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A *Salmonella* type III secretion effector interacts with the mammalian serine/threonine protein kinase PKN1

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Summary

Essential to salmonellae pathogenesis is an export device called the type III secretion system (TTSS), which mediates the transfer of bacterial effector proteins from the bacterial cell into the host cell cytoplasm. Once inside the host cell, these effectors are then capable of altering a variety of host cellular functions in order to promote bacterial survival and colonization. SspH1 is a *Salmonella enterica* serovar Typhimurium TTSS effector that localizes to the mammalian nucleus and down-modulates production of proinflammatory cytokines by inhibiting nuclear factor (NF)-κB-dependent gene expression. To identify mammalian binding partners of SspH1 a yeast two-hybrid screen against a human spleen cDNA library was performed. It yielded a serine/threonine protein kinase called protein kinase N 1 (PKN1). The leucine-rich repeat domain of SspH1 was demonstrated to mediate this interaction and also inhibition of NF-κB-dependent gene expression. This suggested that PKN1 may play a role in modulation of the NF-κB signalling pathway. Indeed, we found that expression of constitutively active PKN1 in mammalian cells results in a decrease, while depletion of PKN1 by RNA interference causes an increase in NF-κB-dependent reporter gene expression. These data indicate that SspH1 may inhibit the host's inflammatory response by interacting with PKN1.

Introduction

Salmonellae are Gram-negative bacterial pathogens that can cause a variety of serious diseases in humans and animals, ranging from self-limiting gastroenteritis to the potentially life-threatening systemic illness typhoid fever (Pegues *et al*., 2005). One strategy of salmonellae to survive within animal hosts is their ability to subvert an array of host cellular functions by delivering bacterial proteins, called effectors, directly into the host cells' cytoplasm (Galan, 2001). After translocation these effectors localize to specific compartments within host cells and target specific host signalling pathways, often via their enzymatic activities. Salmonellae encode two separate effector transport systems, termed type III secretion systems (TTSS), within *Salmonella* pathogenicity islands (SPI) 1 and 2 that function at different times during the infection. The SPI1 TTSS translocates effectors across the plasma membrane and mediates invasion of the host cell, while the SPI2 TTSS secrets proteins across the vacuolar membrane and promotes intracellular survival and replication.

The *Salmonella enterica* serovar Typhimurium effector protein SspH1 can be delivered into mammalian cells by both the SPI1 and the SPI2 TTSS; however, it appears to be constitutively expressed (Miao *et al*., 1999). Once inside the host cell, it localizes to the nucleus, inhibits nuclear factor (NF)-κB-dependent gene expression, and contributes to down-modulation of proinflammatory cytokine production by *S. enterica* serovar Typhimurium (Haraga and Miller, 2003). SspH1 belongs to a family of bacterial proteins that contains leucine-rich repeats (LRR) of the LPX type (Miao *et al*., 1999). This family includes at least 15 effectors from six bacterial genera, including the *Salmonella* SspH2 and SlrP, the *Yersinia* YopM and the *Shigella* IpaH9.8. The LRR is a motif that typically mediates protein–protein interactions in eukaryotes (Buchanan and Gay, 1996). Since no enzymatic activities have been found for these LPX repeat proteins, it is plausible that this effector family simply functions to bind mammalian proteins as its pathogenic mechanism. This manuscript demonstrates that SspH1 interacts with the human serine/threonine kinase protein kinase N 1 (PKN1). Since PKN1 activation can also down-modulate NF-κB-dependent gene expression, we hypothesize that SspH1 inhibits the NF-κB signalling pathway by interacting with PKN1.

Results

SspH1 interacts with the mammalian serine/threonine kinase PKN1

Since SspH1 contains LRR, which is thought to be a protein–protein recognition motif in eukaryotes, we hypothesized that SspH1 interacts with a host protein after

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translocation by the TTSS. To identify mammalian interacting partners SspH1 was assayed by the yeast two-hybrid system. *S. enterica* serovar Typhimurium SspH1 was fused to the DNA binding domain (BD) of the yeast Gal4 transcriptional activator and was coexpressed in yeast with a human spleen cDNA library fused to the Gal4 activator domain (AD). Approximately 4×10^6 transformants were screened by growth on synthetic medium lacking histidine, and the resulting colonies were further tested for expression of two additional reporter genes, *ADE2* and *LacZ*. Eighty-four colonies containing nine different cDNA were selected, none of which encoded a protein that bound to the controls SspH2 and SlrP, when they were expressed in yeast as Gal4 BD–effector protein fusions. One of the identified cDNA encoded PKN1 (also called protein kinase C-like 1 or PRKCL1, and protein kinase C-related kinase 1 or PRK1), a fatty acid- and Rho-activated serine/threonine protein kinase that has a catalytic domain highly homologous to that of protein kinase C (PKC) (for review see Mukai, 2003).

We decided to confirm this interaction by co-immunoprecipitation, because another member of the PKN family, PKN2/PRK2, has been shown to interact with YopM (McDonald *et al*., 2003), a *Yersinia* effector containing the same type of LRR as SspH1. CHO-K1 cells were transiently transfected with vectors expressing haemagglutinin (HA)-tagged SspH1 or another bacterial LPX repeat protein, such as SspH2, YopM, IpaH9.8 and SlrP, as controls, and FLAG-tagged PKN1. The proteins were then immunoprecipitated with anti-FLAG- (data not shown) or anti-HA-agarose antibody conjugate. As shown in Fig. 1A and B, PKN1-FLAG co-immunoprecipitated with SspH1-HA but not with SspH2-HA, HA-YopM, HA-IpaH9.8 and SlrP-HA. Similarly, endogenous PKN1 co-immunoprecipitated with HA-tagged SspH1 but not SseJ, another SPI2 TTSS effector, when these proteins were delivered into HeLa cells by bacterial translocation, instead of transfection (Fig. 1C). The specificity of the SspH1-PKN1 binding was further demonstrated by showing that the highly similar PRK2 did not co-immunoprecipitate with SspH1. [Note that SspH1 migrates on polyacrylamide gels as a doublet, when expressed in mammalian cells by transfection, while it is a single band, when it is delivered into cells by the bacterial TTSS (Fig. 1A and C). We hypothesize that the amino-terminus of this protein is unstable in the absence of a bacterial chaperone, resulting in the appearance of an additional smaller, faster migrating band.] Therefore, these data demonstrate that SspH1 specifically binds PKN1, most likely through a direct protein–protein interaction.

SspH1 interacts with the amino-terminal regulatory domain of PKN1 within three antiparallel coiled-coil folds

PKN1 can be divided into two main domains, an amino-terminal regulatory domain and a carboxy-terminal serine/threonine kinase domain, which is constitutively active in the absence of the regulatory domain (Takahashi *et al*., 1998). To determine which of these two PKN1 regions SspH1 interacts with, SspH1-HA was coexpressed in

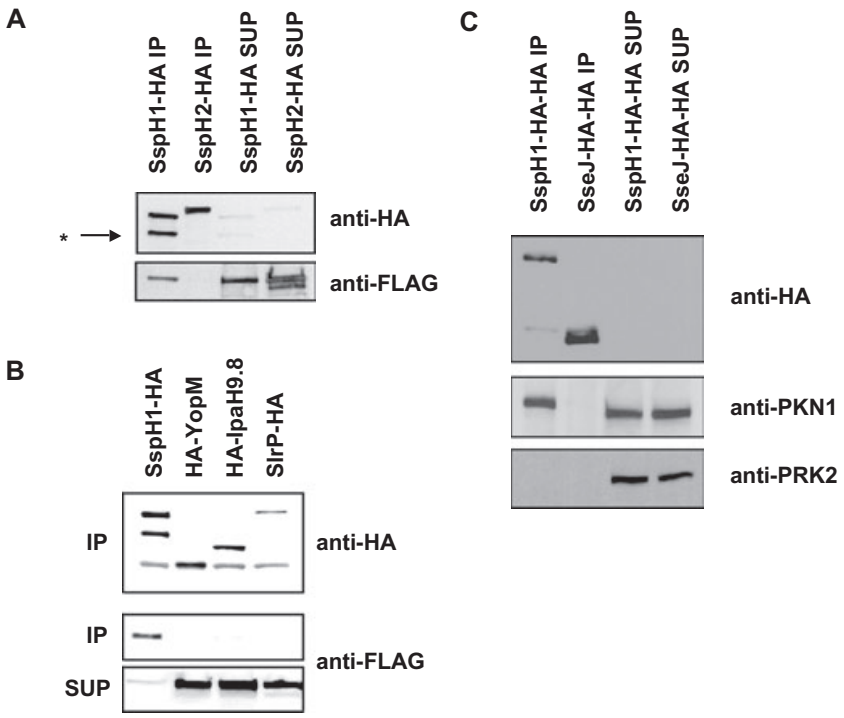

Fig. 1. SspH1 interacts with PKN1.
A. PKN1 co-immunoprecipitates with SspH1 from transfected cells. CHO-K1 cells were transiently transfected with vectors expressing PKN1-FLAG and SspH1-HA or SspH2-HA. Twenty-four hours later the HA-tagged proteins were immunoprecipitated, and the immunoprecipitates (IP) and the supernatants (SUP) were immunoblotted with antibodies to the HA and the FLAG tags. The asterisk (*) denotes a cleaved SspH1-HA product.
B. PKN1 does not co-immunoprecipitate with other LPX repeat proteins. SspH1-HA, HA-YopM, HA-IpaH9.8 or SlrP-HA were coexpressed in CHO-K1 cells with PKN1-FLAG and were immunoprecipitated. The IP and the SUP were probed with antibodies specific for the HA and the FLAG tags.
C. Endogenous PKN1, but not PRK2, co-immunoprecipitates with SspH1 from *Salmonella*-infected cells. HeLa were infected with *S. enterica* serovar Typhimurium expressing SspH1-HA-HA or SseJ-HA-HA at an moi of 20 for 17 h. The HA-tagged proteins were immunoprecipitated using an anti-HA-agarose antibody conjugate, and the IP and the SUP were blotted with anti-HA, anti-PKN1 and anti-PRK2 antibodies.

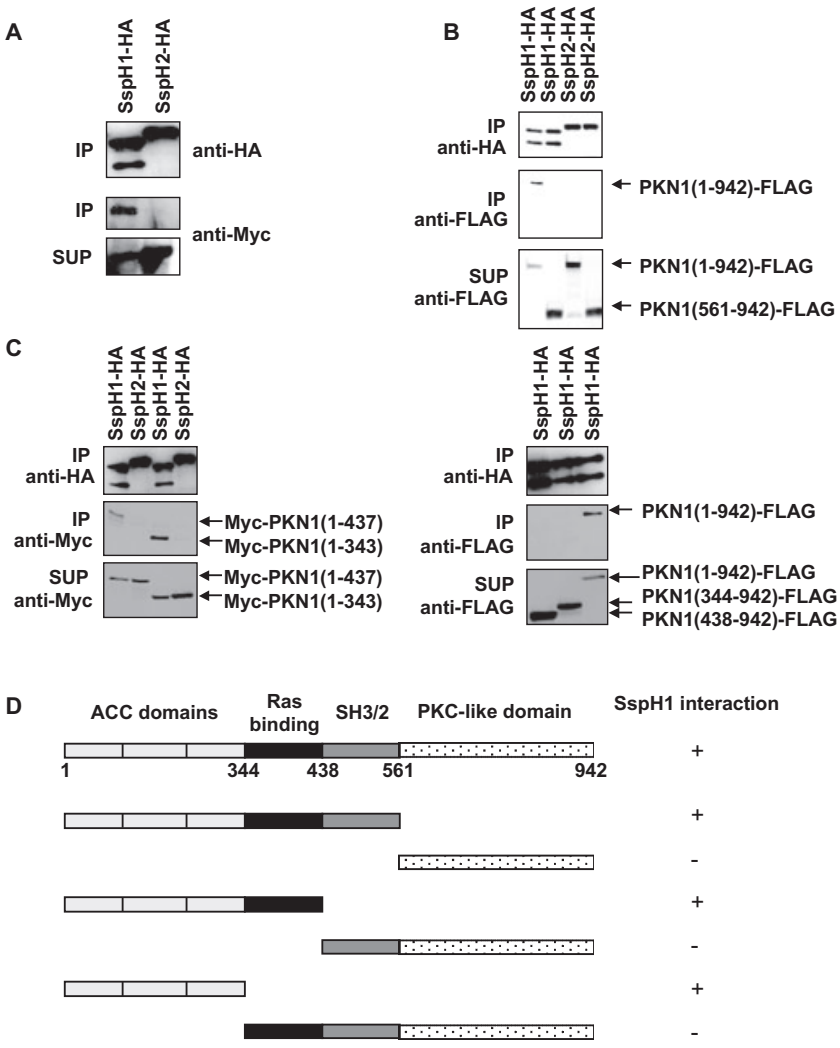

Fig. 2. SspH1 interacts with the ACC domains of PKN1.
A. The amino-terminal regulatory domain of PKN1 co-immunoprecipitates with SspH1. Myc-PKN1(1-560) and SspH1-HA or SspH2-HA were expressed in CHO-K1 cells by transient transfection. Twenty-four hours later the HA-tagged proteins were immunoprecipitated and the IP and the SUP were immunoblotted with anti-HA and anti-Myc antibodies.
B. The carboxy-terminal catalytic domain of PKN1 does not co-immunoprecipitate with SspH1. SspH1-HA or SspH2-HA were coexpressed with PKN1(561-942)-FLAG or full-length PKN1(1-942)-FLAG in CHO-K1 cells and were immunoprecipitated. The IP and the SUP were probed with anti-HA and anti-FLAG antibodies.
C. The ACC domains of PKN1 co-immunoprecipitate with SspH1. CHO-K1 cells were transfected with vectors expressing SspH1-HA or SspH2-HA and Myc-PKN1(1-437), Myc-PKN1(1-343), PKN1(438–942)-FLAG, PKN1(344-942)-FLAG or PKN1(1-942)-FLAG. The HA-tagged proteins were immunoprecipitated and the IP and the SUP were blotted with anti-HA and anti-FLAG or anti-Myc antibodies.
D. Summary of the results in (A)–(C).

CHO-K1 cells with epitope-tagged versions of these PKN1 fragments and was immunoprecipitated using anti-HA-agarose antibody conjugate. We found that SspH1 interacted with the amino- but not the carboxy-terminal portion of PKN1. As shown in Fig. 2A and B, Myc-PKN1(1-560) co-immunoprecipitated with SspH1-HA, while PKN1(561-942)-FLAG did not. The control SspH2 did not precipitate either of these fragments. To further define the region of interaction, the PKN1 amino acid sequence was submitted to Robetta (http://robetta.bakerlab.org), a fully automated full-chain protein structure prediction server, and the sequence was parsed into domains by Ginzu and subsequently modelled by homology or *ab initio* depending on the extent of existing information (Kim *et al*., 2004). Based on this analysis the amino-terminus of PKN1 consists of five domains: three homologous antiparallel coiled-coil (ACC) fold domains (amino acids 1–343), which function as a binding interface to various proteins including the small GTPases RhoA and Rac1 (Maesaki *et al*., 1999), a putative Ras BD (amino acids 344–437) predicted by its similarity to that of the serine/threonine kinase Raf-1 (Terada *et al*., 1999), and a Src homology (SH) 2/3 domain (amino acids 438–560), which provides the structural basis for the autoinhibition of the c-Abl tyrosine kinase (Nagar *et al*., 2003). As shown in Fig. 2C, HA-tagged SspH1, but not the control SspH2, immunoprecipitated Myc-PKN1(1-437) and Myc-PKN1(1-343), while it did not immunoprecipitate PKN1(438-942)-FLAG and PKN1(344-942)-FLAG. These results, which are summarized in Fig. 2D, indicate that SspH1 binds to PKN1 within its three ACC fold domains.

The LRR domain of SspH1 mediates interaction with PKN1 and inhibition of NF-κB-dependent gene expression

Since SspH2 and SlrP do not interact with PKN1 but share high homology to SspH1, chimeric proteins of SspH1 and SspH2 or SspH1 and SlrP were constructed based on

their similar structure to identify the SspH1 domain mediating the interaction with PKN1. DNA sequences encoding the amino-terminal, the LRR, and the carboxy-terminal domains of SspH1 and SspH2 or SlrP were swapped by Strand Overlap Exchange (SOE) PCR and were labelled with an HA tag. The resulting constructs were then coexpressed with FLAG-tagged PKN1 in CHO-K1 cells by transient transfection and those that encoded stable proteins were immunoprecipitated with an antibody to the HA tag. As shown in Fig. 3, PKN1-FLAG co-immunoprecipitated only with those chimeras that contained the LRR domain of SspH1, amino acid residues 206–372. This suggests that the LRR domain of SspH1 mediates the interaction with PKN1.

Since neither SspH2 nor SlrP has an effect on NF-κB-dependent gene expression (Haraga and Miller, 2003), these chimeras were also used to determine whether the LRR domain of SspH1 is the same domain involved in inhibition of NF-κB. Therefore, they were expressed in CHO-K1 cells with a reporter construct to measure NF-κB-dependent gene expression, a plasmid to normalize for transfection efficiency, and a vector expressing mouse CD14, which is part of the lipopolysaccharide (LPS) receptor complex. Cells were then stimulated with LPS or treated with fresh medium, lysed and assayed for reporter gene activity. As shown in Fig. 4, only those cells that expressed chimeras containing the LRR domain of SspH1 had reduced levels of NF-κB-dependent reporter gene expression and activity. This suggests that the LRR domain of SspH1, which mediates interaction with PKN1, is also involved in inhibition of NF-κB. The variation in the level of inhibition by the different chimeras is most likely due to their dissimilar stabilities when expressed in mammalian cells, as shown in Fig. 3. In conclusion, interaction with PKN1 and inhibition of NF-κB activity are both mediated by the LRR domain of SspH1.

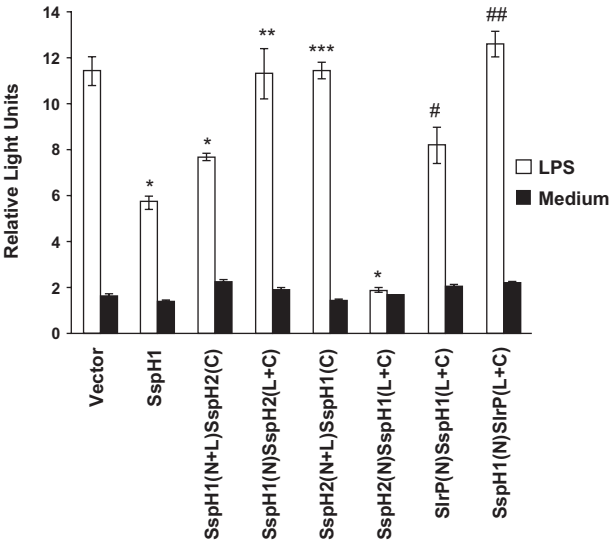

Fig. 4. The LRR domain of SspH1 mediates inhibition of NF-κB-dependent gene expression. CHO-K1 cells were transiently transfected with a plasmid in which expression of firefly luciferase is under the NF-κB-dependent ELAM-1 promoter, a construct in which *Renilla* luciferase is expressed from the constitutive HSV TK promoter, vectors expressing mouse CD14, plus constructs expressing the indicated proteins. Twenty-four hours after transfection the cells were incubated with *E. coli* LPS (open bars) or fresh medium (solid bars) for 5 h, and luciferase activity was measured. Firefly luciferase activity was normalized to the activity of *Renilla* luciferase. Data are representative of one of at least three independent experiments and presented as the mean ± standard deviation of triplicate samples. Statistical significance was evaluated by one-way ANOVA. $^{*}P < 0.001$, $^{**}P = 0.871$, $^{***}P = 0.954$, $^{\#}P = 0.005$ and $^{\#\#}P = 0.071$ versus LPS-stimulated Vector control.

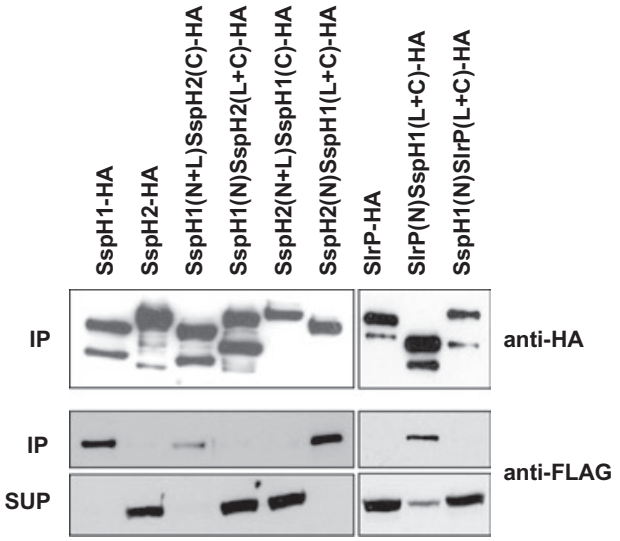

Fig. 3. PKN1 interacts with the LRR domain of SspH1. PKN1-FLAG was coexpressed in CHO-K1 cells with vectors expressing HA-tagged SspH1, SspH2, SlrP, or the indicated SspH1/SspH2 and SspH1/SlrP chimeras. The following abbreviations are used: amino-terminal (N), LRR (L) and carboxy-terminal (C) domain. The proteins were immunoprecipitated using an anti-HA antibody-agarose conjugate, and the IP and the SUP were blotted with anti-HA and anti-FLAG antibodies.

PKN1 activation inhibits NF-κB-dependent reporter gene expression

Since the same domain that interacts with PKN1 also inhibits NF-κB-dependent gene expression, we hypothesized that PKN1 plays a role in modulation of the NF-κB pathway. To test this hypothesis, CHO-K1 cells were transiently transfected with a vector expressing only the kinase domain of PKN1, residues 561–942, which is constitutively active without its regulatory domain (Takahashi *et al.*, 1998), or the same fragment with a mutation in the putative ATP binding site, K644E, which results in a kinase-dead PKN1 (Gao *et al.*, 2000), along with the constructs described above to measure NF-κB-dependent

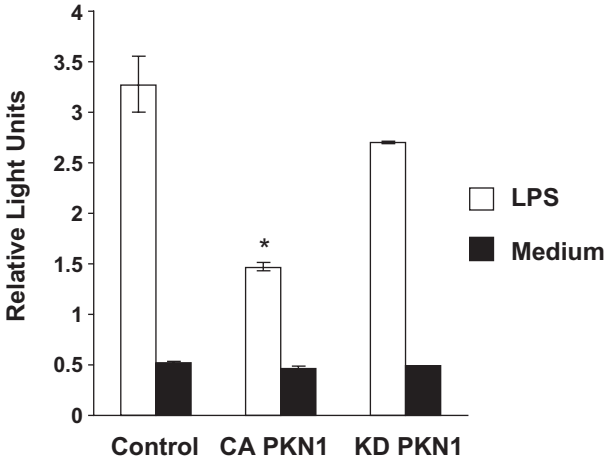

Fig. 5. Constitutively active PKN1 inhibits NF-κB-dependent reporter gene expression. CHO-K1 cells were transiently transfected with only the pELAM-1-*fluc*, pRL-TK and pCDNA3.1/Zeo/mCD14 vectors (Control), or also with a plasmid expressing either constitutively active (CA) or kinase-dead (KD) PKN1. Twenty-four hours later the cells were incubated with *E. coli* LPS (open bars) or fresh medium (solid bars) for 5 h, and luciferase activity was measured. Firefly luciferase activity was normalized to the activity of *Renilla* luciferase. Data are representative of one of at least three independent experiments and presented as the mean ± standard deviation of triplicate samples. Statistical significance was evaluated by one-way ANOVA. $^*P < 0.001$ versus LPS-stimulated Control and KD PKN1.

reporter gene expression. Cells were then stimulated with LPS or treated with fresh medium and assayed for reporter gene activity. As shown in Fig. 5, cells expressing the constitutively active form of PKN1 had reduced reporter gene expression and activity compared to cells expressing the kinase-dead PKN1 or the control cells not containing any PKN1-expressing construct, which suggests that activated PKN1 can inhibit NF-κB.

To measure the effect of endogenous PKN1 on NF-κB, cells were depleted of PKN1 using RNA interference (RNAi). HeLa cells were transfected with PKN1-specific or non-targeting small interfering RNA (siRNA) and the NF-κB reporter construct, were stimulated with tumour necrosis factor (TNF)-α, and reporter gene activity was measured. Depletion of PKN1 was confirmed by immunoblotting the cell lysates with an anti-PKN1 antibody. As shown in Fig. 6, these results agreed with our previous finding using the exogenously expressed constitutively active PKN1. Cells that had reduced levels of PKN1 had increased NF-κB-dependent reporter gene expression and activity, which suggests that PKN1 can inhibit NF-κB-dependent gene expression and may play a role in the NF-κB signalling pathway.

PKN1 is phosphorylated during Salmonella *infection*

To determine whether PKN1 activity is modulated during *Salmonella* infection, the phosphorylation level of PKN1 was examined during infection. Since phosphorylation at threonine (Thr)-774 within the activation loop of PKN1 has been shown to be required for its catalytic activity *in vivo* (Flynn *et al*., 2000), measuring phosphorylation at this residue is thus an indication of PKN1 activity. Intestine-407 cells were infected with *S. enterica* serovar Typhimurium at a multiplicity of infection (moi) of 50 for 0, 0.5, 1, 2, 3 and 4.5 h, the infected cells were lysed, and the lysates were immunoblotted with antibodies specific for unphosphorylated PKN1 and PKN1 phosphorylated at Thr-774. As shown in Fig. 7A, PKN1 becomes phosphorylated approximately 3 h after infection. (Although the phospho-specific antibody also detects PRK2 phosphorylated at its activation loop, based on their size the bands in Fig. 7 represent phosphorylated PKN1, not PRK2.) This occurs approximately 2 h after the invasion-associated signalling cascade, as demonstrated by the phosphorylation level of p38 (Fig. 7A), suggesting that these two events are independent (Hobbie *et al*., 1997). The timing of the PKN1 phosphorylation also indicates that perhaps an effector translocated by the SPI2 TTSS activates PKN1. To determine whether this effector is SspH1, Intestine-407 cells were infected with wild-type *S. enterica* serovar Typhimurium or the same strain deleted for *sspH1* and the phosphorylation levels of PKN1 at Thr-774 were compared in these cells. As shown in Fig. 7B, PKN1 is phosphorylated in cells infected with the *ΔsspH1 Salmo-*

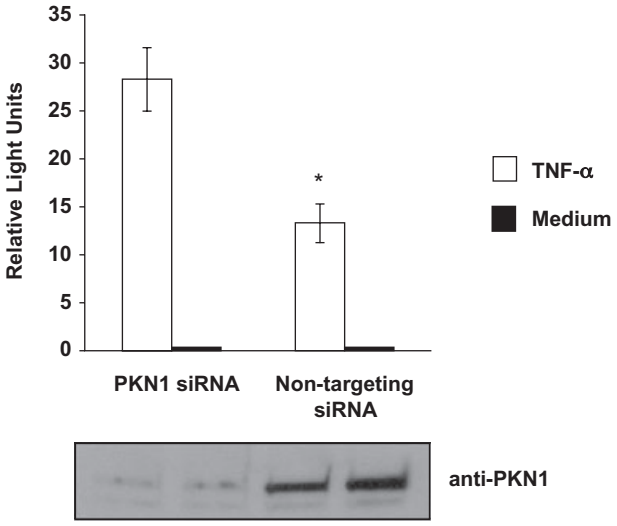

Fig. 6. PKN1 inhibits NF-κB-dependent reporter gene expression. HeLa cells were transfected with PKN1-specific or non-targeting siRNA, pELAM-1-*fluc* and pRL-TK. Twenty-four hours later the cells were stimulated with TNF-α (open bars) or treated with fresh culture medium (solid bars) for 5 h and luciferase activity was measured. Firefly luciferase activity was normalized to the activity of *Renilla* luciferase. Data are representative of one of at least three independent experiments and presented as the mean ± standard deviation of triplicate samples. Statistical significance was evaluated by one-way ANOVA. $^*P < 0.001$. Depletion of PKN1 was confirmed by immunoblotting the cell lysates with an antibody to PKN1.

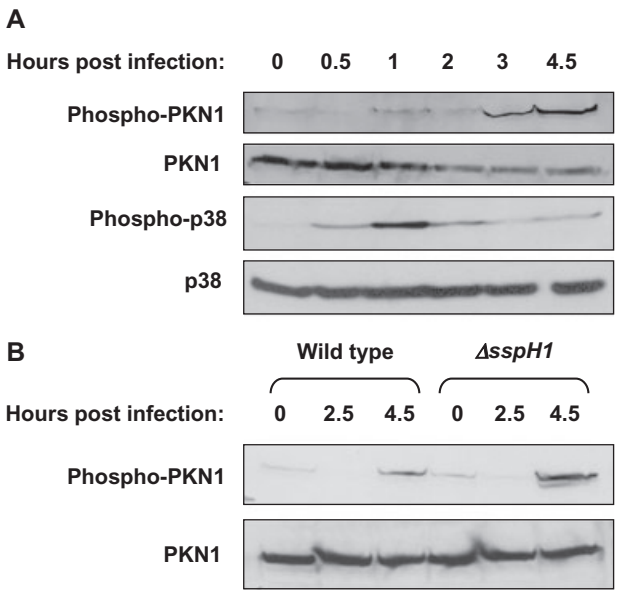

Fig. 7. PKN1 is phosphorylated during *Salmonella* infection.
A. Intestine-407 cells were infected with wild-type *S. enterica* serovar Typhimurium at an moi of 50 for 0, 0.5, 1, 2, 3 and 4.5 h, lysed, and immunblotted with antibodies specific for PKN1, activation loop-phosphorylated PKN1, p38 and phosphorylated p38.
B. Intestine-407 cells were infected with wild-type or *ΔsspH1* *S. enterica* serovar Typhimurium at an moi of 50 for 0, 2.5 and 4.5 h. The infected cells were lysed and blotted with anti-PKN1 and anti-phospho-PKN1 antibodies.

nella similarly to those infected with the wild-type strain. This implies either that SspH1 is not required for phosphorylation of PKN1 during infection or that other effectors or mechanisms are also capable of activating PKN1.

Discussion

Salmonella enterica serovar Typhimurium induces a complex array of mammalian signalling pathways essential for its survival in host tissues. It stimulates rearrangement of the actin cytoskeleton leading to membrane ruffling and internalization of the bacterium, and nuclear responses resulting in the production of proinflammatory cytokines and diarrhoea (Galan, 2001). The bacterium also has the ability to interfere with the maturation of the *Salmonella*-containing vacuole, allowing it to avoid phagocytic killing and to replicate intracellularly, and to down-modulate the host's inflammatory response presumably to persist within its host for prolonged periods (Haraga and Miller, 2003). The ability of the bacterium to stimulate such host responses is dependent on the functions of the SPI1 and the SPI2 TTSS. *S. enterica* serovar Typhimurium encodes at least 27 translocated effectors, many of whose functions and host cellular targets have been determined. In this study we describe the identification of an intracellular binding partner of SspH1, an effector of both TTSS that participates in suppression of proinflammatory cytokine secretion by inhibiting NF-κB-dependent gene expression (Haraga and Miller, 2003). We demonstrate by the yeast two-hybrid assay and co-immunoprecipitation experiments that SspH1 specifically interacts with the human serine/threonine protein kinase PKN1.

PKN1 is a versatile molecule that regulates a diverse set of cellular functions. It has been implicated in cytoskeletal regulation, vesicle transport, apoptosis, control of the cell cycle and cell signalling (Kawamata *et al*., 1998; Takahashi *et al*., 1998; 2003; Dong *et al*., 2000; Isagawa *et al*., 2005). It has been shown to be activated by interaction with fatty acids, such as arachidonic acid, and several members of the Rho family of small GTPases, such as RhoA and Rac1, and by cleavage of the amino-terminal regulatory domain by caspase-3 during apoptosis (Mukai *et al*., 1994; Amano *et al*., 1996; Takahashi *et al*., 1998). It has the ability to localize to several compartments within the cell, including the cytoplasm, the plasma membrane, vesicles and the nucleus, under different cellular conditions (Mukai *et al*., 1996; Torbett *et al*., 2003; Zhu *et al*., 2004). For example, in response to heat shock, serum starvation and treatment with sodium arsenite PKN1 has been shown to translocate into the nucleus. It is interesting to speculate whether infection with *Salmonella* may present a similar stress response resulting in the nuclear localization of PKN1 and its consequent interaction with SspH1. In fact, since SspH1 contains no classical nuclear localization signal and is too large for nucleocytoplasmic diffusion, it may be transported into the nucleus via PKN1.

We found that SspH1 binds to the amino-terminal regulatory domain of PKN1 within a region containing three ACC fold repeats. The ACC fold domain has been described as a well-defined structure containing two long intertwined alpha-helices and a short amino-terminal helix folded back onto them to form a hydrophobic core that contributes to the stability of the structure (Maesaki *et al*., 1999). Although ACC domains are also present in other Rho effector proteins, such as PRK2, SspH1 specifically interacts with the PKN1 ACC. In addition, each ACC fold in PKN1 is unique in that each has different affinities for different Rho members. For example, while ACC1 binds to RhoA and Rac1 with similar high affinities, ACC2 interacts selectively with Rac1, and ACC3 does not bind either of these two GTPases (Flynn *et al*., 1998; Owen *et al*., 2003). ACC1 has also been shown to contain a pseudosubstrate site that inhibits the kinase activity of PKN1 (Kitagawa *et al*., 1996). In fact, yeast two-hybrid and *in vitro* binding assays have demonstrated that the amino-terminal regulatory region of PKN1 can interact with the carboxy-terminal catalytic half of the molecule and this inhibits PKN1 kinase activity. These data suggest that an autoinhibitory intramolecular contact may keep PKN1 in a

closed inactive conformation and that binding by activated Rho may act to disrupt this interaction, thereby unmasking the active catalytic domain of PKN1. This hypothesis is supported by the observation that Rho binding is required for activation of PKN1 by phosphoinositide-dependent protein kinase-1 (PDK1), a known activator of PKN1 (Flynn *et al*., 2000). We hypothesize that SspH1 could induce a similar conformational change in PKN1 that is permissive for binding to PDK1 or another activator of PKN1.

SspH1 contains three main domains, a LRR domain containing eight LRR that separates an amino- and a carboxy-terminal domain. Since the LRR is a short repeating sequence motif that is thought to present a structural framework for the formation of protein–protein interactions, we were not surprised to find that this domain was mediating the interaction with PKN1 (Buchanan and Gay, 1996). However, the SspH1–PKN1 interaction is specific and is not simply the result of the 'stickiness' of the curved horseshoe structure of the LRR, because four other TTSS effectors containing very similar LRR, SspH2, SlrP, IpaH9.8 and YopM, did not bind to PKN1. Interestingly, the *Yersinia enterocolitica* YopM, an effector composed almost entirely of LRR, was recently reported to also interact with two serine/threonine kinases, PRK2 and ribosomal S6 protein kinase 1 (RSK1) (McDonald *et al*., 2003). It is possible that other effectors containing the LPX type of LRR also interact with different serine/threonine kinases. Their functional differences may lie within their specificities for the interacting host kinase. To date no host targets have been identified for SlrP, IpaH9.8 and the LRR domain of SspH2, and it is tempting to speculate that the function of their LRR domains is to bind kinases. LPX repeat-containing proteins may be a unique family of TTSS effectors in that they do not have enzymatic activities. They may simply function as protein binding and localizing molecules, altering mammalian function by changing the subcellular localization of kinases. With the exception of YopM, these effectors have additional domains besides the LRR that may promote localization and/or bind other molecules, such as kinase substrates. Therefore, this family of proteins in bacterial pathogenesis may have evolved to bind kinases to redirect their subcellular localization and/or to bring them in contact with specific substrates that would not otherwise be accessible.

The fact that the LRR domain of SspH1 is also involved in inhibition of NF-κB-dependent gene expression further supports the idea that interaction with a serine/threonine kinase is important for the function of an effector of the LPX repeat protein family. Unfortunately we were not able determine whether suppression of NF-κB activity by SspH1 requires PKN1, because sufficient depletion of PKN1 by RNAi took 48 h and SspH1 expression significantly declined after 24 h, most likely due to cell toxicity. In addition, we were not able to use translocated SspH1 for this experiment, because *S. enterica* serovar Typhimurium possesses several other effectors that can also inhibit the NF-κB pathway and our attempts to identify all of them have not been successful. However, we did find that activated PKN1 could also inhibit NF-κB-dependent gene expression. Whether or not SspH1 directly activates PKN1 is not yet clear. The observation that the *sspH1* mutant *S. enterica* serovar Typhimurium could also induce phosphorylation of PKN1 does not rule out the possible importance of SspH1 in PKN1 regulation. As there are multiple effectors capable of down-modulating proinflammatory signalling, *S. enterica* serovar Typhimurium may translocate several effectors, whose actions, one way or another, trigger activation of PKN1.

Experimental procedures

Bacterial strains, mammalian cell lines, culture and infection conditions

The *S. enterica* serovar Typhimurium strain CS401, which is a spontaneous streptomycin-resistant derivative of ATCC (American Type Culture Collection) 14028 and carries a *phoN*::Tn*10d* insertion, and its mutant derivative with non-polar deletion of *sspH1* (EM124) have been described previously (Miao *et al*., 1999; Rakeman *et al*., 1999). AH58, which is CS401 transformed with the SspH1-HA-HA-expressing plasmid pAH41 (Haraga and Miller, 2003), and JAF119, which is CS401 harbouring the SseJ-HA-HA-expressing plasmid pJAF111 (Freeman *et al*., 2003), have also been published. Human Intestine-407, HeLa and CHO-K1 cells were obtained from the ATCC and were cultured in Dulbecco's modified Eagle's medium (Intestine-407 and HeLa) or RPMI 1640 (CHO-K1) supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, at 37°C with 5% CO_2. For infection of mammalian cells bacterial strains were grown in Luria–Bertani broth overnight, and the cultures were diluted 1:50 and were grown for 3 h at 37°C with aeration. Bacteria were added to mammalian cells at the indicated moi in antibiotic-free culture medium for 1 h, after which the infected cells were washed three times with phosphate-buffered saline (PBS) and were incubated with culture medium containing 50 μg ml^{-1} gentamicin for the indicated length of time.

Plasmid constructions

pAH47 and pAH48 were constructed by inserting the *Shigella flexneri ipaH9.8* and the *Yersinia pestis yopM*, respectively, into the mammalian expression vector pCMV-HA (BD Biosciences). 3′ HA-tagged chimeric *S. enterica* serovar Typhimurium *sspH1*(1-1116)*sspH2*(1372-2367), *sspH1*(1-617)*sspH2*(637-2367), *sspH2*(1-1371)*sspH1*(1117-2103), *sspH2*(1-636)*sspH1*(618-2103), *slrP*(1-567)*sspH1*(618-2103) and *sspH1*(1-617)*slrP*(568-2298) open reading frames were generated by SOE PCR and were inserted into pTIGZ (Underhill *et al*., 1999), resulting in pAH11, pAH12, pAH13, pAH14, pAH18 and pAH25 respectively. pAH51, pAH53 and pAH55 were made by PCR amplifying the first 1680, 1311 and 1029 base pairs of human *PKN1*, respec-

tively, and inserting them into pCMV-Myc (BD Biosciences). 3′ FLAG-tagged 1312–2826 and 1030–2826 base pair fragments of human *PKN1* were PCR amplified and inserted into pRc/CMV (Invitrogen) to yield pAH54 and pAH56 respectively.

Transfections

Mammalian cells were transiently transfected by electroporation. Briefly, DNA in 50 μl of PBS was mixed with 5×10^6–10^7 cells in 0.2 ml of culture medium in a 0.4 cm gap electroporation cuvette (Bio-Rad). The cells were pulsed in a Bio-Rad Gene Pulser set at 260 V and 960 μFD and were washed with 5 ml of culture medium, before seeding them into 60 mm, 100 mm or 24-well tissue culture dishes. After allowing the cells to adhere to the dish for 3 h, the culture medium was changed again and left on until assaying.

Yeast two-hybrid screen

The pGBD-U plasmid pEM118 expressing the Gal4 DNA BD fused to SspH1 (Miao *et al*., 2003) was expressed in the *Saccharomyces cerevisiae* strain PJ69-4 (James *et al*., 1996). The resulting yeast strain then was transformed with Human Spleen MATCHMAKER cDNA Library (BD Biosciences), which expresses the yeast Gal4 AD fused to cDNA library inserts. Co-transformants were plated onto medium lacking uracil, leucine and histidine and containing 3 mM 3-aminotriazole. HIS3+ colonies were further tested for growth on adenine-deficient medium and for production of β-galactosidase by the filter assay (James *et al*., 1996). Clones expressing all three reporter genes, *HIS3*+ *ADE2*+ *LacZ*+, were then cured of the bait *sspH1* plasmid on plates containing 5-fluoro-orotic acid and the target cDNA plasmids were rescued. To verify the interactions fresh PJ69-4 was retransformed with each target plasmid and was mated with yeast expressing the bait, and the resultant diploids were screened for expression of the *HIS3*, *ADE2* and *LacZ* genes. PJ69-4 harbouring pEM119, a plasmid expressing a Gal4 BD–SspH2 fusion protein, and pEM125, which expresses Gal4 BD–SlrP, were used as controls (Miao *et al*., 2003).

Immunoprecipitations and immunoblotting

CHO-K1 cells were transfected with 5 μg of pRc/CMV/PKN-FL expressing human PKN1-FLAG (Takahashi *et al*., 1998), pRC/CMV/PKN/AF3-FL expressing PKN1(561–942)-FLAG, pAH51, pAH53, pAH54, pAH55 or pAH56, and 4 μg of pNeo/Tak expressing the tetracycline transactivator (Underhill *et al*., 1999), along with 5 μg of pAH8, pAH9, pAH10, pAH47 or pAH48, expressing SlrP-HA, SspH1-HA, SspH2-HA, HA-IpaH9.8 and HA-YopM respectively (Haraga and Miller, 2003). Transfected cells were seeded into 60 mm or 100 mm tissue culture dishes. Alternatively, HeLa cells were seeded into 100 mm tissue culture dishes and were infected with *S. enterica* serovar Typhimurium strain AH58 or JAF119 at an moi of 20. Twenty-four hours after transfection or 17 h after infection the cells were washed twice with PBS and were lysed with 0.5 ml of lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris pH 8.0 and 5 mM EDTA) supplemented with Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics) on ice for 30 min. The lysates were centrifuged at 10 000 r.p.m. for 30 min at 4°C, and the supernatants were incubated with mouse monoclonal anti-HA-agarose antibody conjugate or anti-FLAG M2 affinity gel (Sigma) for 4 h on a rotator at 4°C. The supernatants were collected and the precipitates were washed six to seven times with lysis buffer before SDS-polyacrylamide gel electrophoresis and immunoblotting.

HA-, FLAG- and Myc-tagged proteins were detected with HA.11 (Covance), anti-FLAG M2 (Sigma) and c-Myc mouse monoclonal antibodies (BD Biosciences) respectively. p38 was probed with anti-p38 MAPK rabbit polyclonal antibody (Cell Signaling Technology), while PKN1 and PRK2 were detected with anti-PKN1 goat (Santa Cruz Biotechnology) and anti-PRK2 rabbit polyclonal antibodies (Cell Signaling Technology) respectively. Phosphorylated PKN1 and p38 were blotted with rabbit anti-phospho-PRK1 (Thr774)/PRK2 (Thr816) and anti-phospho-p38 MAPK (Thr180/Tyr182) antibodies respectively (Cell Signaling Technology). The secondary anti-goat, anti-mouse and anti-rabbit horseradish peroxidase antibody conjugates were from Amersham Biosciences.

Luciferase assays

CHO-K1 or HeLa cells were transfected with 3 μg of pELAM-1-*fluc* expressing firefly luciferase from the NF-κB-dependent endothelial cell-leukocyte adhesion molecule (ELAM)-1 promoter (Schindler and Baichwal, 1994), 0.3 μg of pRL-TK expressing *Renilla* luciferase from the constitutive herpes simplex virus (HSV) thymidine kinase (TK) promoter (Promega), and the indicated plasmids. In addition, cells stimulated via toll-like receptor (TLR)-4 were also transfected with 5 μg of pCDNA3.1/Zeo/mCD14 expressing mouse CD14 (Underhill *et al*., 1999). RNA interference was performed by co-transfecting HeLa cells with 5 μg of PKN1-specific si*GENOME SMART*pool Reagent or 5 μg of siCONTROL Non-Targeting siRNA (Dharmacon). For testing the effect of PKN1 on NF-κB-dependent gene expression, pRC/CMV/PKN/AF3-FL (Takahashi *et al*., 1998), which is a mammalian expression vector for the constitutively active PKN1(561-942)-FLAG fragment, and pRC/CMV/PKN/AF3(K664E)-FL (Gao *et al*., 2000), which expresses the kinase-dead fragment PKN1(561-942, K644E)-FLAG, were used at 5 μg per transfection. Transfected cells were incubated for 24 or 48 h (RNAi) before assaying. CHO-K1 cells were incubated with 100 ng ml^{-1} *Escherichia coli* LPS (Sigma) or fresh medium, while HeLa cells were stimulated with 50 ng ml^{-1} TNF-α (Endogen) or medium alone for 4–5 h. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) in a Berthold LB9501 luminometer. Firefly luciferase activity was normalized to the activity of *Renilla* luciferase.

Statistical analyses

Values are expressed as the mean ± standard deviation of triplicate samples. Differences among groups were statistically analysed by a one-way analysis of variance (ANOVA) using the ANOVA1 function of MATLAB (The MathWorks, Natick, MA). A value of $P < 0.05$ was considered significant.

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References

Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., *et al.* (1996) Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. *Science* **271:** 648–650.

Buchanan, S.G., and Gay, N.J. (1996) Structural and functional diversity in the leucine-rich repeat family of proteins. *Prog Biophys Mol Biol* **65:** 1–44.

Dong, L.Q., Landa, L.R., Wick, M.J., Zhu, L., Mukai, H., Ono, Y., and Liu, F. (2000) Phosphorylation of protein kinase N by phosphoinositide-dependent protein kinase-1 mediates insulin signals to the actin cytoskeleton. *Proc Natl Acad Sci USA* **97:** 5089–5094.

Flynn, P., Mellor, H., Palmer, R., Panayotou, G., and Parker, P.J. (1998) Multiple interactions of PRK1 with RhoA. Functional assignment of the Hr1 repeat motif. *J Biol Chem* **273:** 2698–2705.

Flynn, P., Mellor, H., Casamassima, A., and Parker, P.J. (2000) Rho GTPase control of protein kinase C-related protein kinase activation by 3-phosphoinositide-dependent protein kinase. *J Biol Chem* **275:** 11064–11070.

Freeman, J.A., Ohl, M.E., and Miller, S.I. (2003) The *Salmonella enterica* serovar Typhimurium translocated effectors SseJ and SifB are targeted to the *Salmonella*-containing vacuole. *Infect Immun* **71:** 418–427.

Galan, J.E. (2001) *Salmonella* interactions with host cells: type III secretion at work. *Annu Rev Cell Dev Biol* **17:** 53–86.

Gao, Q., Kumar, A., Srinivasan, S., Singh, L., Mukai, H., Ono, Y., *et al.* (2000) PKN binds and phosphorylates human papillomavirus E6 oncoprotein. *J Biol Chem* **275:** 14824–14830.

Haraga, A., and Miller, S.I. (2003) A *Salmonella enterica* serovar Typhimurium translocated leucine-rich repeat effector protein inhibits NF-kappa B-dependent gene expression. *Infect Immun* **71:** 4052–4058.

Hobbie, S., Chen, L.M., Davis, R.J., and Galan, J.E. (1997) Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol* **159:** 5550–5559.

Isagawa, T., Takahashi, M., Kato, T., Jr, Mukai, H., and Ono, Y. (2005) Involvement of protein kinase PKN1 in G(2)/M delay caused by arsenite. *Mol Carcinog* **43:** 1–12.

James, P., Halladay, J., and Craig, E.A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144:** 1425–1436.

Kawamata, T., Taniguchi, T., Mukai, H., Kitagawa, M., Hashimoto, T., Maeda, K., *et al.* (1998) A protein kinase, PKN, accumulates in Alzheimer neurofibrillary tangles and associated endoplasmic reticulum-derived vesicles and phosphorylates tau protein. *J Neurosci* **18:** 7402–7410.

Kim, D.E., Chivian, D., and Baker, D. (2004) Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Res* **32:** W526–W531.

Kitagawa, M., Shibata, H., Toshimori, M., Mukai, H., and Ono, Y. (1996) The role of the unique motifs in the amino-terminal region of PKN on its enzymatic activity. *Biochem Biophys Res Commun* **220:** 963–968.

McDonald, C., Vacratsis, P.O., Bliska, J.B., and Dixon, J.E. (2003) The *Yersinia* virulence factor YopM forms a novel protein complex with two cellular kinases. *J Biol Chem* **278:** 18514–18523.

Maesaki, R., Ihara, K., Shimizu, T., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1999) The structural basis of Rho effector recognition revealed by the crystal structure of human RhoA complexed with the effector domain of PKN/PRK1. *Mol Cell* **4:** 793–803.

Miao, E.A., Scherer, C.A., Tsolis, R.M., Kingsley, R.A., Adams, L.G., Baumler, A.J., and Miller, S.I. (1999) *Salmonella typhimurium* leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. *Mol Microbiol* **34:** 850–864.

Miao, E.A., Brittnacher, M., Haraga, A., Jeng, R.L., Welch, M.D., and Miller, S.I. (2003) *Salmonella* effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. *Mol Microbiol* **48:** 401–415.

Mukai, H. (2003) The structure and function of PKN, a protein kinase having a catalytic domain homologous to that of PKC. *J Biochem (Tokyo)* **133:** 17–27.

Mukai, H., Kitagawa, M., Shibata, H., Takanaga, H., Mori, K., Shimakawa, M., *et al.* (1994) Activation of PKN, a novel 120-kDa protein kinase with leucine zipper-like sequences, by unsaturated fatty acids and by limited proteolysis. *Biochem Biophys Res Commun* **204:** 348–356.

Mukai, H., Miyahara, M., Sunakawa, H., Shibata, H., Toshimori, M., Kitagawa, M., *et al.* (1996) Translocation of PKN from the cytosol to the nucleus induced by stresses. *Proc Natl Acad Sci USA* **93:** 10195–10199.

Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., *et al.* (2003) Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell* **112:** 859–871.

Owen, D., Lowe, P.N., Nietlispach, D., Brosnan, C.E., Chirgadze, D.Y., Parker, P.J., *et al.* (2003) Molecular dissection of the interaction between the small G proteins Rac1 and RhoA and protein kinase C-related kinase 1 (PRK1). *J Biol Chem* **278:** 50578–50587.

Pegues, D.A., Ohl, M.E., and Miller, S.I. (2005) *Salmonella* species, including *Salmonella typhi*. In *Principles and Practice of Infectious Diseases*. Mandell, G.L., Bennet, J.E., and Dolin, R. (eds). New York: Churchill Livingstone, pp. 2636–2654.

Rakeman, J.L., Bonifield, H.R., and Miller, S.I. (1999) A HilA-independent pathway to *Salmonella typhimurium* invasion gene transcription. *J Bacteriol* **181:** 3096–3104.

Schindler, U., and Baichwal, V.R. (1994) Three NF-kappa B binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. *Mol Cell Biol* **14:** 5820–5831.

Takahashi, M., Mukai, H., Toshimori, M., Miyamoto, M., and Ono, Y. (1998) Proteolytic activation of PKN by caspase-3 or related protease during apoptosis. *Proc Natl Acad Sci USA* **95:** 11566–11571.

Takahashi, M., Gotoh, Y., Isagawa, T., Nishimura, T.,

Goyama, E., Kim, H.S., *et al.* (2003) Regulation of a mitogen-activated protein kinase kinase kinase, MLTK by PKN. *J Biochem (Tokyo)* **133:** 181–187.

Terada, T., Ito, Y., Shirouzu, M., Tateno, M., Hashimoto, K., Kigawa, T., *et al.* (1999) Nuclear magnetic resonance and molecular dynamics studies on the interactions of the Ras-binding domain of Raf-1 with wild-type and mutant Ras proteins. *J Mol Biol* **286:** 219–232.

Torbett, N.E., Casamassima, A., and Parker, P.J. (2003) Hyperosmotic-induced protein kinase N 1 activation in a vesicular compartment is dependent upon Rac1 and 3-phosphoinositide-dependent kinase 1. *J Biol Chem* **278:** 32344–32351.

Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M., and Aderem, A. (1999) The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* **401:** 811–815.

Zhu, Y., Stolz, D.B., Guo, F., Ross, M.A., Watkins, S.C., Tan, B.J., *et al.* (2004) Signaling via a novel integral plasma membrane pool of a serine/threonine protein kinase PRK1 in mammalian cells. *FASEB J* **18:** 1722–1724.

