REVIEWS

Structural insight into effector proteins of Gram-negative bacterial pathogens that modulate the phosphoproteome of their host

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Abstract: Invading pathogens manipulate cellular process of the host cell to establish a safe replicative niche. To this end they secrete a spectrum of proteins called effectors that modify cellular environment through a variety of mechanisms. One of the most important mechanisms is the manipulation of cellular signaling through modifications of the cellular phosphoproteome. Phosphorylation/dephosphorylation plays a pivotal role in eukaryotic cell signaling, with ~500 different kinases and ~130 phosphatases in the human genome. Pathogens affect the phosphoproteome either directly through the action of bacterial effectors, and/or indirectly through downstream effects of host proteins modified by the effectors. Here we review the current knowledge of the structure, catalytic mechanism and function of bacterial effectors that modify directly the phosphorylation state of host proteins. These effectors belong to four enzyme classes: kinases, phosphatases, phospholyases and serine/threonine acetylases.

Keywords: bacterial effector kinases; bacterial phosphatases; bacterial phospholyases; bacterial acetylases; structure-function relationship; noneukaryotic-like kinases

Introduction

Pathogenic bacteria have evolved diverse mechanisms to invade and establish a replicative niche within eukaryotic cells, and in higher eukaryotes in particular. These mechanisms include, but are not limited to, quorum sensing, biofilm formation, cell adhesion, secretion of toxins and other virulence proteins to the host cell. Most Gram-negative pathogens utilize either Type 3 or Type 4 secretion systems (T3SS and T4SS),[1,2] which act as macromolecular syringes allowing bacteria to inject a set of effector proteins inside the host cell. These effectors modify normal cellular processes to sustain bacterial proliferation. T3SS is evolutionary related to the bacterial flagellum, and T4SS to the conjugation apparatus. Depending on the bacteria replication cycle, the number of secretion systems varies from one [T3SS—*Shigella,* enteropathogenic *Escherichia coli* (EPEC); T4SS—*Legionella*], two [T3SS—*Yersinia*, *Salmonella*, enterohaemorrhagic *E. coli* (EHEC)], and up to three (T3SS—*Burkholderia pseudomallei*). The multiple secretion systems are activated at different stages of infection. For example, *Salmonella* Pathogenicity Island 1 (SPI1) T3SS is turned on early, during bacterial internalization. The SPI2 T3SS is activated later during infection and delivers effectors necessary for the formation of the *Salmonella*-containing vacuole (SCV), where the bacterium

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survives and replicates, and *Salmonella*-induced filaments (SIFs).[3]

Although the effectors are tailored precisely to the bacterial life cycle and the host cellular environment, nevertheless common themes occur among various pathogens.[4] Some of the effectors are enzymes, for example, proteases, phosphatases, glycosylases, acetylases, or lipases, others mimic functions of host proteins, yet others are transcription factors or protein–protein interaction partners.[5] The effectors interfere in various host cellular processes, such as cytoskeleton rearrangement, signaling, cellular adherence, transcription, vesicular trafficking, membrane biogenesis, apoptosis and metabolism. Some of the characterized effectors display new folds and catalyze previously undiscovered reactions. The number of effectors secreted by pathogens varies greatly from ~20 in EPEC,[6] to ~60 in EHEC[7] and as many as ~300 in *Legionella*.[8] The unusual abundance of effectors in *Legionella* is thought to be related to the diversity of *Legionella* primary hosts, that is amoebas, in which the bacterium replicates.[9]

Phosphorylation/dephosphorylation plays a pivotal role in eukaryotic cell signaling, with ~500 different kinases in the human genome, representing around 1.7% of all genes.[10] There are ~30 catalytic domains of dual-specificity phosphatases, which are paired with multiple specificity domains, and ~100 tyrosine phosphatases.[11] Although the ability of pathogenic bacteria to influence host phosphorylation was known for a long time, it is only recently that extensive phosphoproteome studies shed light on substantial changes in the host phosphoproteome caused by the pathogens. Tracking of phosphorylation changes in HeLa cells during 2 h after *Shigella flexneri* infection using a label-free quantitative proteomics approach showed that the changes in the phosphorylation profile are substantial and dynamic, changing with time.[12] Gene ontology mapping of the proteins differentially phosphorylated during infection classified them by function into signal transduction (50 proteins), actin cytoskeleton (40), exocytosis, endocytosis and intracellular transport (24), RNA processing (24), and cell cycle (23). The consensus phosphorylation motifs suggested that the changes were most likely associated with ACG kinases AKT, PKA/PHC/PKG, RSK, CamKII, as well as MAPK, CDC2, CDK, CK2 and ATM/ATR/DNAPK. The mTOR pathway was overrepresented in the phosphoproteome and important for activation of both S6 and AKT kinases.[12] Only few *Shigella* effectors are known to interfere with host protein phosphorylation (OspF (PDB: 3I0U), OspG; see below). Comparison of the phosphoproteome of HeLa cells infected with wild-type or Δ*ospF Shigella* strains identified ~140 proteins that showed differences in their phosphorylation,[12] providing evidence for the global role of this effector.

The phosphoproteome of *Salmonella*-infected HeLa cells was investigated 20 minutes post-infection[13] and in RAW264.7 microphages 8 h postinfection[14] using SILAC quantitative mass spectrometry. At 20 minutes post-infection ~500 proteins showed changes in their phosphorylation, with the nuclear and membrane fractions enriched in proteins with decreased phosphorylation. Gene Ontology mapping showed that peptides with increased phosphorylation participate in apoptosis, transmembrane transport, nuclear organization and cell proliferation, while peptides with decreased phosphorylation are involved in cytoskeleton organization, protein complex assembly and cell polarity. The phosphorylation consensus sequences indicated increased activity of PKC/PKG, AKT, PIM family kinases, S6 kinase, mTOR, Src, ERK, and others. Comparing the effects of *Salmonella* and *Shigella* at early post-infection times identified 57 common phosphorylated proteins, indicating overlap of the molecular mechanisms of these two pathogens in epithelial cell invasion.[14]

Similarly to Δ*ospF Shigella*, the Δ*sopB* (inositol phosphate phosphatase) *Salmonella* strain showed a 35% decrease in the number of proteins with an altered phosphorylation pattern,[13] even though the mechanism of these two proteins are different. Enrichment of peptides with a RxRxxS motif suggested significant involvement of AKT, Rsk and p70S6K kinases. An observed increase in BAD phosphorylation at Ser99, which inactivates its proapoptotic functions, and an increase in Ser126 and Ser129 phosphorylation of syntaxin 7, which promotes membrane fusion in the endosomal pathway, may be linked to the action of these kinases during *Salmonella* infection.

The *Salmonella* SPI2 T3SS is activated at a longer post-infection time. This T3SS is essential for the pathogen survival in macrophages but less crucial for survival in epithelial HeLa cells. Out of 442 proteins in RAW264.7 macrophages and 606 in Hela cells with changed phosphorylation profile there were 355 common proteins.[14] There were however substantial differences in overall functions of the affected proteins. As deducted from the GO terms, proteins affected in macrophages are involved in protein transport, actin regulation and immune signaling, processes that were frequently associated with SPI2 effectors. In epithelial cells the most impacted proteins were involved in apoptosis and regulation of gene expression, in line with previous reports that in this type of cell apoptosis is attenuated. A thorough analysis of phosphorylation patterns in macrophages revealed that the ERK1/2, CDK. PKA, PKC, and MAPK kinases are the key regulators in macrophages in the late stages of infection affected by the SPI2 effectors.

In light of such dramatic rearrangement of host cell phosphoproteome, the question "What are the mechanisms by which pathogens modify host cell phosphorylation?" becomes paramount. These mechanisms may be classified as direct, caused by the bacterial effectors or indirect, caused by the downstream effects of host proteins modified by the effectors. Four direct mechanisms of bacterial effectors that influence host cell phosphoproteome have been discovered:

- Phosphorylation. Kinases reversibly phosphorylate Ser and Thr residues of the host cell proteins.
- Phosphate hydrolysis. Phosphatases dephosphorylate phosphoSer and phosphoThr residues.
- Phosphate elimination. PhosphoSer- and phosphoThr-lyases eliminate the phosphate and convert Ser and Thr to dehydroalanine and methyldehydroalanine, respectively.
- Acetylation. Acetylases irreversibly acetylate specific Ser or Thr residues in kinases.

The role of these bacterial effectors in host-pathogen interactions was discussed in a recent review.[15–17] Here we review the structural information available for the above four classes of bacterial effectors and their catalytic mechanisms.

Bacterial Effector Kinase Family

Analysis of gene and protein sequences showed presence of eukaryotic-like proteins among bacterial effectors, indicating that they were acquired through lateral transfer from their hosts late during evolution.[18,19] Several bacterial effector kinases belong to this category including *Salmonella* Typhimurium SteC,[20] *Yersinia pestis* YpkA,[21] and *Legionella pneumophila* LegK1, LegK3, LegK4.[22] In addition, bacteria evolved their own effector kinases. These kinases belong to the kinase superfamily but are more similar to nonregulatory kinases. They include NleH1 (PDB: 4LRJ) and NleH2 (PDB: 4LRK) from pathogenic *E. coli*,[23] OspG from *Shigella*,[24] SboH from *Salmonella bongori*[25] and YspK from *Yersinia*.[26] Bioinformatics analysis shows that although the kinase domain is much smaller than the average human kinase, it contains all the essential elements characteristic of kinases [Fig. 1(a)]. Of the eleven motifs characteristic of regulatory kinases,[27] the bacterial effector kinase domain spans motifs I–VIII but lacks the activation loop. Recent structural studies of NleH1, NleH2, and OspG revealed the structural organization of these kinases[28–31] and allowed building a structure-based phylogenetic tree, which indicated that the bacterial effector kinases form a new family within the kinase superfamily[28] [Fig. 1(b)]. Although Hervet *et al*.[32] argued that *Legionella* LegK2 lacks the activation loop [Fig. 1(a)], the classification of LegK2 kinase in one of these two classes is presently uncertain. So far three-dimensional structures are known only for the noneukaryotic-like kinases.

Noneukaryotic-like kinases

Functional aspects. Functional aspects of bacterial effector kinases were reviewed recently,[17] therefore our discussion of functional aspects of effector kinases in this review is brief. NleH1 and NleH2 are homologous effector serine/threonine protein kinases (STPK) (84% sequence identity) from pathogenic *E. coli* strain.[37] Related *Citrobacter rodentium* possesses only one copy of NleH.[33] These proteins are composed of a C-terminal kinase module (~160 amino acids) and an N-terminal segment of ~140 amino acids with the "telltale" signature of an intrinsically disordered domain. The first 25 amino acids are believed to contain the T3SS secretion signal.[34]

The clearest function of NleHs is the inhibition of the NF-κB pathway.[35] NleH1 was shown to bind the ribosomal protein 3 (RPS3) and prevent its phosphorylation by IKKβ.[36] As a consequence, the RPS3 is not translocated to the nucleus to serve as a subunit for NF-κB transcriptional complexes. The kinase activity of NleH1 is also essential for this effect, since the active site mutant of NleH1 is not able to inhibit the NF-κB pathway.[35] However, the phosphorylation target of NleH1 is not yet known. NleH2, despite being homologous to NleH1, has apparently an opposite effect and mildly activates the NF-κB pathway.[37] In addition, NleHs were shown to bind PDZ2 domain of the Na+/H+ Exchanger Regulatory Factor 2 (NHERF2) *via* four C-terminal residues[38] and deletion of this motif blocked the NF-κB inhibition activity of NleH1.[37] Finally, NleHs were able to inhibit apoptosis by binding to the N-terminal segment of Bax Inhibitor 1.[23]

OspG is a *Shigella* effector kinase composed of only the kinase module (170 amino acids) and an N-terminal secretion signal (25 amino acids). Intriguingly, OspG also suppresses the NF-κB pathway but does it by stabilizing IκB.[24] As with NleHs, the kinase activity was shown to be important for this biological function, however the phosphorylation target is also unknown. OspG binds ubiquitin[39] and a series of E2~Ub conjugates,[24] however, the relevance of this interaction for the NF-κB inhibition is unclear.

The effector kinase domain. The ~160 residues long effector kinase module of this protein family is significantly smaller than the eukaryotic regulatory kinases that are over 300 amino acids long. The module is composed of a mostly β-stranded N-terminal domain and a C-terminal mixed α/β domain, joined together by a short connector[28] [Fig. 2(a)].

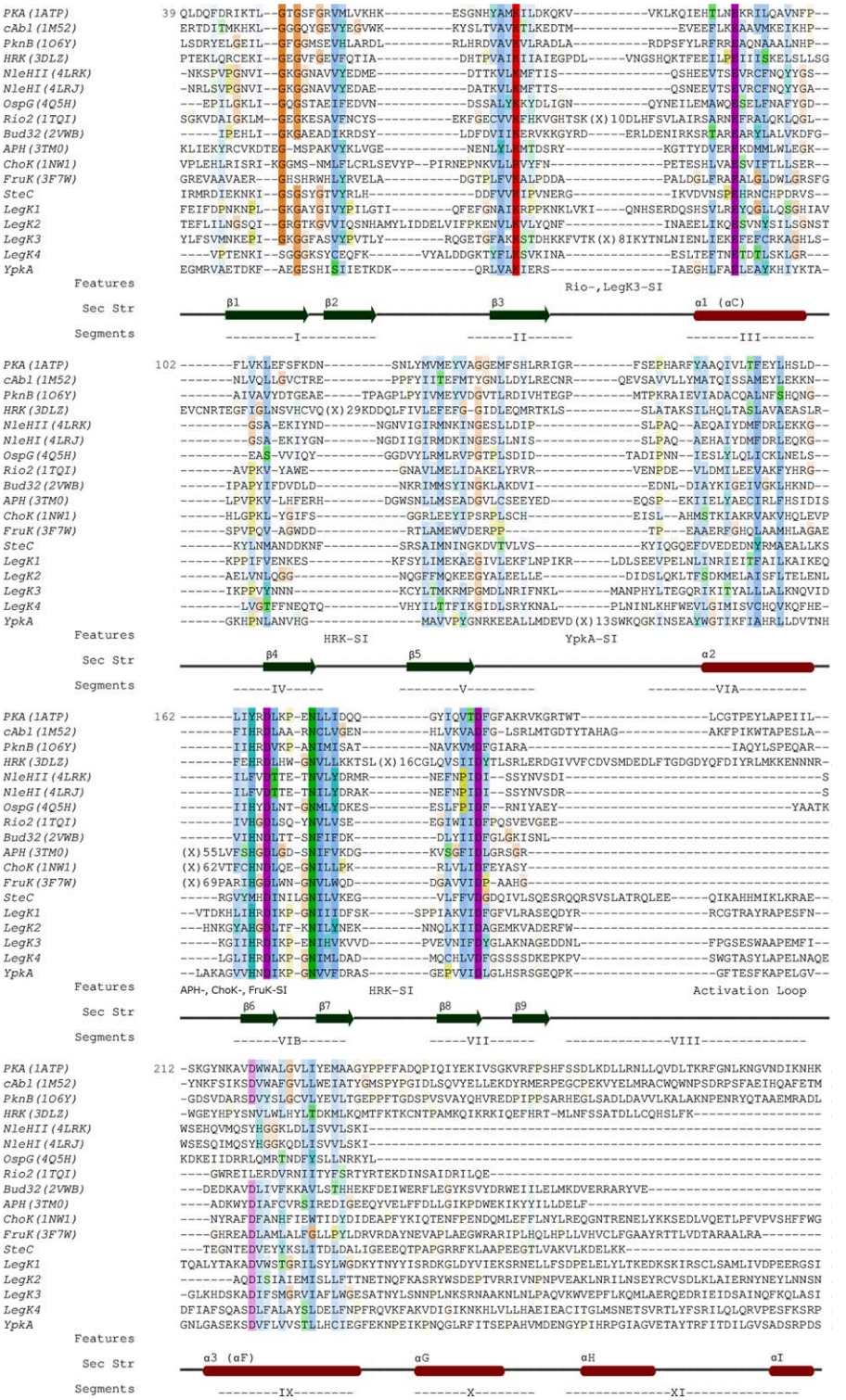

Figure 1. (A) Structure-based sequence alignment of several mammalian kinases and effector kinases with known structures. Highly conserved residues are color coded: hydrophobic residues are blue, His and Tyr—cyan, polar residues—green, Arg and Lys—red, Asp and Glu—magenta, Gly—orange, and Pro—olive. Higher color saturation reflects higher level of conservation. Other effector kinases were aligned based on their sequences. PKA—human protein kinase A, cABL—mouse cABL kinase, PknB—*Mycobacterium tuberculosis kinase PknB,* HRK—atypical human kinase haspin, NleHI and NleHII—*E. coli* O157:H7 kinases, OspG—*Shigella flexneri* kinase, Rio2—*Archaeoglobus fulgidus* Rio2 kinase, Bud32—*Methanocaldococcus jannaschii* Bud32 kinase, APH—*Enterococcus faecalis* 3′,5"-Aminoglycoside Phosphotransferase, CHOK—*Caenorhabditis elegans* choline kinase, FruK—*Thermobifida fusca* fructosamine-3-kinase, SteC—*Salmonella* Typhimurium kinase, LegK1, LegK2, LegK3, LegK4—*Legionella pneumophila* kinases, YpkA—*Yersinia pestis* kinase. The secondary structure elements are marked below the sequences together with the motifs I-XI [27]; (B) evolutionary tree built based on structure conservation with program Multiseq.[154]

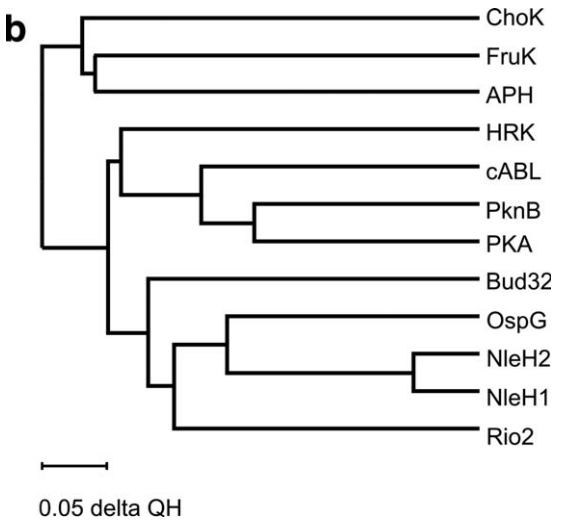

Figure 1. (Continued)

The N-terminal domain contains motifs I to IV,[27] covering the glycine loop and the catalytic Lys, which is properly oriented *via* a salt bridge with a neighboring Glu. The C-terminal domain includes motifs V-IX that include the catalytic loop and the DFG-motif. Motif VIII, which comprises the activation loop, is very short in NleHs[28] and slightly longer in OspG.[29,31] The catalytic site is situated in a deep crevice between the two subdomains, next to the ATP and Mg^{2+} binding site. Site-directed mutagenesis showed the importance for catalysis of Lys169 (motif II), Glu183 (motif III), Asp249 (motif VIB), and Asp268 (motif VII)[28] in NleH2 and of Lys53 (motif II) and Asp 138 (motif VIB) in OspG.[39] Similarly to other kinases, NleHs and OspG possess two arrangements of hydrophobic residues called the catalytic and regulatory spines.[40,41] The catalytic spine is formed by eight resides from both domains of the kinase that upon ATP binding create a contiguous stack with the adenine ring in the middle. The regulatory spine contains four hydrophobic residues that interact with each other only in the active conformation of regulatory kinases.

The shorter activation loop is devoid of the regulatory phosphorylation site, which poses the question if these kinases are constitutively active or are activated differently than the eukaryotic regulatory kinases. The crystal structures showed that the NleH1 and NleH2 kinase domains were already in the active conformation both in the apo- and AMPPNP-bound structures.[28,30] Not only does the catalytic machinery align well with that of protein kinase A in the active conformation [Fig. 2(b)] but also the two hydrophobic spines are fully assembled. The adenine ring complements the catalytic spine upon ATP binding. The nucleotide binding proceeds with minimal structural rearrangements. The biochemical data support the notion that NleHs do not require a special activation mechanism. Even when

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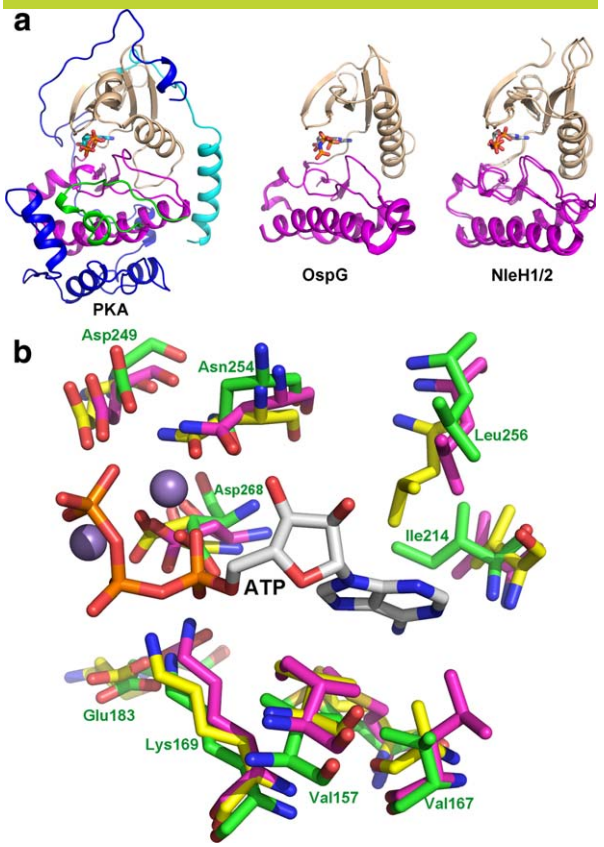

Figure 2. (A) Cartoon representation of kinase domains of OspG and NleH1/2 showing their overall similarity. The human protein kinase A is shown for comparison. The N-terminal lobe is painted wheat, the C-terminal is magenta and the activation loop in green. The N-terminal extension of PKA is in cyan and the C-terminal extension that embraces the N-terminal lobe is in dark blue; (B) superposition of the residues forming the ATP binding site. The carbons in PKA are painted yellow, in OspG they are magenta, in NleH2 they are green. The ATP (white carbons) and two Mn^{2+} ions (spheres) were taken from the PKA structure (PDB code 1ATP). The residue numbers are provided based on NleH2 structure. This and the following figures were prepared using PyMol (www.pymol.org). An interactive view is available in the electronic version of the article.

purified from *E. coli*, NleHs are phosphorylated with an average of 2–3 phosphate groups per molecule, which was attributed to autophosphorylation.[28] The activity assays with radioactive [γ-^{32}P]ATP showed that both full-length NleHs could autophosphorylate and that both the NleHs full-length and kinase domains could phosphorylate the generic kinase substrate myelin basic protein (MBP). Mapping the phosphorylation sites has shown that most of them were located in the N-terminal unstructured segment of NleH.[28]

OspG was shown to bind Ub and the UbcH7~Ub conjugate.[24,39] By itself OspG showed

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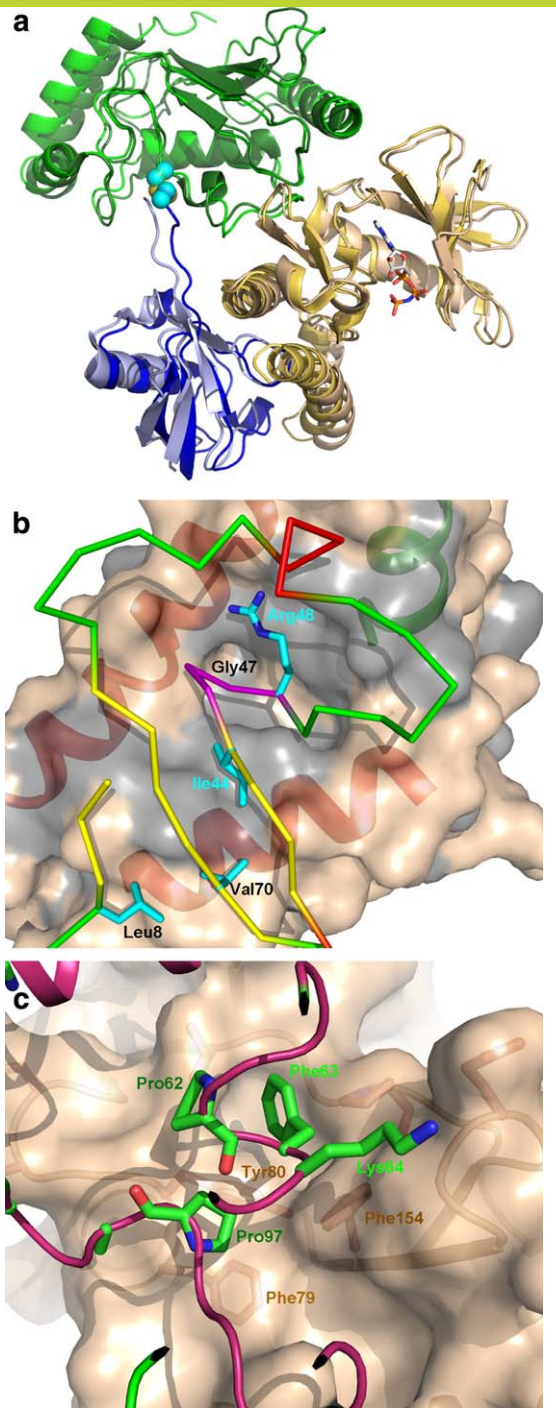

Figure 3. (A) Cartoon representation of OspG-E2∼Ub complexes. The UbcH5 and UbcH7 complexes are superimposed and are nearly identical. OspG is painted wheat, UbcH5/7 is green and Ub is blue. The darker shades show the UbcH7 complex; (B) the OspG-Ub interface. The OspG is shown as molecular surface colored wheat with gray marking hydrophobic residues. The Ub faces OspG with the Ile44 side and inserts a loop culminating with GlyXX into a deep depression in OspG surface; (C) the UbcH7 residue contacting OspG surface. Phe63 plays a key role in these interactions. An interactive view is available in the electronic version of the article.

low intrinsic activity against another generic kinase substrate—histones. However, the activity increased 8- and 20-fold upon binding to Ub and the UbcH7∼Ub conjugate, respectively,[29,31,39] indicating a novel activation mechanism. Moreover, the affinity toward nonhydrolysable ATP-γS was higher when OspG was complexed with the UbcH5∼Ub (PDB: 4BVU) conjugate.[31] The structures of the complexes of OspG with UbcH7∼Ub and UbcH5∼Ub have shown that UbcH5/7∼Ub conjugate binds to two sites on OspG, leaving the active site open for the interaction with the kinase substrate[29,31] [Fig. 3(a)]. These observations suggest that the interactions of OspG with Ub and the ubiquitin conjugating enzyme shift the conformation of OspG toward a fully active conformation.[31] Another possible explanation is that Ub binding affects the catalytic machinery of OspG to adopt productive conformation, as some Ub residues are less than 8 Å away from the catalytic loop.[29] Another indication of conformational flexibility of OspG is the difficulty of obtaining crystals of this protein by itself or in the presence of ATP. These structures are essential to understand the details of the activation mechanism.

Currently, the phosphorylation targets of effector kinases remain unknown. However, recently the v-crk Sarcoma Virus CT10 Oncogene-like Protein (CRKL) was proposed as the first NleH1 target.[42]

Structural aspects of protein–protein interactions. Notwithstanding a relatively small size, the effector kinases were shown to participate in several protein–protein interactions.[23,36,38] No structural information on NleH-host protein complexes is yet available, and complex formation with previously identified host target proteins has proved to be challenging (Grishin AM, unpublished results). However, the structures of OspG with ubiquitin-conjugating enzyme conjugated with ubiquitin (E2∼Ub) have recently been determined.[29,31] Several ubiquitinated E2s were initially identified by a yeast two-hybrid screen as the host proteins binding to OspG.[24] Subsequently, it was shown that OspG also interacts with ubiquitin and polyubiquitin chains.[39] The binding constant of OspG to UbcH7-Ub (580 n*M*) was 15 times higher than to Ub (9 μ*M*) and 150 times higher than to UbcH7 (∼90 μ*M*). Importantly, the UbcH5/7∼Ub increased the OspG kinase activity more than Ub alone. Taken together, it was concluded that the E2∼Ub conjugate is the OspG cellular binding target.[29]

UbcH5/7∼Ub associates with OspG in an open conformation, essentially the same as observed when bound to a HECT-type E3s,[43] and with the same surfaces on E2s and Ub involved in both these interactions. Ub binds OspG through the well-known Ile44 hydrophobic patch (Leu8, Ile44, and Val70) and UbcHs through α-helix 1, loop4 and loop

$7^{29,31}$ [Fig. 3(b,c)]. These two surfaces have been previously characterized as involved in the recognition and functionality of the ubiquitination machinery.[44–49] The OspG Ub-binding surface is a large concave hydrophobic surface formed between the C-terminal and the penultimate α-helices and the shortened "activation" loop [Fig. 3(b)]. In addition to hydrophobic contacts a salt bridge between $Lys48^{Ub}$ and $Asp177^{OspG}$ is formed. Comparison with NleHs and PKA showed that the OspG C-terminal helix rotates away from the penultimate helix, with one end moving by ~7 Å, to create this surface.[29,31] Whether this conformation of the C-terminal α-helix is induced by ubiquitin binding or is a feature of OspG will be answered by structure determination of OspG alone. UbcH5/7 binds through key residues Pro62, Phe63 and Pro97 to a hydrophobic patch on OspG involving residues from both lobes (Phe79, Tyr80, Leu99, Pro102, and Phe154) on the opposite side to the active site [Fig. 3(c)]. Residues located around this central hydrophobic zone formed additional polar interactions.[29,31]

Effects of protein–protein interactions. OspG binding to the same surface of E2~Ub conjugate as do the E3 ligases suggested that OspG might interfere with the host E3 functions. Indeed, the addition of OspG to a parkin autoubiquitination assay completely inhibited the autoubiquitination *in vitro* at 1:1 OspG:UbcH7 (PDB: 4Q5H) ratio.[29] Whether OspG affects the host ubiquitination machinery *in vivo* is unknown. However, several mutations disrupting OspG:E2 or OspG:Ub interfaces significantly reduced the inhibition of the NFκB pathway. For example, the OspG Leu190Asp/Leu191Asp double mutation, which targeted the interactions of the C-terminal α-helix with the rest of the molecule, rendered OspG incapable of binding to Ub and blocking IκB degradation.[39] Similarly, the OspG Cys127Arg mutation on the Ub-binding interface not only blocks the activation of the kinase activity by UbcH5~Ub conjugate but also results in the mouse infection phenotype, similar to the deletion of OspG altogether.[31] Moreover, OspG Phe154Arg and Leu99Arg/Pro102Glu mutations within the E2 binding interface also affected OspG function. The Leu99Arg/Pro102Glu mutant was no longer activated by E2~Ub while the Phe154Arg mutant was only marginally activated. The mutations also affected stability of OspG *in vivo*; the mutants, but not the wild-type OspG, were degraded quickly in a proteasome-dependent manner.[29]

Legionella kinase LegK2. LegK2 is a 62-kDa translocated effector of T4SS.[50] Kinase domain of LegK2 resides in the N-terminal region (aa 77–232) and has all the consensus sequences in Hanks subdomains I-VII, required for catalytic activity.[32] LegK2 undergoes autophosphorylation and phosphorylates the general eukaryotic protein kinase substrate MBP *in vitro* in the presence of Mn^{2+} ions. LegK2 is the only *Legionella* kinase that proved to be indispensable for virulence toward amoeba. Kinase activity of LegK2 is required for efficient recruitment of ER to Legionella-containing vacuoles, necessary for intracellular replication of bacteria.[32] Substrates of LegK2 have not been identified yet, and functions of the C-terminal nonkinase domain of this effector remain to be elucidated.

Eukaryotic-like Ser/Thr protein kinases

Structural aspects of Legionella kinases. *L. pneumophila* strains encode three translocated eukaryotic-like serine/threonine protein kinases LegK1, LegK3 and LegK4.[32,51] Multiple sequence alignments of kinase domains from *L. pneumophila* Paris with other prokaryotic and eukaryotic STPKs [Fig. 1(a)] revealed that LegK1 and LegK3 cluster in the group of eukaryotic protein kinases, close to STPKs from pathogenic anaerobic amoeba *Entamoeba histolytica*.[18] This observation, together with the lower GC content (33%) of the LegK3 gene (lpp2626) compared to the average of 38% for protein-coding genes of *L. pneumophila* Paris, suggested it was acquired by a horizontal gene transfer (Garcia-Vallve *et al*. 2003).[52] However, there is no direct evidence for a lateral transfer of LegK1 genes from eukaryotic organisms. The same phylogenetic analysis demonstrated that the STPK domain of LegK4 is closely related to PknG (from *M. tuberculosis*) and to the *Y. pseudotuberculosis* translocated STPK YpkA.[18]

Alignments with several prokaryotic and eukaryotic protein kinases revealed that all three kinases harbor highly conserved residues in subdomains I-IX [Fig. 1(a)].[27,53] These include the glycine rich loop in subdomain I, the invariant Lys in subdomain II, which anchor and orients ATP in the kinase active site, and the conserved Glu (Lys in case of LegK3) in subdomain III that stabilizes this interaction. The HRDxKxxN motif in the catalytic loop and the conserved $D^{F}/_{Y}G$ triplet in subdomain VII participate in chelating Mg^{2+} ions. All the three kinases have an activation loop (subdomain VIII) with the conserved APE motif, which plays a significant role in substrate recognition.

Based on *in vitro* studies, LegK1 is expected to be constitutively active or use an activation loop-independent activation mechanism, since mutations of both Ser252 and Tyr256, the only potential phosphorylation sites in the activation loop, did not affect the enzyme activity.[51] LegK3 and LegK4 undergo autophosphorylation and phosphorylate the general eukaryotic protein kinase substrate MBP, as demonstrated by *in vitro* kinase assay. LegK1 phosphorylates specifically its known substrate IkB, but not MBP. All three kinases

require Mg^{2+} ions for kinase activity while LegK4 is also able to use Mn^{2+} with the same efficiency.[32]

Physiological role of Legionella eukaryotic-like kinases. Separate inactivation of LegK1, LegK3 or LegK4 kinase genes did not influence bacterial growth in liquid medium or cellular morphology, nor did it affect *Legionella* virulence toward amoeba.[32] However, overexpression of one of the kinases, LegK1, in mammalian HEK293T cells significantly upregulated the NF-κB pathway, as demonstrated by NF-κB luciferase reporter studies.[51,54] The kinase activity of LegK1 was responsible for this effect, since the mutation of a conserved catalytic Asp223 from the catalytic loop to an alanine completely abolished NF-kB reporter activation.[54] Recombinant LegK1 triggered phosphorylation in cell-free extracts and also phosphorylated IκB on Ser32 and Ser36 *in vitro*. LegK1 efficiently phosphorylated four other IκB family members (IκBβ, p100, p105, and IκBϵ), which harbor the characteristic DSGXXS/T phosphomotif.[51] Further truncation studies demonstrated that the N-terminal pre-kinase region of LegK1 was indispensable for IκBα phosphorylation, whereas the C-terminal domain was not strictly required for enzyme activity; full-length LegK1 and a truncated LegK1(1–386) phosphorylated IκBα with a comparable efficiency in radioactive ^{32}P incorporation assay, however deletion of the first 45 residues abolished *in vitro* phosphorylation of IκBα.[51]

The functions of LegK3 and LegK4 remain largely unknown. A *L. pneumophila* mutant deficient in *legK1*, *legK2* and *legK3* genes still induced p38 and SAPK/JNK MAPK activation.[50] In support of this result, expression of LegK1 and LegK3 in mammalian cells did not affect MAPKs activation. According to the same study, overexpression of these proteins did not activate the IFNβ promoter in two different luciferase reporter systems.[51]

Salmonella kinase SteC. SteC is the only secreted protein kinase effector of *Salmonella enterica* serovar Typhimurium and is delivered by SPI2 T3SS[20] across the Salmonella-containing vesicle (SCV) vacuolar membrane. SCVs are surrounded by an actin cytoskeleton meshwork, presumably involved in maintaining the integrity of vacuolar membranes.[55,56] Formation of this network is dependent on SteC and its kinase activity.[20]

The SteC C-terminal kinase domain (aa 201–457) contains the characteristic kinase subdomains I to IX with a ~35 residue long activation loop and is most similar to the human RAF proto-oncogene STPK Raf-1.[20,57] SteC and its kinase domain undergo autophosphorylation and phosphorylate the general kinase substrate MBP *in vitro*.[20] This kinase may play a role in colonization of the chick intestine as demonstrated by gene inactivation studies.[58] Moreover, SteC-null and kinase-deficient mutant strains displayed increased replication in infected cells and in a mouse model of systemic infection, suggesting that SteC could serve to restrain bacterial growth and thus regulate virulence.[59] The mechanism of action of SteC involves actin rearrangement through activation of a signaling pathway containing the MAP kinases MEK and ERK, myosin light chain kinase (MLCK) and Myosin IIB.[59] SteC phosphorylates MEK on Ser200, which leads to MEK autoactivation through autophosphorylation of Ser218 and Ser222.[59]

A recent phosphoproteomics analysis of macrophage RAW264.7 and HeLa cells infected with *Salmonella* identified chaperone HSP27 as another potential substrate of SteC. This chaperone is phosphorylated *in vivo* on Ser15 and on at least six other sites *in vitro*, indicating that it might be a direct substrate of SteC and a part of a parallel route to SteC-induced host actin manipulation.[14] Moreover, the described interaction between the N-terminal non-kinase domain of SteC and the yeast guanine nucleotide exchange factor CDC24, the activator of a small GTPase CDC42, may suggest that both domains of SteC complement each other in actin modulation.[60]

Yersinia protein kinase A (YpkA). YpkA from *Y. pseudotuberculosis* (YopO in *Y. enterocolitica*) is a 729 amino acids multidomain protein with a Ser/Thr protein kinase activity required for full virulence in mouse models of infection.[61,62] The eukaryotic-like STPK domain encompasses residues 150–400. Upon translocation to a host cell YpkA is targeted to the inner side of the plasma membrane by a membrane localization domain (MLD, amino acids 20–90).[63,64] This hydrophobic region is shielded inside the bacterium by the chaperon SycO to prevent aggregation.[64] Recently, a phosphoinositide-binding domain (PBD) with conserved membrane localization GKxYxnF motif was mapped in the same region (aa 32–89).[65]

A Ser/Thr kinase domain of YpkA contains subdomains I-XI[61] (Fig 1 1). YpkA is expressed in *Yersinia* in a catalytically inactive form and requires G-actin binding and autophosphorylation for activation.[66,67] Multiple autophosphorylation sites have been identified in the N-terminal and kinase domains of YpkA,[67,68] however the detailed activation mechanism and the role of phosphorylation of the activation loop is not yet known.

Heterotrimeric G protein was identified as a direct substrate of YpkA kinase. Phosphorylation of Ser47 in the consensus sequence, GXXXXGK(S/T) of the diphosphate binding loop disrupted GTP binding and prevented G protein activation.[69] Residues 40–49 within the YpkA membrane-localization domain were shown to be critical for Gαq binding and phosphorylation.[68] Recently, vasodilator-stimulated phosphoprotein (VASP) was shown to be phosphorylated by YpkA *in vitro* and *in vivo*, predominantly at S157. The STPK and actin-

This figure also includes an iMolecules 3D interactive version that can be accessed via the link at the bottom of this figure's caption.

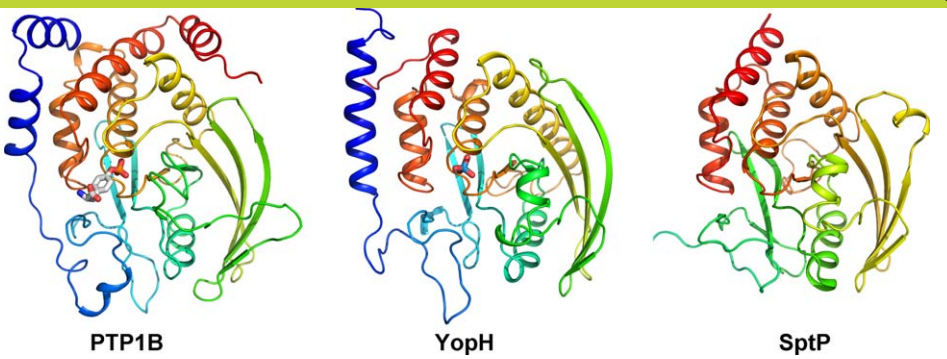

Figure 4. The structures of bacterial phosphatases YopH and SptP are compared to the human protein phosphatase 1B. An interactive view is available in the electronic version of the article.

binding domains of YpkA were involved in its interaction with VASP, while GDI domain was dispensable.[70]

Phosphatases

Two bacterial effector phosphatases, YopH (PDB: 1YTW) and SptP (PDB: 1G4W), have been structurally characterized and are described below.

Y. pestis protein tyrosine phosphatase YopH

Functional aspects of YopH. YopH is indispensable for *Yersinia* virulence,[71,72] it subdues host defenses by inhibiting phagocytosis and disrupting focal adhesion in macrophages.[73,74] It also interferes with T and B lymphocyte activation and thus blocks adaptive immune response.[75] *In vivo* studies in a mouse infection model demonstrated that YopH inactivated PRAM-1/SKAP-HOM and the SLP-76/Vav/PLCγ2 signal transduction axes, which might be critical for bacterial survival.[76] Several direct substrates of YopH were identified in different cell types: p130Cas (p130Crk-associated substrate), paxillin and focal adhesion kinase FAK in HeLa cells,[74,77] Fyn-binding protein and murine SKAP-HOM in macrophages,[78,79] Lck, LAT, and SLP-76 in T-cells,[80,81] p85 (regulatory subunit of the PI3K) in HEK293.[82]

Structure of YopH. YopH consists of two domains, the smaller N-terminal domain (aa 1–130) is connected by a an intrinsically disordered region to the larger C-terminal PTPase domain (aa 163–468). The PTPase domain bears a remarkable structural similarity to human PTP1B (PDB: 1PTY)[83] despite only ~20% sequence identity.[84] It contains all the invariant residues present in eukaryotic PTPases.[85] The catalytic domain forms an eight-stranded mixed β-sheet, flanked by five α-helices on one side and two α-helices on the other side[86] (Fig. 4). The P-loop (phosphate binding), located between β-strand and α-helix, adheres to the signature PX($^{I}/_{V}$)($^{I}/_{V}$)HCSAGXGR($^{T}/_{S}$)G PTPase motif. This motif includes the catalytically essential His402, nucleophile Cys403[87] and the GXGXXG sequence, similar to the nucleotide-binding motif of dehydrogenases and kinases.

The X-ray structures of the apo YopH PTPase domain and its complexes with various ligands mimicking intermediate and transition states[88] together with kinetic, mutagenesis and computational studies (reviewed in Refs. [89,90) allowed deciphering of the PTPase mechanism. The nucleophilic displacement reaction proceeds in two steps: (i) phosphoryl transfer to a functional group on the enzyme and (ii) hydrolysis of the resultant phosphoenzyme intermediate (E-P) and regeneration of the active enzyme. Upon substrate binding the flexible WPD loop (aa 350–360) moves to a closed conformation bringing Asp356 to the substrate where it acts as a general acid and transfers a proton to the scissile oxygen of the substrate initiating phosphate dissociation. Deprotonated Cys403 in the active site serves as a nucleophile to accept a phosphoryl group.[89,90] Arg409 stabilizes the transition state forming a bidentate hydrogen bond with two nonbridging oxygens of the phosphate group. The phosphoryl transfer proceeds most likely *via* a dissociative transition state.[91] During the following E-P hydrolysis step the invariant Asp356 residue acts as a general base to activate a nucleophilic water[92] that is positioned by Gln446 along the S-P bond direction. The hydroxyl group of Thr410 interacts with the scissile bond and initiates E-P hydrolysis.[93] Although YopH and human PTP1B follow the same catalytic mechanism, their catalytic rates vary significantly; YopH is 20-fold more active than PTPB1.[94] Recent solution NMR relaxation studies revealed that the WPD loop dynamics determines the overall reaction rate, since WPD loop closure during the phosphopeptide cleavage step is tightly synchronized with the protonation of the leaving group.[95]

Crystal structures of YopH complexed with model peptide substrates based on an autophosphorylation site of EGFR[85] revealed a second binding site within the PTPase catalytic domain.[96,97] This site binds the YopH substrate p130Cas in a

phosphotyrosine-dependent manner and is involved in targeting YopH to Cas *in vivo*.[97]

The N-terminal noncatalytic domain of YopH (YopH-NT) has dual properties: in bacterial cell it binds chaperone SycH, which enables its translocation,[98,99] while in a host cell acquires a globular conformation and serves as a substrate binding domain.[100–102] The phosphotyrosine substrate binding specificity of YopH-NT is similar to that of eukaryotic SH2 domains, which recognize pYXX(X)P amino acid sequences. However, YopH-NT shares no structural homology with eukaryotic analogs.[102] The secretion segment (aa 2–17) and translocation domain (aa 18–71)[103] make an integral part of the YopH-NT compact fold composed of four α-helices and two β-hairpins.[100,102]

Salmonella Typhimurium tyrosine phosphatase SptP

Structural aspects of SptP. SptP is a T3SS effector required for full virulence of *S*. Typhimurium *in vivo*.[104] It is a modular protein with two functional domains: the C-terminal PTPase domain and the N-terminal domain similar to the *Pseudomonas aeruginosa* exotoxin *S*[105] (21% sequence identity) and the *Yersinia* spp. cytotoxin YopE.[106,107] The PTPase domain of SptP shares 30% sequence homology with YopH and is very similar structurally and mechanistically to this enzyme. SptP phosphatase domain (residues 300–543) follows the canonical tyrosine phosphatase fold, with the exception of the first helix, which is missing. Although the active site of SptP is well conserved, this phosphatase has distinct surface properties with a shape and charge distribution different from YopH, consistent with different substrates and cellular effects of these proteins.[108]

Upon synthesis in bacterial cell, the N-terminal domain of SptP forms a tight complex with its specific chaperone SicP.[109] Since this binding is crucial for SptP stability and translocation,[110,111] translation of SptP is strictly coupled to SicP expression.[112]

SptP function. The N-terminal domain of SptP demonstrated a potent GAP activity, inhibiting signaling through Rho GTPases Rac-1 and Cdc42. This GAP activity of SptP is responsible for downregulation of the actin-cytoskeleton rearrangements stimulated in host cells by T3SS effectors SopE and SopE2.[111] However, both domains are required for another observed activity of SptP, inhibition of ERK activation.[113,114] Several substrates of SptP phosphatase domain were identified, including AAA+ ATPase VCP, which when dephosphorylated by SptP, promoted membrane fusion events that might be necessary for SCV maintenance and maturation.[115] SptP was also reported to suppress mast cell degranulation by dephosphorylating tyrosine kinase Syk and the vesicle fusion protein NSF.[116]

Phosphoserine/Phosphothreonine Lyases

Fold and catalytic mechanism

Structure. A family of homologous proteins, encompassing *Shigella* OspF, *Salmonella* SpvC (PDB: 2PIW), *Chromobacterium* VirA (PDB: 3BO6) and *Pseudomonas syringae* HopAI1, were shown to irreversibly eliminate the phospho-group from the threonine in the Thr-X-Tyr motif in the activation loop of MAP kinases.[117] Although the first studies annotated OspF as a phosphatase, the subsequent analysis revealed that the action of OspF, SpvC and HopAI1 reduces molecular weight by 98 Da, corresponding to the elimination of H_3PO_4, and therefore resulting in a Cα=Cβ dehydroalanine/dehydrothreonine residue.[117–119]

The structures of OspF (PDB code 3I0U, Singer *et al*., unpublished), SpvC[120,121] and VirA (PDB code 3BO6, Brennan *et al*., unpublished) are very similar and demonstrate a unique fold. The lyase domain of ~240 residues consists of a central seven-stranded β-sheet with its convex side covered by nine α-helices [Fig. 5(a)]. A long and positively charged groove extends along the concave side of the β-sheet, necessary for binding of pT and pY residues. Activity studies revealed that OspF and SpvC have almost identical substrate specificity, with preference p38>ERK2>ERK5 and very low activity on JNK.[120] Consistent with the evolutionary conserved function, the *P. syringae* HopAI1 lyase inactivates two plant MAP kinases, MPK3 and MPK6, that are involved in plant defense systems.[122]

Apart from the direct enzyme-substrate interaction, OspF, SpvC and VirA but not HopAI1, have mimicked a D-motif—a protein–protein interaction module, encompassing the first N-terminal 26 residues, which bind to a negatively-charged patch and a hydrophobic groove on the surface of a MAP kinase.[120] The N-terminal D-motif is widely used by the MAP kinase kinases to activate MAP kinases and contains a well-established consensus sequence $(^{R}/_{K})_{2-3}$-X_{2-6}-Φ-X-Φ, where Φ is a hydrophobic residue. This motif binds to a groove between α5, α6, β7, and β8 on the C-terminal lobe of ERK.[123] OspF constructs with truncated or deleted D-motif lose their ability to co-purify with ERK2, suggesting its high functional importance.[120]

Substrate biding and catalysis. The structure of the SpvC complexed with the diphosphorylated peptide of the same sequence as the substrate's activation loop allowed the catalytic mechanism to be resolved.[120,121] The peptide adopts a U-shape conformation with the pThr-Glu-pTyr residues located at the bottom of the positively charged groove in SpvC

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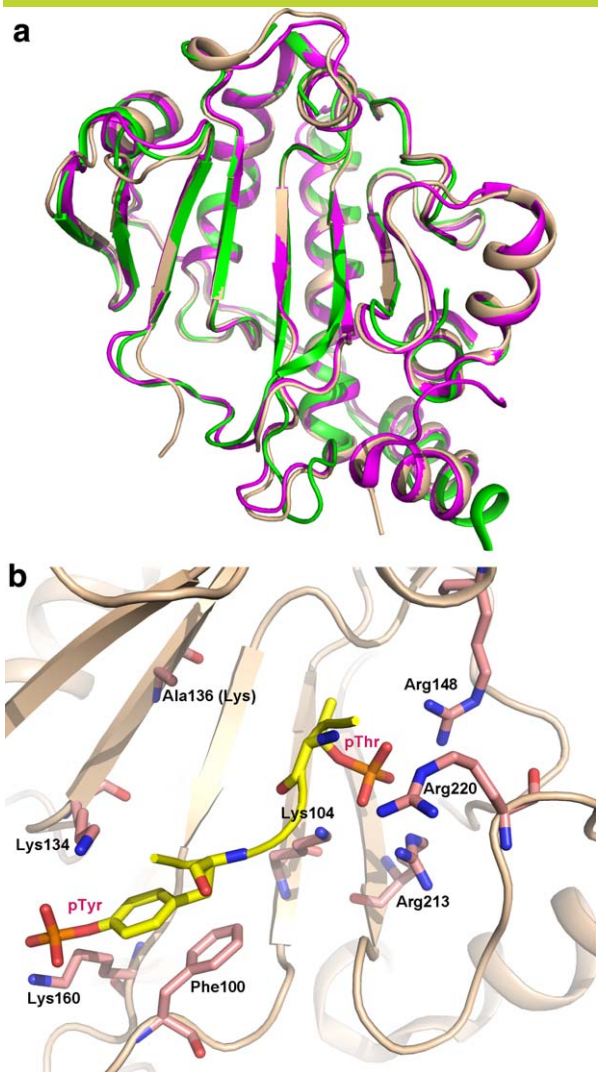

Figure 5. (A) Superposition of phosphothreonine lyases *Salmonella* SpvC (wheat), *Shigella* OspF (magenta) and *Chromobacterium* VirA (green) shown in cartoon representation. Their structures are strikingly similar; (B) the substrate binding site of SpvC with pThr and pTyr-containing peptide captured in a K136A mutant. An interactive view is available in the electronic version of the article.

[Fig. 5(b)]. The substrate pTyr is located in the positively charged pocket mainly formed by Lys134 and Lys169, neutralizing phosphate group, and with Phe100 stacked against the tyrosine ring. Mutations of Lys160 and Phe100 affect significantly catalytic efficiency while mutation of Lys134 is less severe. The phosphate group of the substrate pThr binds in a deep cavity formed by Arg148, Arg213, Arg220, and Lys104 and is almost completely shielded from the solvent. Mutation of any of the arginines to a glutamate or to a lysine abolished SpvC activity. This phosphoresidue binding site mimics the environment of this residue in the activated form of MAP kinases where the pThr and pTyr residues are surrounded by positively charged residues.[124]

Lys136 and His106 were proposed to act as the catalytic residues for the β-elimination reaction of the phosphothreonine residue.[120,121] Lys136 acts as a catalytic base to abstract proton from the C atom of phosphothreonine, while His106 acts as a catalytic acid to donate a proton to the leaving phosphate group. This hypothetical mechanism was substantiated by mutational studies and by QM/MM and DFT modeling calculations.[125–127]

Although the middle residue of the pThr-Xaa-pTyr motif is exposed to solvent, its conformational flexibility dramatically affects catalytic efficiency.[120] The best substrate, p38, has a Gly in this position, ERK2 has a Glu, while the poorest substrate, JNK, has a Pro. The mutation of this Pro in the JNK peptide to Gly rendered the latter a much better substrate. The comparison of the conformation of the activation loop in the activated state of the kinase and the conformation of the substrate peptide in the SpvC active site revealed a drastic conformational change in residues N-terminal to the phosphotyrosine [Fig. 5(b)], which may explain the need for the flexible residue between the two phosphorylated residues.[120]

Functional role. Mice infected with the mutant *Salmonella* Typhimurium strain lacking the *spvC* gene showed pronounced colitis and inflammation compared to the wild-type bacteria.[128] Deletion of the *ospF* gene resulted in significant changes in the differential host cell phosphoproteome.[12] In addition to the expected inhibition of gene expression, the deletion affected the phosphorylation status of many proteins involved in transcription, chromatin modification and RNA processing. Additionally, proteins related to the actin cytoskeleton, microtubules, apoptosis and cell cycle were affected as well. Thus OspF massively impacts host protein phosphorylation by targeting the central MAP kinase pathway—p38 and ERK. Recently, OspF was shown to bind chromatin at the promoter region of the *IL8* gene and to interact with the C-terminal chromoshadow domain of the heterochromatin protein 1 (HP1γ) involved in the recruitment of epigenetic regulators.[129] Moreover, the presence of OspF led indirectly to the inhibition of phosphorylation of the regulatory Ser83 of HP1γ, which becomes phosphorylated by ERK-dependent MSK1. In addition, OspF affects chromatin remodeling through binding to the retinoblastoma protein (RB).[130] OspF contains a LxCxE RB-binding motif (I^{178}MCLE^{182}), which is located on a long α–helix on the convex surface of OspF molecule. The mutation in this motif not only disrupted the binding to RB but also reduced the capacity of OspF to repress IL-8 secretion *in vivo*.[130]

The impact of OspF on non-MAP kinase dependent pathways remains largely unknown. So far it

has been shown that OspF can activate TGF-beta activated kinase (TAK1) by disrupting the negative feedback loop regulation between p38 and TAK1, which in turn enhanced NF-κB, JNK and c-Jun signaling.[131]

Acetylases

The YopJ/HopZ/AvrRxv family of bacterial effector acetylases is widely distributed among animal and plant pathogens and plant symbionts. YopJ/YopP (*Yersinia*), AvrA (*Salmonella*), AvrBsT (*Xantomonas campestris*), YopP (*Bartonella*), VopJ (*Vibrio*), and HopZ1–3 (*P. syringae*) are some of the members of this family. *Yersinia* infection is accompanied by a profound inhibition of several MAP kinases and NF-κB pathway.[132] These effects were attributed to YopJ, which was shown to downregulate ERK, p38 and JNK by decreasing the level of their phosphorylation[132–134] and by preventing phosphorylation and subsequent degradation of IκB.[135] Subsequently, YopJ was found to bind and inhibit activation of a number of MAP kinase kinases—MKK1, MKK3, MKK4, and MKK5, as well as IKKβ, a component of the IκB kinase complex.[136]

There is as yet no crystal structure for any member of this family but a limited structural insight can be derived from amino acid sequence analysis, which indicates the similarity of YopJ to adenovirus cysteine protease AVP and ubiquitin-like-specific cysteine protease 1 (ULP1), belonging to the proteolytic Clan CE.[137] The deduced catalytic triad, consisting of Cys, His, and Glu, was confirmed by mutagenesis. Similar results were also demonstrated by mutations of *Xantomonas campestris* AvrBsT residues. Initially, YopJ was considered to be a protease/deubiquitinase.[66] However, later it was shown that YopJ acetylated the Ser/Thr within the activation loop of MEK1, MEK2,[138] TAK1[139] as well as IKKα and IKKβ.[140] Furthermore, the YopJ activity was acetyl-CoA dependent. The YopJ docking site on the kinase was localized through mutagenesis and functional studies of yeast MKK Pbs2 to α-helix G (within motif X).[141]

YopJ and AvrA are activated by inositol hexakisphosphate (IP_6), thus indicating that IP_6 is likely a cofactor common for this acetylase family.[142] AvrA also inhibits the NF-κB pathway[143,144] but by different means than YopJ since it does not block IKK phosphorylation. Moreover, AvrA inhibits the JNK pathway at the level of MKK4/7 but not the p38 and ERK pathways.[144,145] AvrA can be downregulated by phosphorylation within the N-terminal translocation motif by one of the kinases of the ERK pathway,[144] suggesting an intriguing interplay between AvrA and kinase cascades.

The substrate specificity of YopJ/HopZ/AvrRxv family members varies. The AopP effector from *Aeromonas salmonicida* could inhibit the NF-κB pathway downstream from IKK activation, while it did not interfere with the MAP kinase pathway,[146] while VopA from *Vibrio parahaemolyticus* could inhibit MAK kinase pathway but not the NF-κB pathway.[147] Although the acetylation of Ser and Thr residues in the activation loop was considered to be the main mechanism of inhibition, VopA could acetylate a conserved lysine residue preceding the catalytic loop in MAP kinases, important for ATP binding.[148] This modification rendered MKK6 incapable of binding ATP. YopJ could only acetylate a Lys residues located in the activation loop.[138] Moreover YopJ, VopA, and PopP2 (*Ralstonia solanacearum*) were shown to autoacetylate.[140,148] The autoacetylation site for PopP2 was located at a lysine conserved in every member of the family. *R. solanacearum* is a plant pathogen and the PopP2 lysine mutant was not recognized by the plant defense system, suggesting a role for this Lys in acetyl-CoA recognition.[149]

Conclusions

Processes within eukaryotic cells are highly coordinated and are directed through a complex system of interconnected signaling pathways. Many of these signals involve adding or removing a phosphate group by dedicated enzymes, events that are recognized downstream by specialized recognition domains. Bacterial pathogens invading host cells rearrange cellular processes to adapt the initially hostile environment into one that allows bacterium to survive and prosper. To accomplish this task pathogens secrete effector proteins that modify cellular processes toward the needs of the pathogen. Since phosphorylation/dephosphorylation plays a key role in cellular signaling, pathogens have evolved sophisticated systems that affect the phosphorylation state of the key cellular signaling components thus directly and indirectly modifying the entire cellular phosphoproteome. Progress in recent years has significantly increased our understanding of the mechanisms by which pathogens subvert cellular processes and has led also at the same time to better understanding of the normal cellular processes.

Obviously, progress in our understanding of molecular mechanisms of pathogen/host warfare provides new avenues for developing new therapies. The importance of the secretion systems and in particular T3SS, T4SS as the syringes injecting bacterial effectors into the host cell and thus being the first step in infection was long recognized. These systems were targeted for drug development[88,150] and progress in their development has been recently reviewed.[151] Other promising pharmaceutical targets have also been identified and their usefulness for drug development is being investigated.[152] Moreover, the tools evolved by bacteria represent a unique evolution–tailored set of proteins capable of modifying the desired cellular processes and the full potential of these tools is yet to be discovered. Application of bacterial effectors YopH and OspF to

rewire signaling cascades was recently demonstrated in the response of to external stimuli. Similarly, *Jurkat* T cells were modified to mitigate off–target activities during adoptive immunotherapy.[153]

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