase signalling pathways. A role for the N-terminal extension of MKK4 has been recently revealed [23]. Through sequential interaction with upstream and downstream components of the JNK<sup>MAPK</sup> pathway, this sequence organizes the assembly of the signalling modules required to ensure response specificity to cytokine stimulation. Interestingly, an N-terminally truncated mutant of MKK4 that is unable to respond to cytokine stimulation is still able to transduce stress stimuli, indicating that the integrity of the N-terminal region is not required for kinase activity but rather for signalling specificity [23]. Given that, on LF cleavage, MKK4 loses about the same sequence as the one missing from this truncated mutant, it can be predicted that LF cleavage of MKK4 does not impair MKK4 enzymic activity but rather modifies the overall ability of the JNK<sup>MAPK</sup> pathway to participate in appropriate stress responses. Additional evidence that LF proteolysis does not result in a loss of the functional activity of MAPKKs but rather in a modification of their specificity comes from the recent finding that LF-cleaved MEK1 can be phosphorylated and can induce Golgi fragmentation during mitosis [40].

Several MAPKK family members have distinct isoforms, mostly resulting from the alternative splicing of exons encoding the N-terminal extension [19], where the LF cleavage site and important protein-protein interaction determinants map.

Although the functional role of these isoforms is presently obscure, the identification of LF-cleavage sites indicates that specific isoforms of MKK3, MKK6 and MKK7 are insensitive to cleavage, because proteolysis occurs at sites that are absent from these isoforms (MKK3a [32], MKK6 [29] and isoforms  $\alpha$  of MKK7 [30]). For MKK3 and MKK7, cleavage by LF results in the conversion of longer isoforms to products almost identical in sequence with the shorter isoforms MKK3a and MKK7 $\alpha$ . Future work will clarify whether the production and accumulation of these isoforms on challenge with toxin is correlated with the cytotoxic activity of LF.

We have shown previously that a truncated mutant of MEK2 lacking the cleavage sequence (MEK2 $\Delta$ 1-33) does interact efficiently with LF<sup>E687A</sup> in the yeast two-hybrid system [17], indicating that the presence of the cleavage sequence in MEK2 is not required for LF-substrate interaction. Here we have shown that LF cleaves different MAPKKs at sites divergent in primary sequence, and at more than one peptide bond within the same substrate. Analysis of the sequences flanking the cleaved MAPKKs peptide bonds suggests that the LF active site is able to accommodate peptide segments that are substantially different in structure. Taken together, these results indicate that, as in the clostridial neurotoxins [14,15], important determinants of LF recognition are located at substrate regions distant from the site of proteolysis.

We are currently investigating the molecular basis of LF-substrate specificity: to this end, we have co-crystallized LF in complex with one of its MAPKK substrates and are attempting to resolve the structure of this complex by X-ray diffraction. We hope that this structure will provide detailed information on the structural determinants that confer substrate recognition specificity.

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