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Review

Anthrax toxins

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Abstract. Though its lethal effects were ascribed to an exotoxin almost half a century ago, the pathogenesis of anthrax has yet to be satisfactorily explained. Subsequent work has led to the molecular identification and enzymatic characterization of three proteins that constitute two anthrax toxins. Protective antigen binds an as yet unknown cell receptor and mediates the entry of the other two components to the cytoplasm via the endosomal pathway. Edema factor, so named for its ability to induce edema, is a Ca^{2+}/calmodulin-dependent adenylate cyclase. Lethal factor, the dominant virulence factor associated with the toxin, proteolytically inactivates mitogen-activated protein kinase kinases, key players in signal transduction. We describe the fascinating work that has led to these discoveries and discuss their relevance to our understanding of the pathogenesis of anthrax.

Key words. Anthrax; mitogen-activated protein kinases; bacterial pathogens; edema factor; lethal factor.

The identification of anthrax toxins

Bacillus anthracis is a large Gram-positive bacterium responsible for the disease anthrax. Though its spores cannot usually infect healthy tissues, it can gain entry into a host via an abrasion or cut [1]. Left untreated, the bacteria may proliferate, become invasive, spread to the lymph nodes, and promote a systemic infection that rapidly leads to death of the host. The disease can be induced experimentally in animals by injecting spores subcutaneously. In such animals, lesions form at the site of injection, characterized by a swelling in the skin and subcutaneous tissues as they are infiltrated by a pale gelatinous exudate [2]. The skin in the central portion of the lesion later becomes necrotic, forming a blackened eschar from which anthrax derives its name (Gr. *anthrakos*, coal). Susceptibility to infection by anthrax varies from species to species with monkeys, mice, guinea pigs, and rabbits being less resistant and dogs, rats, and swine being relatively resistant to infection. Thus, the LD_{50} for subcutaneous injections into mice or guinea pigs is on the order of 10–30 spores, whereas that in rats or dogs is greater than 10^5 spores [1]. Resistance to experimental infection by spores may be related to the host's ability to respond to invasion, since Cromartie et al. [2] noted that in resistant animals, the site of infection becomes infiltrated with large numbers of leukocytes, whereas in susceptible animals, only a small number of leukocytes are present. In either case, many of the infiltrating leukocytes were observed to be necrotic, suggesting that these cells may be targets of anthrax activity.

The mechanism by which anthrax kills its host is as yet unknown. Some evidence suggested *B. anthracis* may produce a toxin. Subcutaneous injections of sterile crude extracts of skin lesions resulted in the production

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of similar lesions [3] and crude extracts of bacteria had been found to contain 'aggressions,' so called because, when mixed with sublethal doses of spores, they could cause death and in vitro were found to inhibit phagocytotic activities of polymorphonuclear leukocytes [4]. However, analyses of blood cultures of infected rabbits [5] and guinea pigs [6] indicated that levels of bacteria in the blood rise steadily until the final hours of life when they undergo a marked increase, reaching levels of 10^7 and 10^9 organisms/ml of blood, respectively. This, coupled with repeated failures by many to identify lethal endo- or exotoxins associated with the bacteria or the fluids of an infected animal [4] led early workers to hypothesize that death was a consequence of mechanical obstruction caused by such a large number of organisms. Paradoxically, Bloom et al. [5] found in their analysis of blood cultures of infected rabbits that approximately 15% of injected animals had less than 10^3 bacteria/ml at the time of death.

This enigma led Smith and Keppie [6] to reexamine whether *B. anthracis* causes death through overproliferation. When they measured the number of bacteria in the blood of infected guinea pigs they found that it increased from approximately 10^6 to 10^9 bacteria/ml in the 12 h preceding death. By treating infected guinea pigs with streptomycin, they were able to prevent bacteremia and if the antibiotic was administered before the number of bacteria increased beyond 10^7/ml, then the animals could be saved. Interestingly, if streptomycin was administered after this critical threshold, then death was invariably the outcome. These results clearly indicated that bacteremia was essential for death, but they also showed that the fate of the infected animals was determined when the number of bacteria present in the blood was two orders of magnitude lower than at the time of death (a point which will be returned to later). Thus, mechanical blockage could not adequately explain why infected animals died. Rather, a chain of events set in motion at an earlier stage of the infection led to death independent of bacterial proliferation.

Smith and Keppie [6] then went on to demonstrate that sterile-filtered serum derived from infected guinea pigs could induce edema, when injected subcutaneously, or death when injected intravenously. Thus, the deadly effects of anthrax were in fact mediated by an exotoxin. They attributed previous failures to demonstrate the existence of an exotoxin to the higher concentration of toxin present in plasma versus peritoneal exudates, which they had previously used as a source of the toxin, and to the greater effectiveness of intravenous as opposed to intraperitoneal injection of the toxin.

A preliminary characterization of anthrax toxin suggested that it consisted of two components, since the addition of anti-serum raised to a previously identified immunizing agent (protective antigen, PA) [7] could prevent the production of edema and abolish its killing power. Since PA by itself was non-toxic, it followed that it must associate with a second, toxic component. This conclusion was later confirmed when Smith et al. [8] found that if they ultra-centrifuged plasma from infected guinea pigs, the lethal activity of the resulting fractions was diminished when compared to the original plasma preparation. However, if the supernatant was mixed with the pellet then the toxic activity was restored. The pellet and the supernatant, therefore, contained two components, named factors I and II, respectively, which synergistically united to form anthrax toxin. Interestingly, PA was also synergistic with preparations of factor I, though not to the same extent as factor II, suggesting that it may be related to, or a component of factor II. The later development of media and techniques that allowed the production of toxin in vitro [9, 10] greatly aided the subsequent purification and characterization of each of these factors [11–13].

In an elegant series of experiments, Stanley and Smith [14] found that while crude preparations of factor I plus purified factor II or purified factor I plus crude factor II caused skin edema in rabbits and were toxic when injected into mice, purified factor I plus purified factor II could cause skin edema but was not lethal. Thus, a third factor, or factor III, was lost during purification. In fact, factor III was found to be retained on a chromatographic column during purification of factor I as well as in fractions discarded during the purification of factor II. Factor III alone or in combination with factor I was shown to be non-toxic. However, when combined with factor II it was toxic for mice but produced no skin edema in rabbits. Preparations consisting of all three factors were synergistic for toxicity but produced edema of decreased size, indicating that factor III could competitively inhibit factor I in the production of edema. Shortly thereafter, similar results were obtained by Beall et al. [15] using different methods of purification.

Although these three factors have come to be equated with anthrax toxins, it is noteworthy that toxin produced in vivo differs from that produced and purified from cultures of *B. anthracis* in vitro in that it causes death more rapidly [16, 17]. Thus, additional factors produced during the course of infection may enhance the lethal effects of anthrax toxins.

In the years following this work, the previous terminology was supplanted by another to reflect the activities of these factors; factor I became known as edema factor (EF), factor II came to be equated with PA, and factor III was referred to as lethal factor (LF). More recently, the combination of EF plus PA has been referred to as edema toxin (EdTx), and that of LF plus PA as lethal toxin (LeTx) [18].

The components of anthrax toxins

Protective antigen

PA (Swiss Prot accession number P13423, Gen Bank accession number M22589), previously referred to as factor II, is the most extensively characterized component of anthrax toxin. The gene for PA is encoded at the *pag* locus on the plasmid pXO1 (formerly known as pBA1) [19]. The gene has been cloned [20] and sequenced [21] and found to contain a 2319-bp-long open reading frame of which 2205 bp encode an A/T-rich (69%) cysteine-free, 735-amino-acid (83-kDa) secreted protein.

Although PA is a component of anthrax toxin, by itself it is not toxic (see above). The earliest indication of its function came from a series of experiments performed by Molnar and Altenbern [22] using partially purified toxin components. They noted that PA was removed from the circulation within 1–2 h of its injection into Fischer rats. By contrast, LF remained in the bloodstream for at least 4 h following its injection. If LF or PA + LF was injected shortly after PA then the injected animal died within 70 min. However, if LF or PA + LF were injected 1 h after PA, then the time to death after injection of the toxic component was delayed. Moreover, if LF was injected more than 2 h after PA, it exhibited no toxicity. A similar result was obtained if PA + LF was injected 4 h after PA. These results indicate that PA is bound and sequestered by a component of the host tissues and that, to exert its toxic effects, LF must interact with PA prior to its sequestration. This mode of action closely resembles that of A-B binary toxins in which the component having the enzymatic

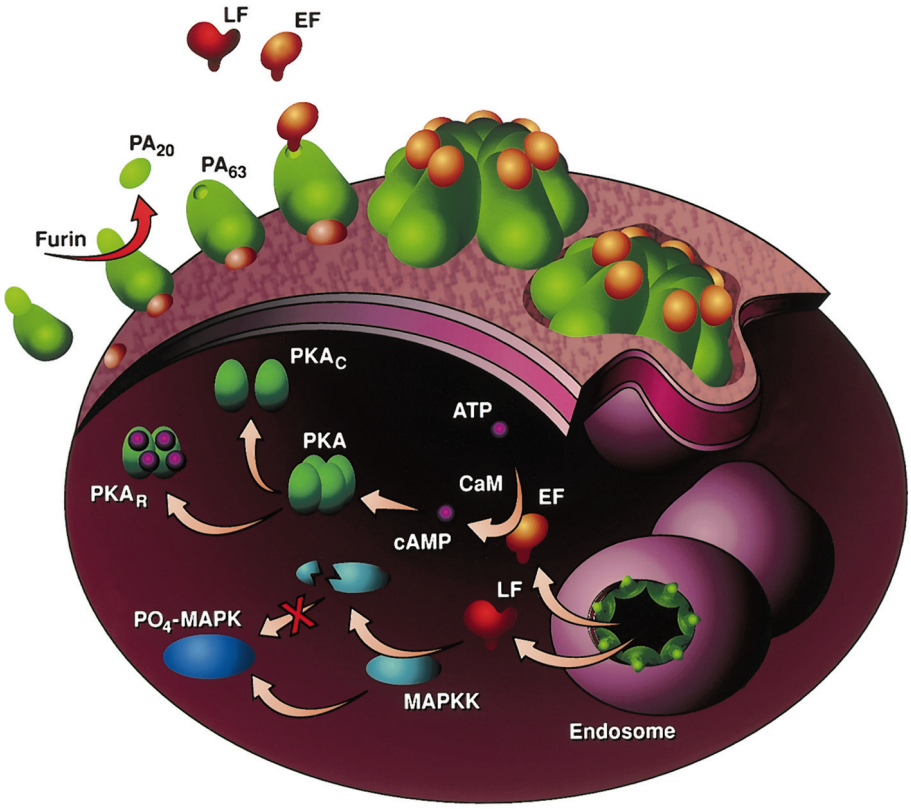

Figure 1. An outline of anthrax toxin activity. Protective antigen (PA) binds an unidentified, cell surface receptor where it is proteolytically modified by a furin-like protease leaving PA_{63} still bound to the cell. This allows PA_{63} to heptamerize and bind either edema factor (EF) or lethal factor (LF). Entry of this complex into the cell is mediated by the endosomal pathway. Exposure of PA_{63} to the acidic endosomal environment causes it to undergo a conformational change, resulting in the formation of a channel through which EF and LF may gain entry to the cytoplasm. EF is a Ca^{2+}/calmodulin (CaM)-dependent adenylate cyclase that rapidly converts ATP to cyclic AMP (cAMP). One of the principal cellular roles of cAMP the regulation of protein kinase A (PKA) activity. Inactive PKA is a tetramer composed of two regulatory (PKA_R) and two catalytic (PKA_C) subunits. The binding of four cAMP molecules to the regulatory subunits causes dissociation of the PKA tetramer and consequently the activation of PKA_C. By contrast, LF proteolytically inactivates mitogen-activated protein kinase (MAPK) kinase (MAPKK), thereby preventing the activation of the MAPK pathway.

activity ('A' domain) gains entry to the cytosol via a cell-binding component ('B') [23]. Thus, PA is envisioned to bind a cell surface receptor and translocate LF (or EF; see below) to the cytosol (fig. 1). Direct evidence for this hypothesis was not obtained until 1982 when Leppla [24] showed that PA was required for entry of EF into the cytosol of Chinese hamster ovary cells.

Before it is able to translocate EF or LF to the cytosol, PA must first be proteolytically modified. Cleavage occurs at the sequence $RKKR^{167}$, resulting in the removal of a 20-kDa fragment, likely by furin or a similar cell-surface-associated protease [25, 26], yielding PA_{63}. Deletions of or mutations at this site render PA resistant to proteolysis and consequently non-toxic in combination with LF or EF [25, 27]. Removal of the NH_2-terminal fragment is apparently necessary to expose a region of PA which can bind to the other toxin components, since uncleaved PA can bind the cell but is unable to associate with EF or LF [25, 27]. Analysis of COOH-terminal truncations of PA_{63} indicate that the COOH terminus is necessary for cell binding [28]. Similarly, using fragments of PA generated by chymotrypsin and trypsin treatment, Novak et al. [29] were also able to show that the COOH terminus of PA was necessary for cell binding.

PA_{63} can form cation-selective channels in planar phospholipid bilayers whereas intact PA cannot [30]. Observations by non-denaturing gel electrophoresis [31] and electron microscopy [32] indicate that seven molecules of PA_{63} are required to form each channel (32). More recently, X-ray crystallographic analysis of PA has shown that heptamerization is sterically blocked until the removal of the NH_2-terminal 20-kDa fragment [33].

Several experiments indicate that PA mediates entry of LF and EF into the cell via the endosomal pathway. Friedlander [18] showed that by pre-exposing mouse peritoneal macrophages to agents which can dissipate intracellular proton gradients (such as ammonium chloride), it was possible to render them resistant to LeTx. This effect was not caused by a block in toxin internalization, since bound PA was not accessible to antibodies. Subsequent exposure of these cells to an acidic environment could reverse this inhibition. Furthermore, the uptake of EF by cells can be blocked by treatment with the microfilament inhibitor cytochalasin D and this effect may also be overcome via acidification [34]. Thus, it may be concluded that LeTx must pass through an acidic, endocytic vesicle to exert its toxic effects within the cytosol.

Edema factor

EF (Swiss Prot accession number P40136; Gen Bank accession number M24074), previously referred to as factor I, is encoded at the *cya* locus on the pX01 plasmid [19]. The gene has been cloned [35, 36] and sequenced [37] and found to contain a 2400-bp-long open reading frame of which 99 bp encode a hydrophobic 33-amino-acid signal sequence, and 2301 bp encode an A/T-rich (71%) cysteine-free, 767-amino-acid (88.8-kDa) mature secreted protein.

Sequence analysis of EF indicates that residues 1–250 at the NH_2 terminus share homology with the corresponding region of LF. This region of LF has been demonstrated to mediate binding to PA [38] and when fused to heterologous proteins is sufficient to allow their translocation to the cytosol via PA [38].

EF was the first component of anthrax toxin that was shown to have an enzymatic activity. Leppla [24] noted that cholera toxin (CT), which activates eukaryotic adenylate cyclase by ADP-ribosylation, can cause the formation of skin edema similar to that formed by EdTx when injected subcutaneously into rabbits [39]. The effects of CT are readily assayed in CHOK1A cells that undergo characteristic morphological changes shortly after toxin treatment [40]. Leppla [24] observed that EF plus PA induced a similar morphological response. This could be competitively inhibited by the addition of LF [24], reflecting the fact that EF and LF bind the same site on PA. Direct measurement of cAMP in the cells indicated that the addition of EF plus PA could cause as much as a 200-fold increase over non-stimulated levels and that this increase could be prevented by the simultaneous addition of an excess of LF. However, unlike CT, EF appeared not to ADP-ribosylate adenylate cyclase, but was instead found to possess adenylate cyclase activity [24]. In vitro, purified EF has no activity. However, the addition of nanomolar levels of calmodulin can activate EF in a Ca^{2+}-dependent fashion [41].

Analysis of deletion mutants of EF indicate that the first 261 NH_2-terminal residues are dispensable for calmodulin binding and activity [42]. These results are in accord with sequence analyses, which show that residues 265–570 of EF share homology with the catalytic domains of adenylate cyclases of *Bordatella pertussis* [43] and *Bordatella bronchiseptica* [44].

Lethal factor

LF (Swiss Prot accession number P15917; Gen Bank accession numbers M29081, M30210), previously known as factor III, is encoded on the pXOI plasmid [19]. The gene, called *lef*, has been cloned [45] and sequenced [46], and found to contain a 2427-bp open reading frame of which 99 bp encode a 33-amino-acid signal peptide and 2328 bp encode an A/T-rich (70%), cysteine-free, 776-amino-acid (90.2-kDa) mature, secreted protein.

The LF protein may be divided into three domains. As discussed earlier, the NH_2 terminus of LF shares sequence homology with that of EF. Arora and Leppla [38] have demonstrated that amino acids 1–254 are sufficient to mediate the entry of fused proteins of different origins via PA, regardless of whether this region is fused at the NH_2 or COOH terminus. Following the PA-binding region, there is a series of five imperfect repeats of 19 amino acids each [47]. The remaining portion of LF shows no sequence homology with known proteins, a fact which hindered the elucidation of the activity of LF for some time.

Studies of LF activity rely, for the most part, upon an in vitro assay developed by Friedlander [18], who found that several macrophage-derived cell lines were particularly sensitive to LeTx and underwent lysis following its application. Different cell lines display different sensitivities to LeTx. For instance, it causes the lysis of J774A.1 cells, but not IC21 cells [48]. Each of these cell lines can bind PA and proteolytically modify it to PA_{63}, but when LF is directly introduced to the cytoplasms of each cell line by osmotic lysis of pinosomes, only J774A.1 cells lyse [48]. This result has two implications. First, it shows that neither binding of PA nor processing through the endosomal pathway is necessary for LF activity. This was later confirmed when it was shown that the addition of LF to the cytoplasm by Sendai virus envelope fusion [49] or by microinjection, or its addition directly to cytoplasmic extracts is sufficient for its full activity [50]. Secondly, the result suggests that the cell line differences in susceptibility lie downstream of LF entry into the cell and may reflect a differing sensitivity to the enzymatic activity of LF. Similar observations have been made with macrophages isolated from LeTx-sensitive or -insensitive mice [51].

A preliminary determination of the functional domains of the LF protein was performed by Quinn et al. [52] using insertional mutagenesis and the macrophage lysis assay. As expected, they found that insertions in the NH_2 terminus reduced PA-binding activity. Insertions in the repeat region yielded unstable proteins that could not be used for further analyses. However, insertions in the carboxy terminus eliminated toxicity without altering PA binding, suggesting that the catalytic regions are located at the COOH terminus. Careful analysis of the amino acid sequence at the carboxy terminus revealed that amino acids 686–692 (HEFGHAV) contained a motif characteristic of metalloproteases (HEXXH, where X is any amino acid) [53]. The hypothesis that LF was a metalloprotease was supported by observations that (i) protease inhibitors such as bestatin, and chloromethyl ketones of leucine and phenylalanine prevented LeTx induced lysis of J774A.1 cells [53], (ii) LF was found to bind approximately one [53, 54] or more [55] ^{65}Zn atoms per molecule, (iii) substitution of alanine for potential zinc-binding residues histidines 686 or 690, reduced zinc binding as well as toxicity of LF [53], and (iv) substitution of cysteine for glutamine 687 (E687C), an amino acid that forms part of the catalytic site, reduced LF toxicity but not zinc binding [53].

Despite the suggestive hint that LF might be a metalloprotease, the enzymatic substrate of LF remained unknown for several years. Then in 1998, Duesbery et al. [50], and later Vitale et al. [56], identified mitogen-activated protein kinase (MAPK) kinases 1 and 2 (MAPKK1, 2) as substrates for LF.

The MAPK pathway is a key regulatory signal transduction pathway present in eukaryotic cells. Typically, in such pathways, ligand binding at the cell surface initiates a signal, which is then relayed to intracellular effector molecules via a series of phosphorylation reactions [reviewed in 57]. One pathway that activates MAPK was outlined years ago in frog oocytes [58]. Oocytes of frogs undergo oogenesis and initiate meiosis while in the ovaries, but then arrest at prophase of the first meiotic division. Before they may be fertilized they must 'mature' into eggs, i.e., complete the first meiotic division and then arrest again at metaphase of the second meiotic division. This series of events is triggered by the hormone progesterone, which binds an as yet unidentified cell surface receptor [59, 60]. Receptor stimulation leads to the polyadenylation [61–63] and translation of mRNA encoding the *c-mos* proto-oncogene [64]. Mos is a serine/threonine kinase [65] that phosphorylates and activates MAPKK1 (Mek1) at Ser^{218} and Ser^{222} [66–69]. MAPKK1 in turn activates MAPK2 by phosphorylation at Thr^{188} and Tyr^{190}. In frog oocytes the activation of MAPK2 ultimately promotes the activation of $p34^{cdc2}$/cyclin B, otherwise known as maturation-promoting factor (MPF), the key regulator of the transition from prophase to metaphase. Recent evidence suggests that MAPK2 does so by phosphorylating and activating $p90^{rsk}$ [70], which in turn inhibits the activity of Myt1, a negative regulator of MPF [70, 71].

In addition to its role in MPF activation during meiosis, the MAPK pathway has been demonstrated to play additional roles in cell cycle progression. MAPK activation plays a role in G_1 progression [72–74] as well as in both meiotic and mitotic M phase where it is hypothesized to be involved in spindle assembly [75–83], and regulation of the metaphase to anaphase transition [84–89]. Given its key roles in cell division, it is perhaps not surprising that MAPK activation also plays roles in tumorigenesis. Thus, constitutively active MAPKK1 or 2 can transform mammalian cells [90, 91] and are essential for *ras*-mediated experimental metastasis [92].

Multiple variations of this signaling pathway are present in all eukaryotic cells. Thus far, 7 different MAPKKs and 11 different MAPKs have been iden-

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MAPKK1  .......... ....PKKKPT ..PIQLNPA. PDGSAVNGTS SAETNLEALQ KKLEELELDE QQRKRLEAFL TQKQKVG
MAPKK2  .......... ...LARRKPV LPALTINPTI AEGPSPTSEG ASEANLVDLQ KKLEELELDE QQKKRLEAFL TQ.....
MAPKK3  .......... ........SK PPAPNPTPPR NLDSRTFITI GDRNFEVEAD DLVTISELGR GAYGVVEKVR HAQSGTI
MAPKK4  .......... .......AAP SPSGGGGSGG GSGSGTPGPV GSPAPGHPAV SSMQGKRKAL KLNFANPPFK STARFT.
MAPKK5  .......... ...LWLALGP FPAME.NQVL VIRIKIPNSG AVDWTVHSGP QLLFRDVLDV IGQVLPEATT TAF....
MAPKK6  SQSKGKKRNP GLKIPKEAFE QPQTSSTPPR DLDSKACISI GNQNFEVKAD DLEPIMELG. .......... .......
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Figure 2. NH_2-terminal alignment of human MAPKK1–6. The 60 NH_2-terminal amino acids of human MAPKK 1–6 were aligned using the MULTALIN alignment program (accessable via the Internet at http://w3.toulouse.inra.fr/multalin). Conserved elements found proximal to the identified cleavage sites in MAPKK1 and 2 are indicated in black (proline residues) and gray (basic residues).

tified. Functional separation of the components of these pathways in mammalian cells is complicated by their extensive interactions. Elements of each pathway are retained in 'modules' that are coordinated by scaffolding proteins such as MP1 or JIP1 [93–95]. Typically, stimulation at the cell surface activates a module, causing translocation of the MAPK component to the nucleus where it regulates the activities of various transcription factors. However, it should be kept in mind that MAPKs also act at a post-translational level since translation is not required for oocyte maturation. When LF was injected into oocytes, their maturation in response to progesterone was prevented. Analysis of oocyte extracts indicated that LF prevented the activation of MAPK, suggesting that it acted 'upstream' in the pathway [50]. Similar results were obtained for MAPK activity in transformed NIH3T3 cells. However, Vitale et al. [56] have reported that treatment of J774A.1 macrophages with LeTx leads to the transient phosphorylation of MAPK. The reasons for this discrepency are not apparent.

Subtle changes in the electrophoretic mobility of MAPKK from both oocytes and transformed NIH3T3 cells suggested that LF may proteolytically modify its NH_2 terminus. This hypothesis was confirmed using an in vitro approach with purified, recombinant proteins [50]. Subsequent analysis indicated that LF removed residues 1–7 of MAPKK1 (PKKKPTP) [50]. Similarly, LF was found to cleave MAPKK2 between residues 9 and 10, resulting in the loss of NH_2-terminal residues LARRKPVLP [50]. These results were independently confirmed shortly thereafter by Vitale et al. [56] using a yeast two-hybrid approach. Apparently, the removal of this short sequence is sufficient to inactivate MAPKK1 since recombinant MAPKK1 lacking the same seven amino acids also lacked activity [50].

A comparison of the NH_2-terminal sequences of MAPKK1 and 2 shows each contains two proline residues that are interspersed by one or two amino acids and preceded by a series of basic residues (fig. 2). The importance of the basic residues in substrate recognition has not yet been determined. However, site-directed mutagenesis at the cleavage site indicates that both prolines play an important role in cleavage site recognition, since mutation of either Pro^5 or Pro^7 to Ala renders MAPKK1 resistant to cleavage [50] while the combined mutation of both residues to Ala prevents its cleavage. The importance of proline residues at the cleavage site was underscored by Hammond and Hanna [96] who, at the same time, found that LF preferentially acted upon proline-containing peptides. It is interesting to note that in their yeast two-hybrid assay, Vitale et al. [56] identified a cDNA for MAPKK2, which encoded amino acids 31–400, a sequence that lies outside the cleavage site. Thus, additional elements will likely play a role in LF substrate recognition.

Further comparison of the amino acid sequences of MAPKK1 and 2 to those of MAPKK3–6 (fig. 2) shows that there is limited homology between these proteins at their NH_2 termini. However, each contains a pair of proline residues separated by zero to four amino acids, while MAPKK1, 2, 3, and 6 also contain a series of basic residues preceding the prolines. Thus, it is conceivable that additional members of this protein family besides MAPKK1 and 2 may also serve as substrates for LF. MAPKK3, 4, and 6 have been implicated in regulation of p38 (HOG) MAPK [97, 98], which mediates cellular response to stimuli such as osmotic shock or cytokines [99–102] and may also play a role in monitoring spindle assembly during mitosis [103]. MAPKK4 regulates the activity of stress-activated protein kinase (SAPK), which in turn regulates jun [98], while MAPKK5 regulates the activity of erk5 [104] and is activated in response to oxidative stress, hyperosmolarity, and serum treatment [105, 106]. However, the close similarities of the activity profiles of LeTx and PD098059 in the National Cancer Institute's anti-neoplastic drug screen [50] indicate that in tumor cells, the predominant target of LF activity is MAPKK1.

Pathogenicity, a molecular understanding

Despite our detailed understanding of the anthrax toxin, we are unable to fully explain its pathogenicity. It

is known that anthrax LeTx is the dominant virulence factor produced by *B. anthracis*. This was demonstrated by Mock and colleagues [107–109] who showed that strains of *B. anthracis* deficient in the production of LF or PA are not lethal in mice, whereas strains deficient in the production of EF are. However, this should not be interpreted as an indication that EF does not play a role in the pathogenesis of anthrax. Indeed, EdTx has been found to inhibit phagocytosis of opsonized, avirulent *B. anthracis* by polymorphonuclear neutrophils [110] and more generally, elevated cAMP levels have long been noted to inhibit macrophage phagocytosis [111, 112]. Thus, EdTx might be expected to promote bacterial survival during the early stages of infection. Furthermore, Mock's EF- and LF-deficient strains were less able to induce lethality and edema, respectively, than the parental EF/LF-producing strain, a finding which is in agreement with an earlier study [14, 113], and which suggests that EdTx and LeTx may act synergistically. This is perhaps not surprising since elevated cAMP levels have often been noted to antagonize MAPK activation. For instance, following progesterone treatment in frog oocytes, cAMP levels undergo a rapid, transient decrease [114–117]. The resulting decrease in cAMP-dependent protein kinase A (PKA) activity is necessary for the synthesis of Mos protein and the activation of MAPK [118–121]. Similar observations of interaction between PKA and MAPK have been made in a variety of cell types [e.g., 122–126].

During the course of infection, the levels of bacteria in the blood rise steadily until a few hours before death when the numbers of bacteria increase dramatically [5, 6]. Detectable levels of toxin in the blood are not found until late in the infection and appear to be proportional to the number of bacteria present [17]. Physiological observations of the response of a variety of species to infection show few commonalities except that immediately prior to death there is a marked anoxia and respiratory failure [summarized in ref. 17). These observations suggest that, up to a point, the host is able to hold the infection in check. However, hours prior to death, the host undergoes a crisis in which this ability is lost. What follows is a massive bacteremia that results from unabated bacterial proliferation and ultimately respiratory failure. It is important to note that at the time of this crisis, the fate of the host has already been determined since removal of the bacteria will not prevent its death. What, then, has occurred at this crisis point?

The principle target of LeTx is presumed to be macrophages. The evidence for this is as follows. As noted earlier, histopathology of skin lesions induced by injection of spores shows evidence of infiltration with leukocytes, many of which appear necrotic [2]. Also, as first demonstrated by Friedlander [18], macrophages and certain macrophage-derived cell lines abruptly lyse upon treatment with LeTx. The threshold amount of toxin required to lyse murine macrophages (approximately 0.1–1 μg/ml PA, 0.01–0.1 μg/ml LF) [18, 48, 51] is comparable to what one might expect to find in a dying mouse, based upon an estimated LD_{50} of 11 μg PA and 2.2 μg LF [127] and a blood volume of 3–5 ml per mouse. Finally, Hanna et al. [128] have found that mice depleted of macrophages by repeated injections of silica become resistant to LeTx. Susceptibility to the toxin may be restored by the injection of an LeTx-sensitive macrophage-derived cell line but not an LeTx-resistant cell line. These results would seem to indicate that a substance produced by macrophages in response to LeTx and released upon their destruction may be the cause of host death. The destruction of the host's macrophages may form the physiological basis of the crisis mentioned above. In this regard it is extremely important to determine the fate of macrophages during an in vivo infection.

As noted earlier, different animals show varying degrees of susceptibility to anthrax spores. Interestingly, the amount of toxin present in the blood at the time of death (or the amount of injected toxin required for death) appears to be inversely correlated with the ability of that animal to resist infection by spores [reviewed in ref. 17). Thus, guinea pigs, which are relatively susceptible to infection, were found to contain 50 units of toxin/ml blood at the time of death (lethal injection = 1000 units/kg), whereas Fischer rats that are relatively resistant to infection were found to contain less than 8 units of toxin (lethal injection = 15 units/kg) [see ref. 17 for a review]. Similarly, inbred strains of mice, which die quickly following injection of anthrax spores, are relatively resistant to the LeTx, while spore-resistant mice are sensitive to LeTx [127]. These differences are also apparent at the cellular level, as macrophages derived from spore-resistant, toxin-sensitive mice lyse at lower concentrations of LeTx than those derived from spore-sensitive, toxin-resistant mice [[51]]. These observations suggest that macrophages may play a dual role in anthrax infection. Animals resistant to anthrax infection mount a stronger primary defense at the onset of infection. However, this strength will later prove to be the animals' weakness, as lower levels of anthrax toxins will induce lysis of macrophages and release of toxic substances.

Hanna et al. [129] found that cultured macrophages treated with LeTx release superoxide anion at about the time that cell lysis begins, and that macrophage lysis could be prevented by the addition of anti-oxidants. When LeTx resistant cell lines were examined, they were found to be deficient in the production of reactive oxygen intermediates (ROIs). Thus, these observations suggest that the production of ROIs may be involved in the lysis of macrophages.

What is the nature of this toxic substance(s) that is released by macrophages upon their lysis? Noting the similarities between systemic shock caused by anthrax and lipopolysaccharide-mediated bacterial sepsis, Hanna et al. [128] have suggested that the host's response may be a result of cytokine overexpression. In support of this, they have found that treatment of macrophages with sub-lytic doses of LeTx will induce the expression of interleukin (IL)-1 and tumor necrosis factor (TNF-α) in vitro and that passive immunization against IL-1 or injection of human IL-1 receptor into mice protected them from the effects of LeTx. TNF-α was found to be released from the macrophages while IL-1 was retained. Hanna [54] hypothesized that during an infection, IL-1β and other cytokines might accumulate to high concentrations within macrophages at sublytic levels of LeTx, and then when the concentration of toxin reaches a maximum, the macrophages lyse, resulting in a massive release of cytokines into the body.

It is not immediately evident how inactivation of any of the MAPKKs might lead to the pathogenesis observed during anthrax infection. In addition to its role in cell division, MAPK has been suggested to function in immunological responses. Thus, lipopolysaccharide has also been shown to induce activation of the MAPK pathway [130–133]. More recently, activation of the MAPK pathway has been implicated to be essential for phagocytosis in macrophages [134]. Proteolytic inactivation of MAPKK by LF would thus be expected to inhibit the ability of macrophages to defend against bacterial infection. Such an activity may play an important early role in establishing an infection by promoting bacterial proliferation; however, its importance at later stages remains to be demonstrated.

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1 Young G. A. Jr, Zelle M. R. and Lincoln R. E. (1946) Respiratory pathogenicity of *Bacillus anthracis* spores. I. Methods of study and observations on pathogenesis. J. Infect. Dis. **79:** 233–246
2 Cromartie W. J., Bloom W. L. and Watson D. W. (1947) Studies on infection with *Bacillus anthracis*. I. A histopathological study of skin lesions produced by *B. anthracis* in susceptible and resistant animal species. J. Infect. Dis. **80:** 1–13
3 Cromartie W. J., Watson D. W., Bloom W. L. and Heckly R. J. (1947) Studies on infection with *Bacillus anthracis*. II. The immunological and tissue damaging properties of extracts prepared from lesions of *B. anthracis* infection. J. Infect. Dis. **80:** 14–27
4 Keppie J., Smith H. and Harris-Smith P. W. (1953) The chemical basis of the virulence of *Bacillus anthracis*. II. Some biological properties of bacterial products. Br. J. Exp. Pathol. **34:** 486–496
5 Bloom W. L., McGhee W. J., Cromartie W. J. and Watson D. W. (1947) Studies on infection with *Bacillus anthracis*. VI. Physiological changes in experimental animals during the course of infection with *B. anthracis*. J. Infect. Dis. **80:** 137–144
6 Smith H. and Keppie J. (1954) Observations on experimental anthrax: demonstration of a specific lethal factor produced in vivo by *Bacillus anthracis*. Nature **173:** 869–870
7 Gladstone G. P. (1946) Immunity to anthrax: protective antigen present in cell-free culture filtrates. Br. J. Exp. Pathol. **27:** 394–418
8 Smith H., Tempest D. W., Stanley J. L., Harris-Smith P. W. and Gallop R. C. (1956) The chemical basis of the virulence of *Bacillus anthracis*. VII. Two components of the anthrax toxin: their relationship to known immunising aggressins. Br. J. Exp. Pathol. **37:** 263–271
9 Harris-Smith P. W., Smith H. and Keppie J. (1958) Production in vitro of the toxin of *Bacillus anthracis* previously recognized in vivo. J. Gen. Microbiol. **19:** 91–108
10 Thorne C. B., Molnar D. M. and Strange R. E. (1960) Production of toxin in vitro by *Bacillus anthracis* and its separation into two components. J. Bacteriol. **79:** 450–455
11 Stanley J. L., Sargeant K. and Smith H. (1960) Purification of factors I and II of the anthrax toxin produced in vivo. J. Gen. Microbiol. **22:** 206–218
12 Sargeant K., Stanley J. L. and Smith H. (1960) The serological relationship between purified preparations of factors I and II of the anthrax toxin produced in vivo and in vitro. J. Gen. Microbiol. **22:** 219–228
13 Fish D. C., Klein F., Lincoln R. E., Walker J. S. and Dobbs J. P. (1968) Pathophysiological changes in the rat associated with anthrax toxin. J. Infect. Dis. **118:** 114–124
14 Stanley J. L. and Smith H. (1961) Purification of factor I and recognition of a third factor of the anthrax toxin. J. Gen. Microbiol. **26:** 49–66
15 Beall F. A., Taylor M. J. and Thorne C. B. (1962) Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. J. Bacteriol. **83:** 1274–1280
16 Fish D. C. and Lincoln R. E. (1968) In vivo-produced anthrax toxin. J. Bacteriol. **95:** 919–924
17 Lincoln R. E. and Fish D. C. (1970) Anthrax toxin. In: Microbial Toxins, vol. 3, pp. 361–414, Montie T. C., Kadis S. and Ajl S. J. (eds), Academic Press, New York
18 Friedlander A. M. (1986) Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. J. Biol. Chem. **261:** 7123–7126
19 Mikesell P., Ivins B. E., Ristroph J. D. and Dreier T. M. (1983) Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. **39:** 371–376
20 Vodkin M. H. and Leppla S. H. (1983) Cloning of the protective antigen gene of *Bacillus anthracis*. Cell **34:** 693–697
21 Welkos S. L., Lowe J. R., Eden-McCutchan F., Vodkin M., Leppla S. H. and Schmidt J. J. (1988) Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene **69:** 287–300
22 Molnar D. M. and Altenbern R. A. (1963) Alterations in the biological activity of protective antigen of *Bacillus anthracis* toxin. Soc. Exp. Biol. Med. **114:** 294–297
23 Gill D. M. Seven toxin peptides that cross cell membranes. (1978) In: Bacterial Toxins and Cell Membranes, pp. 291–332, Jeljaszewicz J. and Wadstrom T. (eds), Academic Press, New York

24 Leppla S. H. (1982) Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. USA **79:** 3162–3166

25 Molloy S. S., Bresnahan P. A., Leppla S. H., Klimpel K. R. and Thomas G. (1992) Human furin is a calcium-dependent serine endoprotease that recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. J. Biol. Chem. **267:** 16396–16402

26 Klimpel K. R., Molloy S. S., Thomas G. and Leppla S. H. (1992) Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc. Natl. Acad. Sci. USA **89:** 10277–10281

27 Singh Y., Chaudhary V. K. and Leppla S. H. (1989) A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action in vivo. J. Biol. Chem. **264:** 19103–19107

28 Singh Y., Klimpel K. R., Quinn C. P., Chaudhary V. K. and Leppla S. H. (1991) The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. J. Biol. Chem. **266:** 15493–15497

29 Novak J. M., Stein M.-P., Little S. F., Leppla S. H. and Friedlander A. M. (1992) Functional characterization of protease-treated *Bacillus anthracis* protective antigen. J. Biol. Chem. **267:** 17186–17193

30 Blaustein R. O., Koehler T. M., Collier R. J. and Finkelstein A. (1989) Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. Proc. Natl. Acad. Sci. USA. **86:** 2209–2213

31 Leppla S. H. (1991) The anthrax toxin complex. In: Sourcebook of Bacterial Protein Toxins, pp. 277–302, Freer A. A. (ed.), Academic Press, London

32 Milne J. C., Furlong D., Hanna P. C., Wall J. S. and Collier R. J. (1994) Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J. Biol. Chem. **269:** 20607–20612

33 Petosa C., Collier R. J., Klimpel K. R., Leppla S. H. and Liddington R. C. (1997) Crystal structure of the anthrax toxin protective antigen. Nature **385:** 833–838

34 Gordon V. M., Leppla S. H. and Hewlett E. L. (1988) Inhibitors of receptor-mediated endocytosis block the entry *Bacillus anthracis* adenylate cyclase toxin but not that of *Bordetella pertussis* adenylate cyclase toxin. Infect. Immun. **56:** 1066–1069

35 Mock M., Labruyere E., Glaser P., Danchin A. and Ullmann A. (1988) Cloning and expression of the calmodulin-sensitive *Bacillus anthracis* adenylate cyclase in *Escherichia coli*. Gene **64:** 277–284

36 Tippetts M. T. and Robertson D. L. (1988) Molecular cloning and expression of the *Bacillus anthracis* edema factor toxin gene: a calmodulin-dependent adenylate cyclase. J. Bacteriol. **170:** 2263–2266

37 Robertson D. L., Tippetts M. T. and Leppla S. H. (1988) Nucleotide sequence of the *Bacillus anthracis* edema factor gene (*cya*): a calmodulin-dependent adenylate cyclase. Gene **73:** 363–371

38 Arora N. and Leppla S. H. (1994) Fusions of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. Infect. Immun. **62:** 4955–4961

39 Craig J. P. (1965) A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature **207:** 614–616

40 Johnson G. S., Friedman R. M. and Pastan I. (1971) Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine-3′:5′-cyclic monophosphate and its derivatives. Proc. Natl. Acad. Sci. USA **68:** 425–429

41 Leppla S. H. (1984) *Bacillus anthracis* calmodulin-dependent adenylate cyclase: chemical and enzymatic properties and interactions with eucaryotic cells. In: Advances in Cyclic Nucleotide and Protein Phosphorylation Research, vol. 17, pp. 189–198, Greengard P. (ed.), Raven, New York

42 Labruyere E., Mock M., Ladant D., Michelson S., Gilles A.-M., Laoide B. et al. (1990) Characterization of ATP and calmodulin-binding properties of a truncated form of *Bacillus anthracis* adenylate cyclase. Biochemistry **29:** 4922–4928

43 Escuyer V., Duflot E., Sezer O., Danchin A. and Mock M. (1988) Structural homology between virulence-associated bacterial adenylate cyclases. Gene **71:** 293–298

44 Betsou F., Sismeiro O., Danchin A. and Guiso N. (1995) Cloning and sequence of the *Bordetella bronchiseptica* adenylate cyclase-hemolysin-encoding gene: comparison with the *Bordetella pertussis* gene. Gene **162:** 165–166

45 Robertson D. L. and Leppla S. H. (1986) Molecular cloning and expression in *Escherichia coli* of the lethal factor gene of *Bacillus anthracis*. Gene **44:** 71–78

46 Bragg T. S. and Robertson D. L. (1989) Nucleotide sequence and analysis of the lethal factor gene (*lef*) from *Bacillus anthracis*. Gene **81:** 45–54

47 Leppla S. H. (1995) Anthrax Toxins. In: Handbook of Natural Toxins: Bacterial Toxins and Virulence Factors in Disease, vol. 8, pp. 543–572, Moss J, Iglewski B., Vaughan M. and Tu A. T. (eds.), Dekker, New York

48 Singh Y., Leppla S. H., Bhatnagar R. and Friedlander A. M. (1989) Internalization and processing of *Bacillus anthracis* lethal toxin by toxin-sensitive and -resistant cells. J. Biol. Chem. **264:** 11099–11102

49 Swain P. K., Sarkar N. K., Sharma M., Goel S., Singh R. P. and Singh Y. (1997) Cytotoxicity of anthrax lethal factor microinjected into macrophage cells through sendai virus envelopes. Indian J. Biochem. Biophys. **34:** 186–191

50 Duesbery N. S., Webb C. P., Leppla S. H., Gordon V. M., Klimpel K. R. and Copeland T. D. (1998) Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science **280:** 734–737

51 Friedlander A. M., Bhatnagar R., Leppla S. H., Johnson L. and Singh Y. (1993) Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. Infect. Immun. **61:** 245–252

52 Quinn C. P., Singh Y., Klimpel K. R. and Leppla S. H. (1991) Functional mapping of anthrax toxin lethal factor by in-frame insertion mutagenesis. J. Biol. Chem. **266:** 20124–20130

53 Klimpel K. R., Arora N. and Leppla S. H. (1994) Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol. Microbiol. **13:** 1093–1100

54 Hanna P. (1998) Anthrax pathogenesis and host response. Curr. Top. Microbiol. Immunol. **225:** 13–35

55 Kochi S. K., Schiavo G., Mock M. and Montecucco C. (1994) Zinc content of the *Bacillus anthracis* lethal factor. FEMS Microbiol. Lett. **124:** 343–348

56 Vitale G., Pellizzari R., Recchi C., Napolitani G., Mock M. and Montecucco C. (1998) Anthrax lethal factor cleaves the n-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. Biochem. Biophys. Res. Commun. **248:** 706–711

57 Lewis T. S., Shapiro P. S., and Ahn N. G. (1998) Signal transduction through MAP kinase cascades. In: Advances in Cancer Research, vol. 74, pp. 49–139, Vande Woude G. F. and Klein G. (eds), Academic Press, San Diego

58 Masui Y. and Markert C. L. (1971) Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J. Exp. Zool. **177:** 129–145

59 Ishikawa K., Hanaoka Y., Kondo Y. and Imai K. (1977) Primary action of steroid hormone at the surface of amphibian oocyte in the induction of GVBD. Mol. Cell. Endocrinol. **9:** 91–100

60 Godeau J. F., Schorderet-Slatkine S., Hubert P. and Baulieu E.-E. (1978) Induction of maturation in *Xenopus laevis* oocytes by a steroid linked to a polymer. Proc. Natl. Acad. Sci. USA **75:** 2353–2357

61 Gebauer F., Xu W., Cooper G. M. and Richter J. D. (1994) Translational control by cytoplasmic polyadenylation of *c-mos* mRNA is necessary for oocyte maturation in the mouse. EMBO J. **13:** 5712–5720

62 Sheets M. D., Wu M. and Wickens M. (1994) Polyadenylation of c-mos mRNA as a control point in *Xenopus* meiotic maturation. Nature **374:** 511–516

63 Barkoff A., Ballantyne S. and Wickens M. (1998) Meiotic maturation in *Xenopus* requires polyadenylation of multiple mRNAs. EMBO J. **17:** 3168–3175

64 Sagata N., Oskarsson M., Copeland T., Brumbaugh J. and Vande Woude G. F. (1988) Function of c-*mos* proto-oncogene product in meiotic maturation in *Xenopus* oocytes. Nature **335:** 519–525

65 Posada J., Yew N., Ahn N. G., Vande Woude G. F. and Cooper J. A. (1993) Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase kinase in vitro. Mol. Cell. Biol. **13:** 2546–2553

66 Nebreda A. R., Hill C., Gomez N., Cohen P. and Hunt T. (1993) The protein kinase *mos* activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cells in vivo. FEBS Lett. **333:** 183–187

67 Shibuya E. K. and Ruderman J. V. (1993) Mos induces the in vitro activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells. Mol. Biol. Cell **4:** 781–790

68 Mansour S. J., Resing K. A., Candi J. M., Hermann A. S., Gloor J. W., Herskind K. R. et al. (1994) Mitogen-activated protein (MAP) kinase phosphorylation of MAP kinase kinase: determination of phosphorylation sites by mass spectrometry and site-directed mutagenesis. J. Biochem. (Tokyo) **116:** 304–314

69 Chen M. and Cooper J. A. (1995) Ser-3 is important for regulating Mos interaction with and stimulation of mitogen-activated protein kinase kinase. Mol. Cell. Biol. **15:** 4727–4734

70 Palmer A., Gavin A.-C. and Nebreda A. R. (1998) A link between MAP kinase and $p34^{cdc2}$/cyclin B during oocyte maturation: $p90^{rsk}$ phosphorylates and inactivates the $p34^{cdc2}$ inhibitory kinase Myt1. EMBO J. **17:** 5037–5047

71 Mueller P. R., Coleman T. R., Kumagai A. and Dunphy W. G. (1995) Myt1: a membrane-associated inhibitory kinase that phosphorylates cdc2 on both threonine-14 and tyrosine-15. Science **270:** 86–90

72 Kahan C., Seuwen K., Meloche S. and Pouyssegur J. (1992) Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts: evidence for thrombin-induced signals different from phosphoinositide turnover and adenylylcyclase inhibition. J. Biol. Chem. **267:** 13369–13375

73 Meloche S., Seuwen K., Pages G. and Pouyssegur J. (1992) Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. Mol. Endocrinol. **6:** 845–854

74 Pages G., Lenormand P., L'Allemain G., Chambard J. C., Meloche S. and Pouyssegur J. (1993) Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc. Natl. Acad. Sci. USA **90:** 8319–8323

75 Ferrell J. E. Jr, Wu M., Gerhart J. C. and Martin G. S. (1991) Cell cycle tyrosine phosphorylation of $p34^{cdc2}$ and a microtubule-associated protein kinase homolog in *Xenopus* oocytes and eggs. Mol. Cell. Biol. **11:** 1965–1971

76 Gotoh Y., Moriyama K., Matsuda S., Okumura E., Kishimoto T., Kawasaki H. et al. (1991) *Xenopus* M phase MAP kinase: isolation of its cDNA and activation by MPF. EMBO J. **10:** 2661–2668

77 Jessus C., Rime H., Haccard O., Van Lint J., Goris J., Merlevede W. et al. (1991) Tyrosine phosphorylation of $p34^{cdc2}$ and p42 during meiotic maturation of *Xenopus* oocyte. Development **111:** 813–820

78 Posada J., Sanghera J., Pelech S., Aebersold R. and Cooper J. A. (1991) Tyrosine phosphorylation and activation of homologous protein kinases during oocyte maturation and mitogenic activation of fibroblasts. Mol. Cell. Biol. **11:** 2517–2528

79 Haccard O., Sarcevic B., Lewellyn A., Hartley R., Roy L., Izumi T. et al. (1993) Induction of metaphase arrest in cleaving *Xenopus* embryos by MAP kinase. Science **262:** 1262–1265

80 Colledge W. H., Carlton M. B., Udy G. B. and Evans M. J. (1994) Disruption of c-*mos* causes parthenogenetic development of unfertilized mouse eggs. Nature **370:** 65–68

81 Hashimoto N., Watanabe N., Furuta Y., Tamemoto H., Sagata N., Yokoyama M. et al. (1994) Parthenogenetic activation of oocytes in c-*mos*-deficient mice. Nature **370:** 68–71

82 Vande Woude G. F. (1994) On the loss of Mos. Nature **370:** 20–21

83 Guadagno T. M. and Ferrell J. E. Jr (1998) Requirement for MAPK activation for normal mitotic progression in *Xenopus* egg extracts. Science **282:** 1312–1315

84 Verlhac M.-H., Kubiak J. Z., Weber M., Geraud G., Colledge W. H., Evans M. J. et al. (1996) Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. Development **122:** 815–822

85 Choi T., Rulong S., Resau J., Fukasawa K., Matten W., Kuriyama R. et al. (1996) Mos/MAPK can induce early meiotic phenotypes in the absence of MPF: a novel system for analyzing spindle formation during meiosis I. Proc. Natl. Acad. Sci. USA **93:** 4730–4735

86 Choi T., Fukasawa K., Zhou R., Tessarollo L., Borror K., Resau J. et al. (1996) The Mos/MAPK pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. Proc. Natl. Acad. Sci. USA **93:** 7032–7035

87 Duesbery N. S., Choi T., Brown K. D., Wood K. W., Resau J., Fukasawa K. et al. (1997) CENP-E is an essential kinetochore motor in maturing oocytes and is masked during Mos-dependent, cell cycle arrest at metaphase II. Proc. Natl. Acad. Sci. USA **94:** 9165–9170

88 Shapiro P. S., Vaisberg E., Hunt A. J., Tolwinski N. S., Whalen A. M., McIntosh J. R. et al. (1998) Activation of the MKK/ERK pathway during somatic cell mitosis: direct interactions of active ERK with kinetochores and regulation of the mitotic 3F3/2 phosphoantigen. J. Cell Biol. **142:** 1533–1545

89 Zecevic M., Catling A. D., Eblen S. T., Renzi L., Hittle J. C., Yen T. J. et al. (1998) Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E. J. Cell Biol. **142:** 1547–1558

90 Mansour S. J., Matten W. T., Hermann A. S., Candia J. M., Rong S., Fukasawa K. et al. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. Science **265:** 966–970

91 Mansour S., Candia J. M., Gloor K. K. and Ahn N. G. (1996) Constitutively active mitogen-activated protein kinase kinase 1 (MAPKK1) and MAPKK2 mediate similar transcriptional and morphological responses. Cell Growth Differ. **7:** 243–250

92 Webb C. P., Van Aelst L., Wigler M. H. and Vande Woude G. F. (1998) Signaling pathways in ras-mediated tumorigenicity and metastasis. Proc. Natl. Acad. Sci. USA **95:** 8773–8778

93 Elion E. A. (1998) Routing MAP kinase cascades. Science **281:** 1625–1626

94 Schaeffer H. J., Catling A. D., Eblen S. T., Collier L. S., Krauss A. and Weber M. J. (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science **281:** 1668–1671

95 Whitmarsh A. J., Cavanagh J., Tournier C., Yasuda J. and Davis R. J. (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation. Science **281:** 1671–1674

96 Hammond S. E. and Hanna P. C. (1998) Lethal factor active-site mutations affect catalytic activity in vitro. Infect. Immun. **66:** 2374–2378

97 Raingeaud J., Whitmarsh A. J., Barrett T., Derijard B. and Davis R. J. (1996) MKK3- and MKK6-regulated gene ex-

pression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol. Cell. Biol. **16:** 1247–1255
98 Derijard B., Raingeaud J., Barrett T., Wu I. H., Han J., Ulevitch R. J. et al. (1995) Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science **267:** 682–685
99 Han J., Lee J. D., Bibbs L. and Ulevitch R. J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science **265:** 808–811
100 Lee J. C., Laydon J. T., McDonnell P. C., Gallagher T. F., Kumar S., Green D. et al. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature **372:** 739–746
101 Rouse J., Cohen P., Trigon S., Morange M., Alonso-Llamazares A., Zamanillo D. et al. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell **78:** 1027–1037
102 Freshney N. W., Rawlinson L., Guesdon F., Jones E., Cowley S., Hsuan J. et al. (1994) Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell **78:** 1039–1049
103 Takenaka K., Moriguchi T. and Nishida E. (1998) Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. Science **280:** 599–602
104 Zhou G., Bao Z. Q. and Dixon J. E. (1995) Components of a new human protein kinase signal transduction. J. Biol. Chem. **270:** 12665–12669
105 Kato Y., Kravchenko V. V., Tapping R. I., Han J., Ulevitch R. J. and Lee J. D. (1997) BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. EMBO J. **16:** 7054–7066
106 Kato Y., Tapping R. I., Huang S., Watson M. H., Ulevitch R. J. and Lee J. D. (1998) Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature **395:** 713–716
107 Cataldi A., Labruyere E. and Mock M. (1990) Construction and characterization of a protective antigen-deficient *Bacillus anthracis* strain. Mol. Microbiol. **4:** 1111–1117
108 Pezard C., Berche P. and Mock M. (1991) Contribution of individual toxin components to virulence of *Bacillus anthracis*. Infect. Immun. **59:** 3472–3477
109 Pezard C., Duflot E. and Mock M. (1993) Construction of Bacillus anthracis mutant strains producing a single toxin component. J. Gen. Microbiol. **139:** 2459–2463
110 O'Brien J., Friedlander A., Dreier T., Ezzell J. and Leppla S. (1985) Effects of anthrax toxin components on human neutrophils. Infect. Immun. **47:** 306–310
111 Okuda K., Takahashi T., Tadokoro I. and Noguchi Y. (1976) Effects of cytochalasin B and dibutyryl cyclic AMP on intraleukocytic bactericidal activity. Jpn. J. Exp. Med. **46:** 101–110
112 Rossi A. G., McCutcheon J. C., Roy N., Chilvers E. R., Haslett C. and Dransfield I. (1998) Regulation of macrophage phagocytosis of apoptotic cells by cAMP. J. Immunol. **160:** 3562–3568
113 Smith H. and Stoner H. B. (1967) Anthrax toxic complex. Fed. Proc. **26:** 1554–1557
114 Morrill G. A., Schatz F., Kostellow A. B. and Poupko J. M. (1977) Changes in cyclic AMP levels in amphibian ovarian follicle following progesterone induction of meiotic maturation. Differentiation **8:** 97–104
115 Speaker M. G. and Butcher F. R. (1977) Cyclic nucleotide fluctuations during steroid induced meiotic maturation of frog oocytes. Nature **267:** 848–849
116 Maller J. L., Butcher F. R. and Krebs E. G. (1979) Early effect of progesterone on levels of cyclic adenosine 3′:5′-monophosphate in *Xenopus* oocytes. J. Biol. Chem. **254:** 579–582
117 Cicirelli M. F. and Smith L. D. (1985) Cyclic AMP levels during the maturation of *Xenopus* oocytes. Dev. Biol. **108:** 254–258
118 Daar I., Yew N. and Vande Woude G. F. (1993) Inhibition of mos-induced oocyte maturation by protein kinase A. J. Cell. Biol. **120:** 1197–1202
119 Matten W., Daar I. and Vande Woude G. F. (1994) Protein kinase A acts at multiple points to inhibit *Xenopus* oocyte maturation. Mol. Cell. Biol. **14:** 4419–4426
120 VanRenterghem B., Browning M. D. and Maller J. L. (1994) Regulation of mitogen-activated protein kinase activation by protein kinases A and C in a cell-free system. J. Biol. Chem. **269:** 24666–24672
121 Matten W. T., Copeland T. D., Ahn N. G. and Vande Woude G. F. (1996) Positive feedback between MAP kinase and Mos during *Xenopus* oocyte maturation. Dev. Biol. **179:** 485–492
122 Miller M. J., Rioux L., Prendergast G. V., Cannon S., White M. A. and Meinkoth J. L. (1998) Differential effects of protein kinase A on Ras effector pathways. Mol. Cell. Biol. **18:** 3718–3726
123 Dousa T. P. (1998) Signaling role of PDE isozymes in pathobiology of glomerular mesangial cells: studies in vitro and in vivo. Cell. Biochem. Biophys. **29:** 19–34
124 Moroo I., Tatsuno I., Uchida D., Tanaka T., Saito J., Saito Y. et al. (1998) Pituitary adenylate cyclase activating polypeptide (PACAP) stimulates mitogen-activated protein kinase (MAPK) in cultured rat astrocytes. Brain Res. **795:** 191–196
125 Webster C. R. and Anwer M. S. (1998) Cyclic adenosine monophosphate-mediated protection against bile acid-induced apoptosis in cultured rat hepatocytes. Hepatology **27:** 1324–1331
126 D'Angelo G., Lee H. and Weiner R. I. (1997) cAMP-dependent protein kinase inhibits the mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking Raf activation. J. Cell Biochem. **67:** 353–366
127 Welkos S. L., Keener T. J. and Gibbs P. H. (1986) Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect. Immun. **51:** 795–800
128 Hanna P. C., Acosta D. and Collier R. J. (1993) On the role of macrophages in anthrax. Proc. Natl. Acad. Sci. USA **90:** 10198–10201
129 Hanna P. C., Kruskal B. A., Allen R., Ezekowitz B., Bloom B. R. and Collier R. J. (1994) Role of macrophage oxidative burst in the action of anthrax lethal toxin. Mol. Med. **1:** 7–18
130 Ding A., Sanchez E. and Nathan C. F. (1993) Taxol shares the ability of bacterial lipopolysaccharide to induce tyrosine phosphorylation of microtubule-associated protein kinase. J. Immunol. **151:** 5596–5602
131 Liu M. K., Herrera-Velit P., Brownsey R. W. and Reiner N. E. (1994) CD14-dependent activation of protein kinase C and mitogen-activated protein kinases (p42 and p44) in human monocytes treated with bacterial lipopolysaccharide. J. Immunol. **153:** 2642–2652
132 Geppert T. D., Whitehurst C. E., Thompson P. and Beutler B. (1994) Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. Mol. Med. **1:** 93–103
133 Buscher D., Hipskind R. A., Krautwald S., Reimann T. and Baccarini M. (1995) Ras-dependent and -independent pathways target the mitogen-activated protein kinase network in macrophages. Mol. Cell. Biol. **15:** 466–475
134 Kugler S., Schuller S. and Goebel W. (1997) Involvement of MAP-kinases and -phosphatases in uptake and intracellular replication of *Listeria monocytogenes* in J774 macrophage cells. FEMS Microbiol. Lett. **157:** 131–136