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Recognition of Antimicrobial Peptides by a Bacterial Sensor Kinase

Martin W. Bader,[1,3] Sarah Sanowar,[6,7] Margaret E. Daley,[5] Anna R. Schneider,[5] Uhnsoo Cho,[4] Wenqing Xu,[4] Rachel E. Klevit,[5] Hervé Le Moual,[6,7] and Samuel I. Miller[1,2,3,*]

[1]Department of Microbiology
[2]Department of Medicine
[3]Department of Genome Sciences
[4]Department of Biological Structure
[5]Department of Biochemistry
University of Washington Medical School
1959 NE Pacific Street
Seattle, Washington 98195
[6]Department of Microbiology and Immunology
[7]Faculty of Dentistry
McGill University
Montreal
Quebec H3A 2B4
Canada

Summary

PhoQ is a membrane bound sensor kinase important for the pathogenesis of a number of Gram-negative bacterial species. PhoQ and its cognate response regulator PhoP constitute a signal-transduction cascade that controls inducible resistance to host antimicrobial peptides. We show that enzymatic activity of *Salmonella typhimurium* PhoQ is directly activated by antimicrobial peptides. A highly acidic surface of the PhoQ sensor domain participates in both divalent-cation and antimicrobial-peptide binding as a first step in signal transduction across the bacterial membrane. Identification of PhoQ signaling mutants, binding studies with the PhoQ sensor domain, and structural analysis of this domain can be incorporated into a model in which antimicrobial peptides displace divalent cations from PhoQ metal binding sites to initiate signal transduction. Our findings reveal a molecular mechanism by which bacteria sense small innate immune molecules to initiate a transcriptional program that promotes bacterial virulence.

Introduction

Multicellular organisms restrict the growth of invading bacteria by both innate and adaptive immunity. One important component of innate immunity is cationic antimicrobial peptides, which are evolutionarily conserved and found in diverse organisms, including amoebas, fruit flies, plants, and mammals (Ganz, 2003; Hancock and Diamond, 2000). Antimicrobial peptides have tremendous sequence diversity, but many share common structural features, including a net positive charge and an amphipathic structure, which promotes their ability to interact with negatively charged biological membranes (Dathe and Wieprecht, 1999). The role of antimicrobial peptides in host resistance to microbial pathogens is now well established. For example, they make up the majority of effectors of the *Drosophila melanogaster* immune system (Hoffmann, 2003), while, in mammals, the peptide CRAMP controls the lesion size resulting from group A *Streptococcus* skin infections (Nizet et al., 2001), and increased expression of human defensin HD-5 in transgenic mice can alter the host-pathogen relationship (Salzman et al., 2003).

Bacteria have developed mechanisms to resist killing by antimicrobial peptides. These mechanisms have been best characterized for *Salmonella typhimurium* but are also present in other bacterial pathogens (Miller et al., 2005; Peschel, 2002). Resistance to antimicrobial peptides is typically acquired by modifications of the bacterial cell surface, such as lipopolysaccharide (LPS). LPS is anchored in the Gram-negative membrane via its lipid A portion, which becomes heavily modified in antimicrobial-peptide-resistant strains (Miller et al., 2005). Lipid A modifications are induced during *Salmonella* invasion of macrophages (Gibbons et al., 2005) and play a role during bacterial virulence (Gunn et al., 2000; Preston et al., 2003; Robey et al., 2001).

Lipid A modifications of a variety of Gram-negative bacteria are regulated by a two-component system termed PhoPQ (Derzelle et al., 2004; Ernst et al., 1999; Guo et al., 1997; Moss et al., 2000; Rebeil et al., 2004). The PhoPQ system constitutes a signal-transduction cascade composed of the membrane bound sensor kinase PhoQ and the cytosolic response regulator PhoP. Activation of the PhoQ kinase leads to autophosphorylation of PhoQ and subsequent phosphorylation of PhoP, which promotes the transcription of a large number of genes, including those that promote antimicrobial-peptide resistance. PhoPQ is essential for *Salmonella* virulence in mice and humans (Fields et al., 1989; Galan and Curtiss, 1989; Hohmann et al., 1996; Miller et al., 1989) and for the virulence of a number of other Gram-negative bacteria (Derzelle et al., 2004; Grabenstein et al., 2004; Llama-Palacios et al., 2003; Moss et al., 2000), highlighting the importance of these genes during bacterial infections.

The *Salmonella* PhoP/PhoQ system is activated in vivo within acidified macrophage phagosomes (Alpuche Aranda et al., 1992). It is repressed in vitro during bacterial growth in high concentrations of the divalent cations Ca^{2+}, Mg^{2+}, or Mn^{2+} (Garcia Vescovi et al., 1996). Repression by these metals is dependent on the PhoQ periplasmic sensor domain, which must transduce signals across the membrane to activate PhoQ phosphatase activity (Castelli et al., 2000; Montagne et al., 2001). The PhoPQ system is active at low cation concentrations in vitro and fully induced during bacterial replication in macrophages. It has therefore been proposed that low-cation conditions exist within the bacterial phagosome and function as a signal for PhoQ activation. Since divalent-cation-mediated repression may only be one part of the PhoQ sensing mechanism, we recently began a search for other signals that activate PhoQ. Since PhoPQ is essential to protect bacteria

*Correspondence: millersi@u.washington.edu

from antimicrobial peptides, we reasoned that such peptides could serve as signals for PhoQ activation (Bader et al., 2003). Previous work demonstrated that the PhoPQ system responds to sublethal concentrations of antimicrobial peptides to promote resistance to peptide-mediated killing. However, as antimicrobial peptides permeabilize membranes, it was unclear whether the mechanism of PhoPQ activation involved an indirect effect of membrane damage or a direct effect of peptide binding to PhoQ.

The studies reported here were initiated to dissect the molecular mechanism governing the response of PhoPQ to antimicrobial peptides and to distinguish it from divalent cation-mediated repression. We found that antimicrobial peptides constitute signals that are directly sensed by the PhoQ sensor kinase, resulting in increased phosphotransfer from PhoQ to PhoP. This study provides evidence that antimicrobial peptides and divalent cations compete for binding to a highly acidic region of PhoQ, suggesting that antimicrobial peptides initiate activation of PhoQ by displacing bound divalent cations. Our results reveal a novel example of a sensor kinase from an animal pathogen that is activated by innate immune effectors.

Results

Antimicrobial Peptides Activate PhoPQ-Regulated Gene Expression at Intermediate Mg^{2+} Concentrations

Antimicrobial-peptide activation of PhoPQ-regulated gene expression was previously demonstrated in growth medium that was undefined for divalent cations. As medium containing high concentrations of divalent cations represses PhoQ-mediated expression, the activation of PhoQ was compared in medium containing both antimicrobial peptides and Mg^{2+}. A strain expressing a PhoQ-regulated protein fusion to *Salmonella* acid phosphatase (PhoN) was chosen for analysis. The activity of this fusion protein was measured in growth medium containing the α-helical antimicrobial peptides LL-37 or C18G and varying concentrations of Mg^{2+}. LL-37 and C18G were used because *Salmonella* likely encounter this class of peptides within macrophages (Rosenberger et al., 2004). Figure 1A shows that sublethal concentrations of antimicrobial peptides significantly induced PhoPQ-dependent gene expression. Stimulation of PhoP activity by LL-37 and C18G occurred at physiologic Mg^{2+} concentrations (1 mM) but was impaired above 5 mM. This indicates that the effects of LL-37 can be competed by Mg^{2+} binding either to the bacterial cell surface or to PhoQ directly. Fusion-protein activities in the presence of C18G and LL-37 were comparable to those observed in the same medium containing low concentrations of Mg^{2+} (10 μM), suggesting that PhoP was fully induced by these peptides.

Activation did not occur in a PhoQ null mutant but was restored when PhoQ was introduced on a plasmid, indicating that the PhoQ sensor kinase is required for signaling (Figure 1B). The induction levels in the complemented strain were similar to a strain expressing a constitutive PhoQ variant, further confirming full induction of PhoQ by antimicrobial peptides.

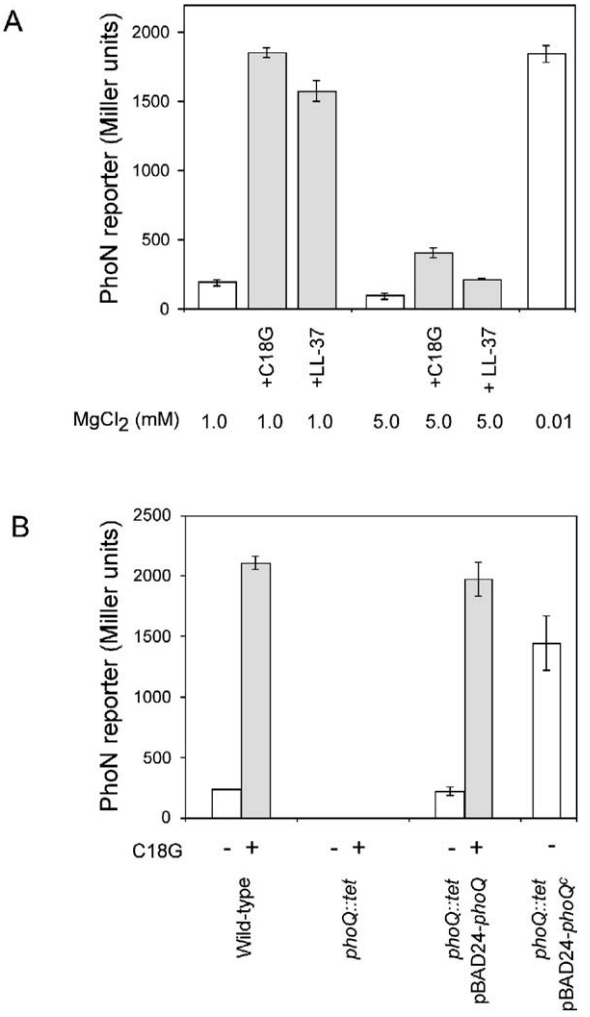

Figure 1. Induction of PhoPQ-Dependent Gene Expression by Antimicrobial Peptides

A protein fusion between PhoP-dependent acid phosphatase (PhoN) and *E. coli* PhoA served as a reporter.

(A) Activation by peptides C18G (5 μg/ml) and LL-37 (5 μg/ml) was measured in N-minimal medium (1 mM or 5 mM $MgCl_2$) and compared to cells grown in 0.01 mM $MgCl_2$.

(B) Activities were measured in LB medium. Strain MB101, which carries a *phoQ::tet* allele, did not exhibit peptide-mediated induction but could be complemented with a plasmid carrying full-length *phoQ* under the control of the arabinose promoter (pBAD24-phoQ). Fusion-protein levels were compared to induction levels in a PhoQ constitutive background ($PhoQ^c$). Values shown are the mean (±SD) of at least three independent experiments performed in duplicate.

The Periplasmic Domain of PhoQ Is Required for the Transcriptional Response to Antimicrobial Peptides

The effect of antimicrobial peptides could be an indirect consequence of membrane permeabilization or a direct effect on the PhoQ protein. As illustrated in Figure 2A, PhoQ is comprised of a periplasmic sensor domain, two transmembrane domains, and a cytosolic catalytic domain that undergoes autophosphorylation upon activation. The PhoQ sensor domain consists of 145 amino acids and plays an important role in cation-mediated repression of the PhoPQ system (Garcia Vescovi et al.,

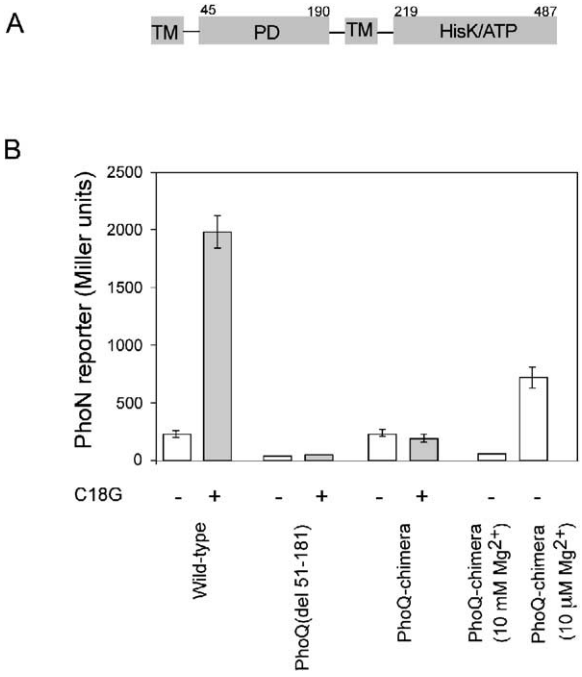

Figure 2. The PhoQ Sensor Domain Is Involved in Responding to Antimicrobial Peptides.

(A) PhoQ consists of two transmembrane domains, a periplasmic sensor domain and a cytosolic domain that contains the catalytic ATP binding domain and the phosphotransfer domain.

(B) The *Salmonella* PhoQ sensor domain is required for peptide-mediated activation. PhoQ lacking the sensor domain, PhoQ(Δ51–181), fails to respond to peptide C18G. A chimeric protein carrying the PhoQ sensor domain from *Pseudomonas aeruginosa* (phoQ-chimera) responds to varying concentrations of Mg^{2+} (10 mM versus 10 μM in N-minimal medium) but fails to mediate C18G-dependent activation of the PhoPQ system. Activities were measured in the presence (gray bars) or absence (white bars) of 5 μg/ml C18G in LB medium. Values shown are the mean (±SD) of three independent experiments performed in duplicate.

1996; Waldburger and Sauer, 1996). Therefore, a strain containing a deleted *phoQ* that expresses a mutant PhoQ(Δ51–181) lacking its periplasmic sensor domain was constructed. The resulting strain failed to induce PhoP-dependent gene activation in the presence of C18G (Figure 2B) or other antimicrobial peptides (data not shown), indicating that the PhoQ periplasmic domain is required for the response to antimicrobial peptides.

We wished to obtain further genetic evidence for a role of the PhoQ sensor domain during growth in the presence of C18G. It has been reported that the periplasmic domain of *Salmonella typhimurium* and other enteric bacterial PhoQ is strikingly different on the primary sequence level from the sensing domain of *Pseudomonas aeruginosa* PhoQ (Lesley and Waldburger, 2001). Despite this difference, both proteins respond equally to growth in Mg^{2+}-limited medium, suggesting that they share a common signaling mechanism. A strain expressing a chimeric protein consisting of *Salmonella typhimurium* PhoQ with the *Pseudomonas aeruginosa* sensor domain was constructed and tested for response to divalent cations and peptides. This strain responded robustly to limitation of cations, consistent with a previous report for *Escherichia coli* PhoQ (Figure 2B) (Lesley and Waldburger, 2001). Interestingly, no increase of PhoN fusion activity was observed in the presence of C18G. Taken together, the results provide strong genetic support for an involvement of the *Salmonella typhimurium* periplasmic domain in antimicrobial-peptide signaling. This indicates that the response is not conserved in PhoQ from the environmental and opportunistic pathogen *Pseudomonas aeruginosa*, although this bacterium may have other mechanisms to respond to antimicrobial peptides (McPhee et al., 2003).

PhoQ Kinase Activity Increases on Exposure of Purified Protein to Antimicrobial Peptides

Though the periplasmic domain is clearly required for PhoQ-mediated signaling on exposure to antimicrobial peptides, it was plausible that the signal was mediated indirectly as a result of outer-membrane permeability or through another protein. In order to distinguish between these possibilities, full-length PhoQ was purified from membranes and then reconstituted into phospholipid vesicles (Figure 3A). Using fluorescently labeled thiol-reactive probes directed against the two cysteine residues at positions 392 and 395 of the PhoQ cytoplasmic domain, we determined that PhoQ exhibits an inside-out orientation (>95%), with the periplasmic domain facing the vesicle inside and the histidine-kinase domain exposed to the assay buffer (data not shown). PhoQ activation by antimicrobial peptides was then measured by the subsequent phosphorylation of purified PhoP (Figure 3B). When PhoQ-containing vesicles were preloaded with 1 μg/ml of C18G, both increased autophosphorylation of PhoQ and phosphotransfer to purified PhoP were observed (Figure 3B). PhoQ activation was concentration dependent and increased to nearly 10-fold in the presence of 5 μg/ml C18G (Figure 3C). The concentration of C18G that exhibited the highest level of PhoQ activity is similar to the concentration that gave robust induction in the in vivo experiments (Figure 1A). Thus, our in vivo data are in good agreement with the in vitro reconstituted system. To assess whether PhoQ activity was due to a nonspecific effect of C18G on the conformation or permeability of phospholipid vesicles generated in this system, C18G was added to the assay buffer at 1 μg/ml but omitted from the vesicle lumen. As shown in Figure 3D, no activation of PhoQ was observed under these conditions. This result strongly argues that the periplasmic domain, which is present on the vesicle inside, is involved in peptide signaling and the observed activation is not due to nonspecific effects of C18G, such as membrane permeabilization. To further substantiate this finding, vesicles were preloaded with both C18G (1 μg/ml) and 5 mM $MgCl_2$, which is known to bind to the sensor domain of PhoQ. Figure 3D shows that the extent of PhoQ-catalyzed phosphate transfer from ATP to PhoP decreased significantly in the presence of 5 mM $MgCl_2$. The competition at very high Mg^{2+} indicates that metal may compete with C18G for binding to the periplasmic domain in the vesicle lumen, further supporting a role for

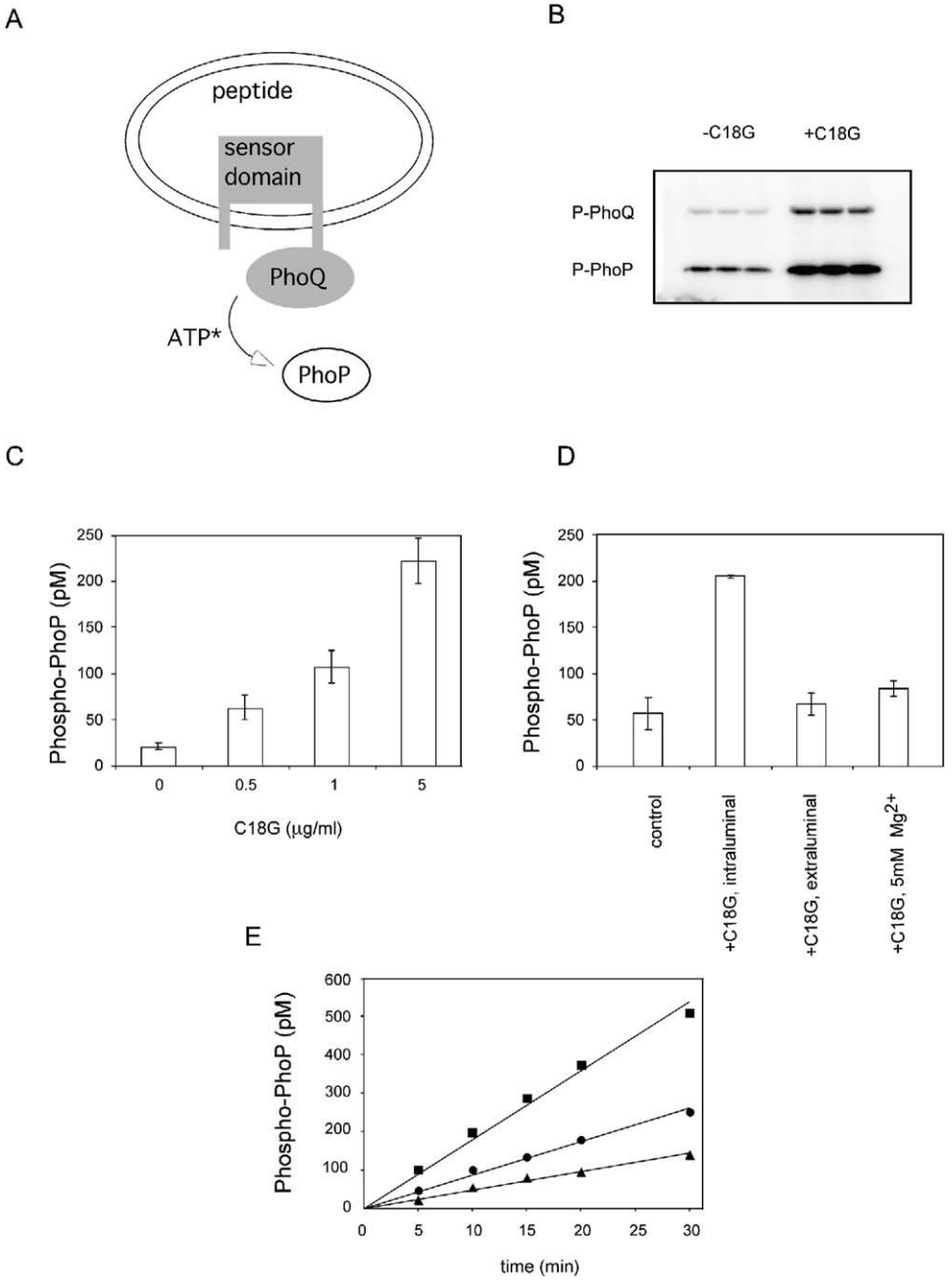

Figure 3. Reconstitution of PhoQ-Mediated Peptide Signaling In Vitro

(A) An in vitro system to study PhoQ activity in membrane vesicles. PhoQ-dependent phosphorylation of purified PhoP can be measured with [γ-^{32}P]ATP.

(B) Antimicrobial peptide C18G stimulates PhoQ autophosphorylation and PhoP phosphorylation. Vesicles were preformed in the presence of 1 μg/ml C18G, washed, and incubated in assay buffer containing [γ-^{32}P]ATP. Purified PhoP was present at 8 M excess to PhoQ (1.5 μM). Autoradiograph of SDS-PAGE is shown.

(C) Activation of PhoQ depends on C18G concentration. Vesicles were preloaded in the presence of increasing concentrations of C18G (0–5 μg/ml), and phosphotransfer to PhoP was determined as under (B).

(D) Activation of PhoQ is initiated by exposure of the sensor domain to peptides. No activation of PhoQ occurred when C18G was omitted from the vesicle lumen and added externally (1 μg/ml). When vesicles were preloaded with 5 mM $MgCl_2$ and 1 μg/ml C18G, activation was significantly reduced. Experiments in (C) and (D) were carried out in triplicate and repeated at least twice. Typical results are shown.

(E) Kinetics of PhoQ-catalyzed phosphotransfer. C18G and LL-37 enhance the initial rates of PhoP phosphorylation. Vesicles were preloaded with no antimicrobial peptide (▲), LL-37 (10 μg/ml, ●), or C18G (1μg/ml, ■). PhoQ-mediated phosphorylation of PhoP was measured at various time points as under (B).

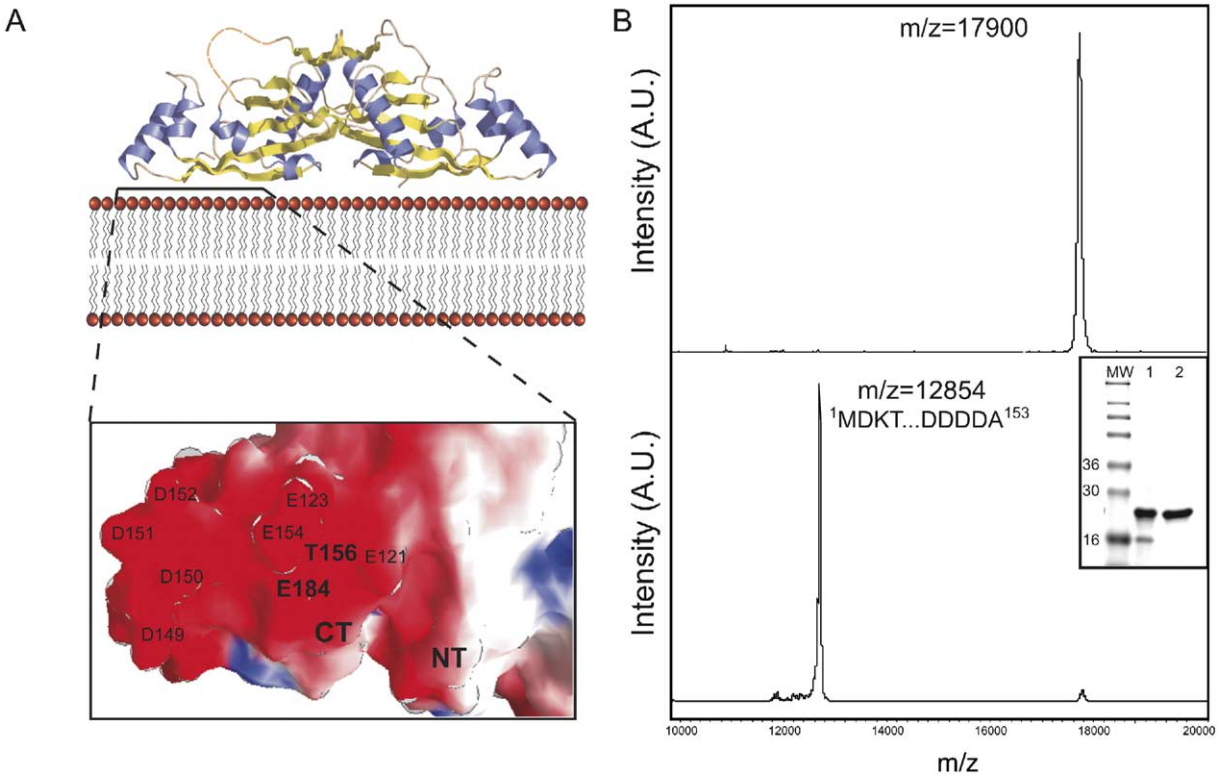

Figure 4. Binding of Divalent Cations to the PhoQ Sensor Domain

(A) The crystal structure of the dimeric PhoQ sensor domain (upper panel) forms a flat surface that comes in close contact to the membrane and binds to phospholipids via divalent-cation bridges (PDB ID code 1YAX). The bottom part of this domain contains a highly negatively charged surface as visualized by GRASP surface representation (lower panel, view from the membrane) and participates in metal binding. Red represents negatively charged residues; NT, N terminus; CT, C terminus.

(B) The acidic surface is involved in metal binding. Fe^{2+}-catalyzed cleavage of the purified periplasmic domain (6 μM) in the presence of $MgCl_2$ (20 mM, upper panel) and absence of $MgCl_2$ (lower panel) was assessed by MALDI mass spectrometry. The mass of the cleavage product observed in the absence of $MgCl_2$ in combination with N-terminal sequencing suggests that cleavage occurred at a specific site in the acidic surface. Fe^{2+}-catalyzed cleavage was also visualized by SDS-PAGE (inset). Lane 1, –$MgCl_2$; lane 2, +20 mM $MgCl_2$.

this domain in signaling. To measure the kinetics of PhoQ activation, initial rates of PhoP phosphorylation were measured in the presence of peptides C18G and LL-37. Figure 3E shows that both peptides lead to an increase in the initial rate of PhoQ-dependent phosphorylation of PhoP. The initial rate obtained with α-helical peptide magainin-2 (10 μg/ml) was similar to the one observed for LL-37 (data not shown). Therefore, PhoQ directly responds to the presence of α-helical peptides since no other component is necessary for PhoQ activation in the in vitro system. Our results further indicate that antimicrobial peptides directly activate PhoQ through interactions with its periplasmic sensor domain.

The Acidic Surface of the PhoQ Periplasmic Domain Binds Divalent Cations

The crystal structure of the PhoQ periplasmic sensor domain reveals a negatively charged surface of PhoQ (Figure 4A), which is predicted to contact the negatively charged membrane through a series of divalent-cation bridges (U.C. and W.X., unpublished data). Divalent cations serve to tether PhoQ to the membrane through ionic bridges between membrane phospholipids and membrane-proximal acidic residues of PhoQ. We sought direct evidence for divalent-cation binding in solution by the PhoQ sensor domain, using Fe^{2+}-catalyzed oxidative cleavage (Figure 4B). Fe^{2+} ions have been shown to interact with Mg^{2+} binding sites in many proteins, and this property can be exploited to obtain information about the position of metal binding sites in proteins. When incubated in the cleavage reaction mixture, the PhoQ sensor domain yielded one major cleavage product as visualized by SDS-PAGE (Figure 4B, inset). No cleavage occurred in the presence of 20 mM $MgCl_2$, consistent with Fe^{2+} binding at one or more PhoQ metal binding sites. The N terminus of the cleavage product was identical with the original construct (i.e., MDKT), and the average mass (determined by MALDI mass spectrometry) was 12,854 Da. Taken together, the results identify the major cleavage product as residues 1–153 with its C-terminal sequence $DDDDA^{153}$ (predicted mass 12,871 Da) and therefore demonstrate binding of magnesium to the acidic surface of the PhoQ sensor domain.

Antimicrobial Peptides and Divalent Cations Bind to an Acidic Surface of PhoQ

Our data suggest that (1) the periplasmic domain is required for peptide sensing and (2) peptides and divalent

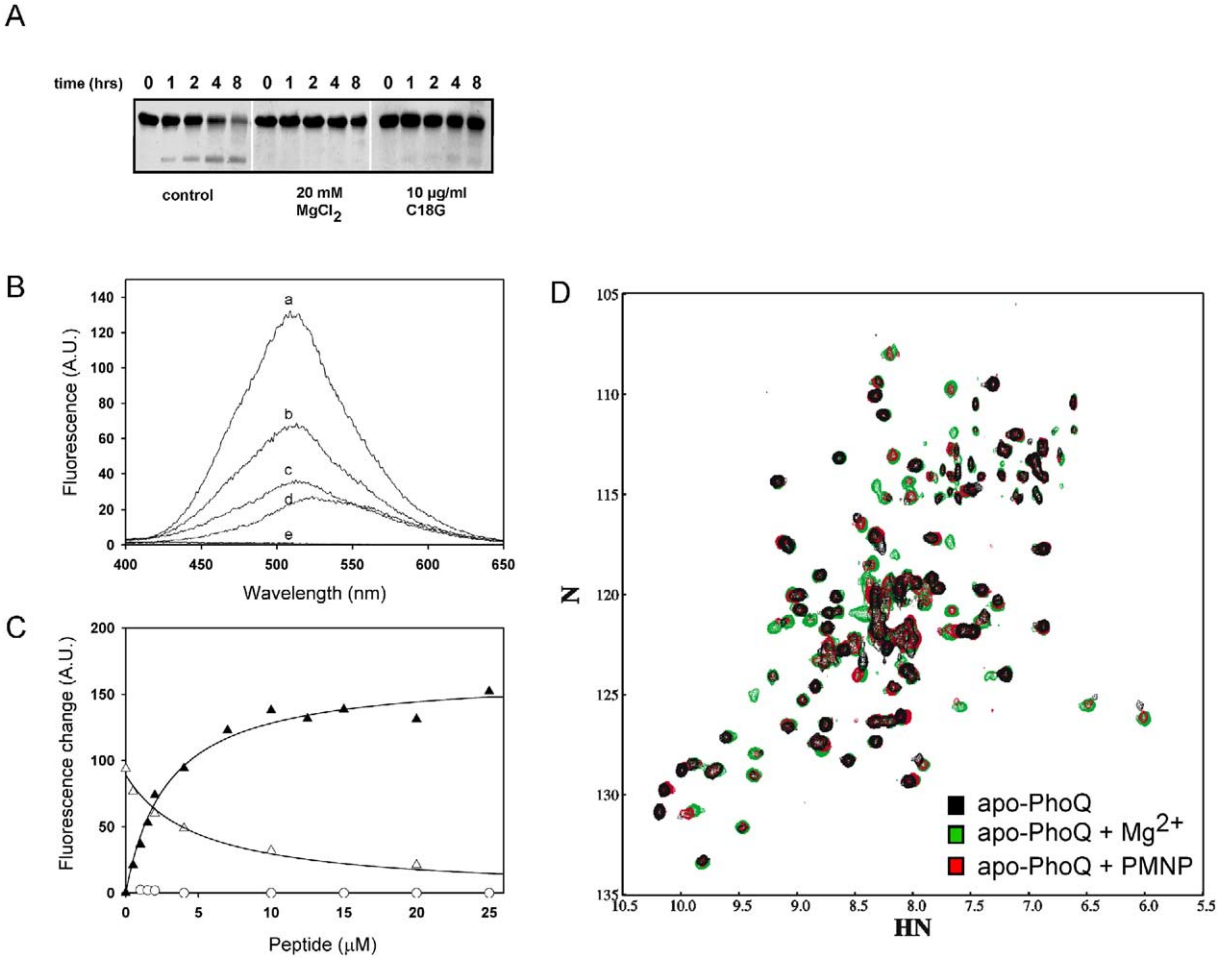

Figure 5. Binding of Antimicrobial Peptides to the PhoQ Sensor Domain

(A) Peptide C18G impairs iron-mediated cleavage of the PhoQ sensor domain. The purified sensor domain was incubated in buffer containing DTT/Fe^{2+} to mediate iron-dependent protein cleavage. Samples were removed at indicated time points and separated by SDS-PAGE. Reactions were also carried out in the presence of 20 mM $MgCl_2$ (middle panel) or 10 μg/ml C18G (right panel). Cleavage is inhibited by both Mg^{2+} and C18G, suggesting the presence of common binding sites.

(B) Dansylated C18G binds to PhoQ. One micromolar dansylated C18G (d) was incubated in 20 mM Tris (pH 7.4), and fluorescence spectra were recorded at an excitation wavelength of 340 nm. PhoQ (2 μM) was then added to the same cuvette (a). The presence of $MgCl_2$ at 1 mM (b) or 5 mM (c) impairs peptide binding. (e) represents protein-only control.

(C) Titration of 1μM PhoQ (▲) and 1 μM CitA (○) with dansylated C18G (dC18G). dC18G fluorescence in the absence of protein was subtracted from the data. The data were fit to a simple equilibrium model. As a control, PhoQ (1 μM) was incubated with 4 μM dC18G and titrated with unlabeled peptide (△).

(D) Superimposed two-dimensional ^1H-^{15}N TROSY-HSQC spectra of the PhoQ sensor domain in its apo form (black) and in the presence of 3.5 mM polymyxin nonapeptide (PMNP, red) or 20 mM $MgCl_2$ (green). All spectra were collected at a protein concentration of 0.8 mM. The peptide bound form reveals a subset of those peaks that appear in the presence of Mg^{2+}, indicating overlapping binding sites for peptide and divalent cations.

cations compete for binding to the periplasmic domain of PhoQ. We utilized three in vitro assays as independent tests for binding of antimicrobial peptides to PhoQ. We determined the extent of iron-mediated cleavage of the PhoQ sensor domain in the presence and absence of C18G peptide. As shown in Figure 4B, hydroxyl radicals generated in the reaction mixture specifically cleave the PhoQ sensor domain. As shown in Figure 5A, when incubated with 10 μg/ml C18G, PhoQ was strongly protected from cleavage, consistent with the proposal that C18G masks Mg^{2+} binding sites, making them inaccessible for iron-mediated cleavage. To gain further evidence for binding of C18G to PhoQ, a fluorescent derivative of the peptide was synthesized (Figure 5B). The dansylated peptide exhibited low fluorescence yield, with λ_{max} = 525 nm when diluted into assay buffer. When incubated with the purified sensor domain, however, the fluorescence yield increased dramatically, with the maximum fluorescence shifting to a lower wavelength (λ_{max} = 514 nm). Such changes are indicative of a more hydrophobic environment of the dansyl group, which can be best explained by binding of the peptide to PhoQ. The presence of 5 mM $MgCl_2$ significantly inhibited the increase in fluorescence in-

tensity of dansylated C18G due to PhoQ (Figure 5B). C18G binding showed saturation behavior at low micromolar peptide concentrations (Figure 5C), with an apparent K_d = 2.8 μM. Such concentrations are sufficient to activate PhoQ enzymatic activity in vivo and in vitro and compete effectively with 1 mM $MgCl_2$ for PhoQ (Figure 1A). Binding of C18G to PhoQ is specific, as the purified sensor domain of the *Klebsiella pneumonia* histidine kinase CitA, which is similar to PhoQ in size, net charge, and three-dimensional structure (PAS fold), did not affect the fluorescence spectra over a range of peptide concentrations tested (Figure 5C). Unlabeled C18G effectively outcompeted dansylated C18G, showing that binding was specific and not due to some nonspecific feature of the dansyl group (Figure 5C). Taken together, our results show that antimicrobial peptides compete effectively with metal to interact directly with the sensor domain of PhoQ at a site that overlaps with the metal binding site cleaved by Fe^{2+} and therefore involves the PhoQ acidic surface.

NMR spectra of the periplasmic domain of PhoQ in the presence of antimicrobial peptides could provide an additional highly sensitive assay for the similarity of conformational changes on binding peptides and divalent cations. Unfortunately, when incubated with C18G or LL-37, PhoQ and antimicrobial peptide formed an insoluble precipitate (at the high concentrations required for NMR), impeding analysis. We therefore turned to polymyxin nonapeptide, a derivative of the antimicrobial peptide polymyxin B that activates PhoQ-mediated signaling in vivo (3.5-fold in the presence of 20 μg/ml). Polymyxin nonapeptide lacks a fatty acid and is therefore less potent than polymyxin, probably as a result of its decreased hydrophobicity and decreased ability to penetrate the outer membrane. Polymyxin nonapeptide has a net positive charge and amphipathicity characteristic of this class of peptides, and it was suitable for NMR analysis. Spectra of PhoQ in the presence and absence of polymyxin nonapeptide are shown in Figure 5D. Remarkably, many of the peaks that appear in the presence of Mg^{2+} (green spectrum) also appear when peptide is added (Figure 5D). The peaks that appear in the spectrum of PhoQ in the absence of peptide and divalent cation (black spectrum, Figure 5D) are not perturbed by the addition of the peptide. This indicates that the stable structured core of the PhoQ dimer does not undergo a peptide-induced conformational change. The spectrum of PhoQ in the presence of peptide contains new peaks that are dispersed throughout the spectrum, indicating that peptide binding induces an additional PhoQ stable structure (red spectrum). Remarkably, the peptide-induced peaks are coincident with a subset of the peaks that appear when Mg^{2+} is added to PhoQ (compare red to green spectra). At the highest peptide concentration used, 10–12 peaks of the ca. 20 that appear with divalent cations are still undetectable or very weak in the PhoQ-peptide spectrum. Addition of 20 mM Mg^{2+} to a sample containing PhoQ and peptide restored the remaining peaks and produced a spectrum indistinguishable from that obtained for PhoQ plus Mg^{2+} in the absence of peptide (data not shown). The NMR spectra provide strong evidence that peptide and divalent cations bind at overlapping sites and that the binding events in solution elicit similar conformational effects that stabilize a region of structure that is intrinsically flexible in the absence of a ligand.

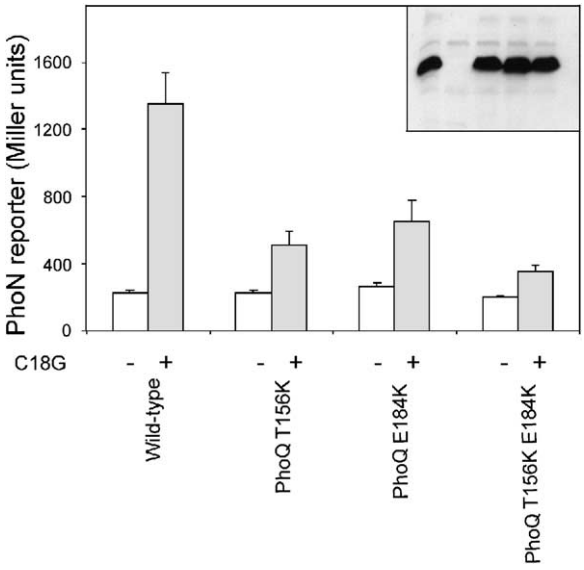

Figure 6. PhoQ Mutants Deficient in Peptide Signaling Map to the Acidic Region

Identification of mutants impaired in peptide-mediated repression. Mutants in the acidic region were screened for a defect in C18G-mediated signaling. Strains were grown to OD_{600} = 0.2 in LB medium and treated with 2.5 μg/ml C18G for 90 min. PhoQ mutants T156K and E184K and a double mutant in these two residues displayed defective C18G-mediated induction of phoN (+). Basal levels of activities were approximately the same (–) for all mutants and wild-type. Values shown are the mean (±SD) of three independent experiments performed in duplicate. Inset: cells were grown in LB medium in the presence of 0.2% arabinose to facilitate detection of PhoQ. Expression levels of wild-type and mutant proteins were determined by Western blot analysis with an antibody against the PhoQ sensor domain. From left to right: wild-type, *phoQ*$^-$, E184K, T156K, T156K E184K.

PhoQ Periplasmic-Domain Mutants Defective in the Response to Antimicrobial Peptides Are in Residues that Participate in the Binding of Divalent Cations

The acidic surface seems well suited to interact with cationic antimicrobial peptides, which have a net positive charge and a tendency to partition into lipid bilayers. We predicted that amino acid changes in the acidic surface would impair the ability of PhoQ to respond to antimicrobial peptides. Most single mutations in the acidic surface that we constructed exhibited only slight effects on peptide-mediated signaling, probably due to the remaining negative charge (data not shown). However, we were able to identify two amino acid substitutions, E184K and T156K, which lie within the acidic surface and exhibit a defect in C18G-induced activation as measured by the PhoN reporter fusion in vivo (Figure 6). The double T156K/E184K mutant exhibited a further decrease in peptide-mediated signaling, suggesting that these amino acid changes act additively. PhoQ T156K/E184K was also impaired in its response to LL-37 (2.4-fold versus 8.2-fold activation with 5 μg/ml LL-

37). Remarkably, both T156 and E184 participate in metal binding in the PhoQ crystal, and, as a consequence, amino acid substitutions in these residues display higher activities at 10 mM concentrations of magnesium (unpublished data). However, it is important to point out that amino acid substitutions in these residues do not change the basal levels of activity at intermediate magnesium concentrations (Figure 6). Thus, the activation defect seen with peptide is not due to decreased synthesis of these proteins, which was also confirmed by Western blot analysis (Figure 6, inset). Taken together, our data indicate that antimicrobial peptides and metals may compete for overlapping binding sites on PhoQ. Peptide binding may initiate signaling through the PhoQ transmembrane domains, leading to increased phosphorylation of PhoP.

Discussion

PhoQ Is an Antimicrobial-Peptide Sensor

Histidine sensor kinases are signaling proteins that play an essential role during many aspects of bacterial physiology, including bacterial infections. Although a number of small molecules that initiate specific responses through the activity of histidine kinases have been identified, the identity of most signals recognized by this class of receptors remains unknown, even though many bacterial genomes contain up to 60 such proteins. These identified small-molecule signals are often metabolic components such as phosphorus, nitrogen, citrate, and quinones but also include the antibiotic vancomycin, plant phenolics, and autoinducer molecules (homoserinelactones) involved in quorum or bacterial-density sensing (Arthur et al., 1992; Georgellis et al., 2001; Gilles-Gonzalez et al., 1991; Kaspar et al., 1999; Lee et al., 1995; Miller and Bassler, 2001). In addition, a specific bacterial peptide pheromone, ComX, is sensed by the sensor kinase ComP to promote competency in *Bacillus* species (Magnuson et al., 1994). In this study, we identify antimicrobial peptides as a direct signal for the activation of the PhoQ histidine kinase. PhoQ plays a prominent role in Gram-negative bacterial virulence, suggesting that antimicrobial peptides could be a signature of host environments for bacterial pathogens. Therefore, PhoQ sensing of antimicrobial peptides, an essential and highly conserved component of innate immunity, activates virulence programs within bacterial pathogens.

We had previously shown that the PhoPQ activation is a prominent part of the response to sublethal concentrations of antimicrobial peptides, but it remained uncertain whether PhoQ sensed peptides directly or responded to a secondary consequence of peptide exposure such as membrane damage (Bader et al., 2003). The work reported herein provides a variety of genetic, biochemical, and structural evidence that the periplasmic domain of PhoQ directly binds antimicrobial peptides to transduce a signal that promotes kinase activity in its cytoplasmic domain. Deletion of the periplasmic domain abolished PhoQ-mediated signaling, and amino acid changes in this domain were defined that lack a response to antimicrobial peptides. Most convincingly, the reconstitution of the PhoPQ system with purified components into membrane vesicles clearly demonstrated that PhoQ and its native lipid environment are the only components required for sensing antimicrobial peptides (Figure 3). In this in vitro system, the net phosphorylation of PhoP by PhoQ was greatly enhanced by various antimicrobial peptides. Since the sensor domain of PhoQ faces the vesicle lumen in this system, we were able to show that α-helical peptides such as C18G and LL-37 only stimulate catalytic activity of PhoQ when exposed to the periplasmic and not the cytosolic domain. Since phosphotransfer begins with phosphorylation of another molecule of PhoQ, binding of peptide may facilitate the interaction of the histidine-kinase domains within PhoQ dimers. Therefore, it is reasonable to conclude that PhoQ functions as a direct sensor with binding of antimicrobial peptide resulting in a conformational change, which has an effect through the membrane on the cytoplasmic histidine-kinase domain.

The PhoQ Acidic Surface Binds Divalent Cations to Repress and Antimicrobial Peptides to Activate Kinase Activity

A central finding of our study is that signaling by antimicrobial peptides is tightly linked to divalent-cation-mediated repression of PhoQ. The PhoPQ system is fully inducible by peptides LL-37 and C18G in the range of physiological Mg^{2+} concentrations (1–2 mM), but induction is severely impaired at higher Mg^{2+} concentrations. A similar effect was observed when 5 mM Mg^{2+} was included in the vesicle lumen in the in vitro reconstituted system, indicating that Mg^{2+} and antimicrobial peptides exert their effects at similar binding sites on PhoQ. Based on these observations and our finding that the PhoQ acidic surface is involved in metal binding, we hypothesized that antimicrobial peptides may compete with metals for the same binding sites. Genetic data support this notion, as two amino acid changes, E184K and T156K, which exhibit decreased responses to peptides C18G and LL-37 (Figure 6), map directly to one of the three PhoQ metal binding sites observed in the crystal structure (unpublished data). E184 is at the very C terminus of the sensor domain, which connects to the second transmembrane domain. Thus, our mutational analysis indicates that peptide binding at this site may trigger subsequent transmembrane signaling.

Three independent approaches (NMR, iron cleavage, and fluorescence spectroscopy) provide strong evidence for direct binding of the antimicrobial peptides C18G and polymyxin nonapeptide to the acidic surface of the PhoQ sensor domain (Figure 6). Binding to the acidic domain was concentration dependent and displayed saturation at micromolar peptide concentrations. The binding constant for peptides of full-length PhoQ may be lower due to the membrane proximity of the acidic surface and the higher local concentration of antimicrobial peptides near the membrane. The molecular mechanism by which the PhoQ sensor domain accommodates a large variety of antimicrobial peptides is currently unknown. It is nevertheless tempting to speculate that the negatively charged surface of the PhoQ sensor domain provides enough plasticity to ac-

commodate a large variety of positively charged peptides despite their structural and sequence diversity.

Taken together, our results provide a mechanism for antimicrobial-peptide signaling through PhoQ and lead us to conclude that metal and peptides act at similar sites. Therefore, it is plausible that antimicrobial peptides function to displace metal directly from the anionic surface and that their amphipathic structure enables an interaction with the membrane that promotes a conformational change in PhoQ, leading to its activation. In PhoQ's role as an antimicrobial-peptide sensor, PhoQ peptide binding and enzymatic activation occur in a micromolar range even in the range of low millimolar magnesium concentrations. Therefore, antimicrobial peptides can compete effectively with metal to bind to PhoQ, and PhoQ can sense antimicrobial peptides even under most physiologic magnesium concentrations.

A Model for PhoQ Activation

The above data, in conjunction with the PhoQ crystal structure, allow us to propose a mechanism by which PhoQ is activated by antimicrobial peptides and repressed by divalent metals (Figure 7). Because of its proximity to the membrane, the negatively charged surface of PhoQ is perfectly suited to sense the presence of membrane-active peptides. It has been shown that many antimicrobial peptides interact electrostatically with negatively charged phosphate groups of outer-membrane lipid A as a first step before inserting into the membrane (Piers and Hancock, 1994; Sawyer et al., 1988). We speculate that, similar to the interaction with lipid A, which causes the displacement of magnesium bound to lipid A phosphate groups, antimicrobial peptides displace divalent cations from PhoQ as part of their activation mechanism. Binding to PhoQ may promote a conformational change in the PhoQ dimer that is then propagated through the membrane (Figure 7). Such a model is supported by our peptide binding studies, which show that cation and peptide binding sites overlap (Figure 5). Our NMR data also demonstrate that both peptide and divalent-cation binding to the PhoQ sensor domain lead to the stabilization of a flexible region of the PhoQ sensor domain. However, the binding of peptides may lead to significant structural distortions in the full-length membrane bound protein by disrupting the interaction of divalent cations bound to the acidic surface. This is supported by mutational and structural analysis that demonstrates that structural integrity at the membrane-proximal side of the PhoQ sensor domain is required to promote a fully repressed conformation (unpublished data). Furthermore, antimicrobial peptides, due to their membrane-active nature, will likely also interact with the inner membrane while binding to the PhoQ acidic domain to initiate signal transduction. Therefore, our current working model is that peptide binding to the acidic surface displaces at least some metal bridges between PhoQ and the membrane. Peptide binding may function as a lever to lift the acidic surface off the membrane, with a resultant change in conformation that leads to signal transduction.

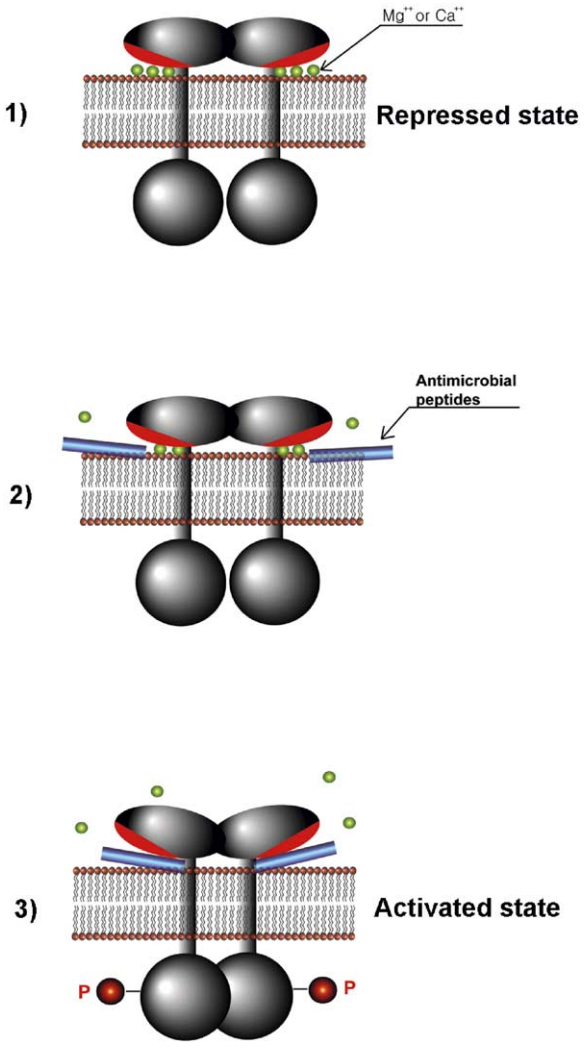

Figure 7. Model of PhoQ Activation by Antimicrobial Peptides

Divalent cations, such as Ca^{2+} or Mg^{2+} (shown as green balls), bind to the acidic surface (red) of the PhoQ sensor domain and form bridges to membrane phospholipids. Metal binding to the acidic surface represses PhoQ activity by locking the PhoQ sensor domain in an inactive conformation (1). Cationic antimicrobial peptides interact with membrane phospholipids and thus come in close contact with Ca^{2+} and Mg^{2+} binding sites of PhoQ. Antimicrobial peptides compete with divalent cations for binding to PhoQ and displace divalent cations from PhoQ (2). This results in a conformational change in the PhoQ sensor domain and subsequent autophosphorylation of PhoQ (red balls) (3).

What Activates PhoQ within Macrophage Phagosomes?

A general principle of bacterial virulence is that microbes sense their presence in host tissues to coordinately express virulence determinants in the correct microenvironment (Mekalanos, 1992). The nature of these microenvironments has long been a topic of debate among those studying bacterial pathogens. Since studies by Pappenheimer in 1936 demonstrated that diphtheria toxin production was increased in growth medium of low iron, it has been known that bacterial pathogenic factors can be regulated in vitro by specific ionic con-

centrations of growth medium (Pappenheimer, 1993). It has been debated whether similar conditions—usually, low iron, Ca^{2+}, or Mg^{2+}; low pH; or changes in temperature or osmolarity—reflect mammalian environments. Evidence from plant pathogenic bacteria has shown that virulence programs are induced by specific plant compounds (Lee et al., 1995), raising the possibility that animal pathogens recognize specific mammalian molecules as signals rather than ionic states of host tissues. Our results suggest one such signal is antimicrobial peptides.

The activity of PhoQ is required for a very central aspect of *Salmonella* pathogenesis: their survival and replication in macrophages (Groisman et al., 1989; Miller et al., 1989). Activation of PhoQ and subsequent phosphorylation of PhoP occurs upon bacterial phagocytosis by macrophages and leads to PhoP-dependent gene expression (Alpuche Aranda et al., 1992). As PhoQ seems to be rather nonspecific in recognizing divalent cations (Garcia Vescovi et al., 1996), it is difficult to imagine how the absence of one cation, such as Mg^{2+}, activates PhoQ under these conditions. Although the idea that phagosomes represent a low-Ca^{2+}/Mg^{2+} environment has been promoted for many years (Garcia-del Portillo et al., 1992; Pollack et al., 1986), this proposal would require that, immediately upon phagocytosis, phagosomes be depleted of all divalent cations, including Ca^{2+}, Mg^{2+}, and Mn^{2+}. Such a scenario is rather unlikely, given that the Ca^{2+} concentration was shown to increase upon acidification of phagosomes and acidification is required for PhoQ-mediated gene expression within macrophages (Alpuche Aranda et al., 1992; Christensen et al., 2002). In addition, recent work suggests that Mg^{2+} may indeed play a much less important role during macrophage infection than has previously been proposed, as phagosomes contain millimolar concentrations of Mg^{2+} at the time of PhoQ-mediated gene transcription (S. Grinstein, personal communication). Such concentrations would be sufficient to severely repress PhoP-dependent gene expression if magnesium were the relevant physiologic signal. Since PhoP is also activated at low pH and activation of PhoQ within macrophage phagosomes requires acidification (Alpuche Aranda et al., 1992; Bearson et al., 1998), it is plausible that phagosome acidification promotes the sensing of antimicrobial peptides by PhoQ or that PhoQ also directly senses varying proton concentrations. This work provides direct evidence and support for the idea that antimicrobial peptides are a direct signal for PhoQ activation in vivo and is consistent with recent work by Finlay's group providing evidence that *Salmonella* resistance to the α-helical macrophage peptide CRAMP is an important function of PhoPQ virulence promotion (Rosenberger et al., 2004). Since LL-37, the human homolog of CRAMP, activates PhoQ, these macrophage peptides may be the actual signal in vivo for induction of the PhoQ virulence program. We therefore propose that antimicrobial peptides and low pH, rather than low Mg^{2+} concentrations, may be the major signals sensed by PhoQ in vivo to activate gene expression within host tissues.

In summary, we have revealed a molecular mechanism by which bacteria sense the presence of antimicrobial peptides. This study provides data indicating the molecular mechanism by which the PhoQ histidine kinase, which is central for the virulence of many Gram-negative bacteria, including animal, plant, and insect pathogens, responds to molecules that are increasingly recognized for their role in innate immunity. Antimicrobial peptides are ubiquitous and ancient host defense molecules, and bacterial species have evolved mechanisms to respond directly to the presence of these peptides in a fashion analogous to the recognition of bacterial ligands by Toll-like receptors.

Experimental Procedures

Growth of Bacterial Strains

For detailed information on bacterial strains and plasmids, see the Supplemental Data available with this article online. Strains were grown in LB or N-minimal media. To study antimicrobial-peptide-mediated induction of the PhoP regulon, the reporter *phoN*::Tn-*PhoA* was chosen (Miller et al., 1989). Strains were grown overnight and diluted 1:100 into fresh medium. When N-minimal media was used, bacteria were washed twice in the same media before inoculation. Strains were then grown to an optical density of 0.2, and antimicrobial peptides were added at the indicated concentrations. After addition of peptide, strains were grown for an additional 60–90 min. Alkaline phosphatase assays were performed using a standard protocol and cultures grown in duplicate at three different occasions.

In Vitro Reconstitution of the PhoP/PhoQ Signaling Cascade

Proteoliposomes were preloaded with phosphorylation buffer (50 mM NaPi [pH 7.5], 200 mM KCl, 0.1 mM EDTA, 5% glycerol) supplemented with various concentrations of antimicrobial peptides C18G and LL37 or 5 mM $MgCl_2$ as indicated. The net phosphorylation of PhoP was measured by incubating proteoliposomes containing 1.5 μM PhoQ with an 8-fold molar excess of PhoP in a 15 μl volume of phosphorylation buffer supplemented with 5 mM $MgCl_2$. Reactions were initiated by the addition of 0.1 mM [γ-^{32}P]ATP (10 Ci/mmol), incubated at 22°C for 20 min, and stopped by the addition of Laemmli loading buffer. Reaction products were heated to 37°C for 3 min and applied to 10% SDS-PAGE gels. Phosphorylated protein was visualized using an FX Scanner (Bio-Rad) and quantified by image analysis using the Quantity One software (Bio-Rad). Intensity units were converted to pM [^{32}P]protein calculated from a standard curve.

Peptide Binding Studies

C18G, dansylated C18G, and LL-37 were synthesized by the UBC peptide synthesis facility (Vancouver, British Columbia) using an Applied Biosystems automated synthesizer. Peptides were HPLC purified after synthesis. Fluorescence spectra of dansylated C18G (dC18G) were recorded at room temperature from 400–650 nm at an excitation wavelength of 340 nm. Peptide dC18G was incubated at increasing concentrations in 20 mM Tris-HCl (pH 7.4) with or without PhoQ sensor domain (1 μM). Samples were incubated for 20 min at room temperature prior to measurements. The fluorescence of unbound peptide was subtracted from the measured fluorescence. Data were fit to a simple equilibrium model $F = F_{max} \times P/(K_D + [P])$, where F is the observed fluorescence change of the sample with protein minus the peptide-only control, F_{max} is the maximal fluorescence change, P is the peptide concentration, and K_D is the dissociation constant for the complex. As a control, binding of dC18G was also tested with purified CitA sensor domain. CitA was purified as described previously (Kaspar et al., 1999).

NMR Spectroscopy

The NMR sample of the apo form of the PhoQ periplasmic domain contained 0.8 mM uniformly ^{15}N-labeled PhoQ in 20 mM imidazole buffer (pH 6.5) with 150 mM NaCl, 0.1 mM EDTA, and 10% (v/v) D_2O. Additional NMR samples were identical but also contained either 20 mM $MgCl_2$ or 3.5 mM polymyxin nonapeptide. NMR experiments were performed at 25°C on a Bruker DMX 500 MHz spectrometer equipped with a triple-resonance, triple-axis gradient

probe. Data were processed and analyzed using the programs NMRPipe/NMRDraw (Delaglio et al., 1995) and NMRView (Johnson and Blevins, 1994).

Iron-Cleavage Assay and MALDI Analysis

PhoQ sensor domain (6 μM) was incubated in 20 mM Tris-HCl (pH 7.4), 20 mM DTT with either 20 mM $MgCl_2$ or 10 μg/ml C18G or alone for 10 min. $FeSO_4$ was then added to a final concentration of 0.05 mM. Samples were taken at indicated time points, mixed with Laemmli buffer, boiled, and separated by SDS-PAGE. For MALDI analysis, samples were mixed with 0.1% TFA and 5% acetonitrile, desalted by ZipTip (Millipore, C18). One microliter of the eluate was mixed with matrix solution and analyzed in a Bruker Biflex mass spectrometer in linear mode with 28% laser power. Approximately 500 shots were recorded per sample. N-terminal sequencing was performed by Proteome Factory (Berlin).

Structure Analysis

A detailed description of the PhoQ-sensor-domain crystal structure will be described elsewhere (U.C. and W.X., unpublished data). The molecular surface representations were generated by GRASP (Nicholls et al., 1991).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/122/3/461/DC1/.

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Accession Numbers

Coordinates for metal bound PhoQ are available at the Protein Data Bank (ID code 1YAX).