G8 build an interaction platform, which stabilizes the kink in the J2/4 junction between the two sensor helices (Fig. 3, B and C). The extensive contacts within the interaction platform include residues from distant parts of the primary structure. This observation underscores the critical role of the platform in stabilizing the TPP-bound state of the riboswitch.

Residue A72 is mutated in a pyrithiamine-resistant strain of *Aspergillus oryzae* (16). The phosphorylated form of the well-known antibiotic pyrithiamine (PTPP) exerts its activity by directly interacting with TPP riboswitches (18). Both A72 and C38 mutations have also been selected in a screen for pyrithiamine resistance in bacteria (18). These nucleotides are key residues within the interaction platform, which stabilizes the three-way junction (Fig. 3, B and C). Biochemical experiments indicate that TPP and PTPP are still able to bind to the riboswitch despite the mutations (18). Our structural data are in agreement with these experiments and show that the TPP binding pocket would remain unaffected by the A72 or C38 mutations. Instead, these mutations likely disturb the correct folding of the three-way junction and prevent the coupling between TPP binding and the formation of the switch helix P1.

The structure of the AtRs riboswitch described here provides structural insights into the TPP-induced mechanism of shifting between its ligand-free “on” and the ligand-bound “off” conformational states. In the *thiM* gene from *E. coli*, where the TPP riboswitch is also found, the “on” state promotes translation of the mRNA, whereas the “off” state inhibits translation. Previous in-line probing experiments on the *E. coli* TPP riboswitch, in which the spontaneous RNA cleavage rate in the presence and absence of the ligand was monitored, revealed reduced cleavage of the “switch” helix P1 residues upon binding of the TPP (1). This indicates that helix P1 is not formed in the “on” state. Based on the structural and biochemical data, we propose a model for the sequence of events leading to the “off” state of the riboswitch (Fig. 4B): (1) TPP binding promotes the parallel disposition of the sensor helices. (2) Consequently, the interaction platform is assembled, forming a strong kink at the J2/4 junction. (3) The folding of the three-way junction reduces the entropic penalty for the formation of the switch helix P1. Therefore, if the formation of the three-way junction is impeded by one of the mutations, the TPP will still be able to bind to the sensor helices but will nevertheless be unable to turn the riboswitch “off.”

The structure of the AtRs riboswitch reveals how thiamine pyrophosphate is recognized with high specificity and high affinity, rationalizes the mechanism of resistance to the well-known antibiotic pyrithiamine, and demonstrates which regions of the riboswitch are critical for the stability of its “off” conformation. The results presented here provide a good starting point for structure-based in vivo and in vitro experiments aimed at studying the mechanism of TPP riboswitch-regulated gene expression in general.

References and Notes

19. Materials and methods, table S1, and fig. S1 are provided as supporting material on Science Online.

Yersinia YopJ Acetyltransferase and the Host Cell Interaction Mediated by YopJ Acetylation of Serine Residues

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**Yersinia species** use a variety of type III effector proteins to target eukaryotic signaling systems. The effector YopJ inhibits mitogen-activated protein kinase (MAPK) and the nuclear factor κB (NFκB) signaling pathways used in innate immune response by preventing activation of the family of MAPK kinases (MAPKKs). We show that YopJ acts as an acetyltransferase, using acetyl-CoA to modify the critical serine and threonine residues in the activation loop of MAPKK6 and thereby blocking phosphorylation. The acetylation on MAPKK6 directly competes with phosphorylation, preventing activation of the modified protein. This covalent modification may be used as a general regulatory mechanism in biological signaling.

The bacterial pathogen *Yersinia pestis* is the causal agent of plague, also known as the Black Death (1). Two related pathogens, *Y. pseudotuberculosis* and *Y. enterocolitica*, cause gastroenteritis (2). All three *Yersinia* species harbor a virulence factor that encodes a type III secretion system and secrete effector proteins, referred to as Yops (*Yersinia* outer proteins) (2). Yops are delivered by this system into an eukaryotic cell.
cell to cripple the host defense system (3, 4). The *Yersinia* species effector protein, YopJ, disrupts signaling essential for eukaryotic cells to elicit an immune response by inhibiting the evolutionarily conserved MAPK and NF-κB signaling pathways (2, 3, 5, 6). YopJ contains a catalytic domain that is similar to Clan CE of cysteine proteases, which includes the adenoviral protease (AVP) family and the ubiquitin-like protein protease (Ulp1) family (7). Mutation of the putative catalytic cysteine residue to an alanine in YopJ (YopJ-C/A) abolishes its ability to inhibit the MAPK and the NF-κB signaling pathways (7). YopJ binds MAPK kinases, including MAPKK1, MAPKK3, MAPKK4, MAPKK5, and the related kinase that activates the NF-κB pathway, IkB kinase β (IKKβ), and prevents their activation (5). The mechanism by which this binding leads to inactivation of these kinases is unknown.

A cell-free signaling system was developed to recapitulate the inhibition of the MAPK and the NF-κB signaling pathways by YopJ (5–8). Mammalian extracellular signal-regulated kinase (ERK) signaling was activated by addition of recombinant B-Raf to a membrane-free cytosolic lysate (cleared lysate), as demonstrated by the appearance of phosphorylated ERK (Fig. 1A). By contrast, activation of ERK signaling was diminished in cleared lysate isolated from cells transfected with YopJ (Fig. 1A). The catalytic activity in YopJ was required for this inhibition. Addition of B-Raf to cleared lysate isolated from cells expressing mutant YopJ-C/A lead to activation of the ERK pathway (Fig. 1A). For activation of the NF-κB pathway, we added a purified active form of recombinant TNF (tumor necrosis factor) receptor–associated factor 6 (TRAF6) (T6RZC) (9). When T6RZC was added to control and YopJ-C/A cleared lysates, the pathway was activated, as indicated by the phosphorylation of IkB (Fig. 1B). However, the addition of T6RZC to YopJ cleared lysate did not result in activation of the NF-κB pathway (Fig. 1B). Similarly, when other exogenous stimuli [including NF-κB-inducing kinase (NIK), MAPK kinase kinase 1, and activated Ras-V12 membranes] were added to the lysates, signaling was blocked only in the YopJ lysates. No obvious changes were observed in the molecular weight or the stability of MAPKK1 and MAPKK2 (MAPKK1,2) or IKKβ in the lysates (Fig. 1C). These observations were consistent with previous genetic, microinjection, and cellular studies on the activity of YopJ and provided a method for analyzing inhibition of signaling by YopJ in vitro (5–8).

To test whether YopJ acted directly on the MAPKks and IKKβ, we coexpressed a representative member of this group of kinases, human MAPKK6 (rMAPKK6), with either active YopJ (rMAPKK6-J) or the catalytically inactive form of YopJ (rMAPKK6-C/A) in bacterial cells. We then assessed whether the various rMAPKK6s could be activated in our in vitro signaling assay. Although both rMAPKK6 and rMAPKK6-C/A were robustly phosphorylated when added to cleared lysate, the rMAPKK6-J was not activated by phosphorylation by the upstream signaling machinery (Fig. 1D). Therefore, coexpression of YopJ with MAPKK6 in bacteria produced a kinase that could not be activated by the upstream signaling machinery.

Studies on the YopJ-inactivated rMAPKK6 were undertaken to determine the biochemical nature of the modifications. Although all the rMAPKK6s were indistinguishable by SDS–polyacrylamide gel electrophoresis (PAGE) and gel filtration (Fig. S1), mass spectrometry revealed that the total mass of rMAPKK6-J was larger than that of either rMAPKK6 or rMAPKK6-C/A. The majority of YopJ-inactivated rMAPKK6 showed an increase in mass of 126 atomic mass units (amu), whereas smaller populations of rMAPKK6-J exhibited increases in mass of 84 amu or 42 amu (Fig. 2A). We hypothesized that YopJ altered the mass of rMAPKK6 by adding single, double, or triple posttranslational modifications equal to a mass of 42 amu.

We analyzed tryptic peptides for all three rMAPKK6s (rMAPKK6, rMAPKK6-J, and rMAPKK6-C/A) by using liquid chromatography followed by tandem mass spectrometry (10). After obtaining a complete data set for all the predicted tryptic peptides, we found that rMAPKK6-J, but not rMAPKK6 or rMAPKK6-C/A, contained two tandem peptides [peptide A, MAPKK6 195 to 210 amino acids, and peptide B, MAPKK6 211 to 224 amino acids] modified by acetylation with a consequent increase of 42 amu for each peptide (Fig. 2, B and C). In another partially cleaved tryptic peptide (MAPKK6 195 to 224 amino acids) that contained both peptides A and B, we observed multiple acetylated sites. Peptide A in the rMAPKK6-J protein was modified by acetylation on Ser207 (Fig. 2B), and peptide B was modified by acetylation on Thr211 (Fig. 2C). In the third peptide, it appeared that Lys410 and Ser207 and/or Thr211 were modified by acetylation. Modification of the lysine contributes to the inefficient cleavage of this peptide by trypsin. Residues 195 to 224 map to the end of β strand 9 and the activation loop in MAPKK6, which contains Ser207 and Thr211, the sites that are phosphorylated to activate MAPKK6. Although the serine and threonine residues are conserved throughout the MAPKK superfamily, the lysine residue is not (Fig. 2D). We predict that this residue is modified in a YopJ-dependent manner because of its...
coincidental location in the activation loop. The observation that YopJ covalently modifies the representative MAPKK, MAPKK6, by acetylation on the same residues that are used for activation of the kinase suggests a mechanism for the inhibition of MAPKks and IKKβ; namely, acetylation prevents phosphorylation.

YopJ can bind and inhibit MAPKks and IKKβ but not IKKα (fig. S2) (5), and all of these kinases contain serine and/or threonine residues in their activation loop that must be phosphorylated to activate the kinase (Fig. 2D) (11). rMAPKK6, coexpressed with YopJ and shown to be acetylated at Ser207 and Thr211 (Fig. 2, B and C), was not phosphorylated by upstream signaling machinery (Fig. 1D). These observations support our hypothesis that YopJ functions to modify the MAPKks without noticeably changing their migration pattern on SDS-PAGE (Fig. 1C and fig. S1).

To determine whether YopJ directly functions as an acetyltransferase, we performed a transferase reaction in the presence of 14C-labeled acetyl–coenzyme A (CoA) (12). rMAPKK6 was modified with the 14C-labeled acetyl moiety only in the presence of recombinant YopJ expressed as a glutathione S-transferase fusion protein (GST-YopJ) and the labeled acetyl donor [14C]acetyl-CoA (Fig. 3A). The 14C label was associated with both rMAPKK6 and GST-YopJ (Fig. 3B). Based on this and analysis of the GST-YopJ protein beads, rMAPKK6 associated with GST-YopJ was the source of the 14C label (Fig. 3C). Thus, YopJ requires both an intact catalytic site and acetyl-CoA to acetylate rMAPKK6. We did not observe any band in reactions that contained only GST-YopJ and [14C]acetyl-CoA, indicating that our charging of YopJ with a [14C]acetyl moiety might be transient, labile, and/or dependent on the presence of a substrate or that the reaction proceeds through direct transfer. Similarly, we have observed that rMAPKK1 was also modified by [14C]acetyl moiety in a YopJ-dependent manner (fig. S4). These experiments show that YopJ acts as an acetyltransferase to modify MAPKks.

To demonstrate that the modification on rMAPKK6 by YopJ prevents activation via phosphorylation, we used our in vitro signaling system. Pretreatment of rMAPKK6 in the presence of both YopJ and acetyl-CoA diminished the ability of the upstream signaling machinery to activate rMAPKK6 by phosphorylation (Fig. 3D). Hence, the acetylation of a MAPKK by YopJ prevents phosphorylation and activation of this kinase.

YopJ protein is delivered into the cytoplasm of a host cell by a type III secretion system, where it inhibits the activation of the MAPKks and IKKβ (5). Previously, adding recombinant YopJ to lysates did not show an inhibitory affect on signaling pathways. However, by using an acetyl-CoA–supplemented cleared lysate, we observed that addition of GST-YopJ but not GST-YopJ-C/A resulted in the inhibition of the NFκB signaling pathway in vitro (Fig. 3E). Thus, as observed during infection, when delivered to a lysate, YopJ uses acetyl-CoA to target and inactivate MAPKks and IKKβ.

The mechanism of YopJ inhibition is elegant in its simplicity. On the basis of current studies on a representative kinase, MAPKK6, we propose that YopJ blocks signaling of the MAPK and NFκB pathways by binding and acetylating critical residues in the activation loop of MAPKks and IKKβ, respectively, thereby preventing these residues from being phosphorylated.

Figure 2. rMAPKK6-J is acetylated on Ser207 and Thr211 residues in its activation loop.

(A) Reconstructed molecular mass profiles of rMAPKK6, rMAPKK6-J, and rMAPKK6-C/A. (B and C) Electrospray ionization (ESI) tandem mass spectrometry (MS/MS) spectra of modified tryptic peptide A [mass-to-charge ratio (m/z) of 902.4 (z = 2)] and peptide B [m/z of 825.9 (z = 2)] from rMAPKK6-J. The b and y ions are marked on the MS/MS spectra. The amino acid sequence for each peptide is shown below (18). Acetylated residues are designated with a red circle. Masses that show an increase of 42 amu are marked with an asterisk. Two ions related to acetylated peptide B were detected, m/z of 825.9 (z = 2) and m/z of 833.9 (z = 2). MS/MS data of both ions indicated that peptide B was modified by acetylation on Thr211. The only difference between these two ions is that Met220 is oxidized in m/z of 833.9. Figure 3C shows the MS/MS spectrum of m/z 825.9. A corresponding figure defining the b and y ions is presented in fig. S3. (D) Alignment of the activation loop of the MAPKK superfamily with conserved serine and/or threonine residues that are indicated by asterisks.
Analysis of the predicted secondary structure of YopJ demonstrated similarities with the protease AVP (13). Because of the similarities between AVP and its distant relative, Ulp1, it was proposed that YopJ might act as an Ulp1-like protease or a general hydrolase (7). Inconsistent with this earlier hypothesis is the observation that YopJ selectively targets MAPKKs and IKKβ without any obvious changes in their migration on SDS-PAGE (2–6, 8) (Fig. 1C and fig. S1). However, because YopJ shares similarities with a family of cysteine proteases, this provides mechanistic insight into the chemistry of YopJ catalysis (7). A likely first step in the reaction is that YopJ is acetylated on Cys<sup>172</sup> by formation of a thioester bond, and in the second step of the reaction this bond is attacked, not by a water molecule, but by a hydroxyl moiety on a serine or threonine residue of MAPKK, resulting in the formation of an acetylated amino acid. The substrate specificity determined by a bacterial effector protein, YopJ, and its interaction with target host proteins, MAPKKs and IKKβ, illustrates a common mechanism used by many bacterial effectors to ensure that their potent activity does not harm the bacterial host (3).

We find that YopJ-dependent acetylation occurs on the critical serine or threonine residues, thereby directly competing with the posttranslational modification, phosphorylation. Although the possibility exists that this is a unique modification developed by pathogenic bacteria to affect signaling in eukaryotic cells, a major characteristic of bacterial effector proteins is that they usurp or mimic a eukaryotic activity and refine this activity to produce an extremely efficient mechanism to combat eukaryotic signaling. Therefore, a more appealing hypothesis is that the modification of phosphorylatable residues by acetylation is a commonly used eukaryotic mechanism that simply has not been detected previously. Our findings support the provocative hypothesis that modification of amino acids other than lysine by acetylation is used to regulate eukaryotic cellular machineries. Enzymes that acetylate lysines have been studied for many years, including the eukaryotic and bacterial N-acetyltransferases that use acetyl-CoA and a catalytic triad, which appears similar to papain-like cysteine proteases (Cys-Glu/Asp-His) (14–16).

Immunoblotting and the interpretation of tandem mass spectrometry data by Mascot are commonly used for the identification of lysine acetylation (17). However, these assays do not detect acetylation of serines and threonines. In view of the current finding, a more careful manual analysis of liquid chromatography followed by tandem mass spectrometry data may be required to determine whether an amino acid other than lysine is modified by acetylation. The characterization of a bacterial effector as a Ser or Thr acetyl transferase presents a previously unknown paradigm to be considered for other biological signaling pathways.

References and Notes
10. Materials and methods can be found on Science Online.
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gin; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Materials and Methods
Figs. S1 to S4

References and Notes
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Yersinia YopJ Acetylates and Inhibits Kinase Activation by Blocking Phosphorylation

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