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A *Yersinia* Effector and a *Pseudomonas* Avirulence Protein Define a Family of Cysteine Proteases Functioning in Bacterial Pathogenesis

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Summary

A *Yersinia* effector known as YopT and a *Pseudomonas* avirulence protein known as AvrPphB define a family of 19 proteins involved in bacterial pathogenesis. We show that both YopT and AvrPphB are cysteine proteases, and their proteolytic activities are dependent upon the invariant C/H/D residues conserved in the entire YopT family. YopT cleaves the posttranslationally modified Rho GTPases near their carboxyl termini, releasing them from the membrane. This leads to the disruption of actin cytoskeleton in host cells. The proteolytic activity of AvrPphB is essential for autoproteolytic cleavage of an AvrPphB precursor as well as for eliciting the hypersensitive response in plants. These findings provide new insights into mechanisms of animal and plant pathogenesis.

Introduction

Yersinia pestis is the causative agent of the plague, also known as Black Death, which killed over one-third of the population of Europe in the fourteenth century (Cornelis et al., 1998). Interest in *Y. pestis* has recently intensified because of the emerging threat of bioterrorism. The pathogenicity of *Yersinia,* like other invasive bacteria, arises in part from its ability to inhibit the host immune system, which insures its extracellular survival and proliferation. *Yersinia* infection results in the inhibition of phagocytosis, the suppression of cytokine responses, and the induction of apoptosis (Cornelis et al., 1998). Central to the mechanism of *Yersinia* pathogenesis is the employment of a specialized type III secretion system that is encoded by a 70 kb virulence plasmid shared by all three pathogenic species of *Yersinia* (Cornelis et al., 1998; Cornelis and Van Gijsegem, 2000). Numerous reports in the last two decades have demonstrated that the type III secretion system is utilized by a wide variety of gram-negative pathogenic bacteria including both animal and plant pathogens (Cheng and Schneewind, 2000; Galan and Collmer, 1999; Hueck, 1998). The *Yersinia* type III secretion system injects six proteins called Yop effectors (YopH, YopE, YopJ/YopP, YopO/YpkA, YopM, and YopT) into host cells that function in concert to thwart the host immune system (Juris et al., 2002).

Plant pathogens such as *Pseudomonas syringae* and *Xanthomonas campestris* also rely on type III secretion systems to cause disease (Collmer et al., 2000). However, the proteins secreted by these bacteria and the contribution of these proteins to disease are not well understood. The majority of proteins known to be secreted in a type III-dependent manner by plant pathogens were identified based on their ability to induce a hypersensitive response (HR) in resistant hosts. The HR is a rapid physiological response in infected plant tissue culminating in programmed cell death at the infection site and is almost always correlated with cessation of pathogen growth. Because these type III effector proteins render the bacteria avirulent on resistant host plants, they have been named avirulence (Avr) proteins. How Avr proteins induce the HR is not understood, but it is known that HR induction is dependent on plant disease resistance (*R*) genes that somehow mediate recognition of specific Avr proteins. A single *R* gene typically mediates the recognition of a single Avr protein, suggesting that *R* gene products may function as receptors for Avr proteins or for specific enzymatic products of Avr proteins (Bonas and Lahaye, 2002). Although Avr proteins were initially defined by their role in inducing plant defense responses, they must contribute in some fashion to pathogenesis on susceptible host plants (i.e., those lacking a cognate *R* gene). Indeed, several Avr proteins have been shown to enhance disease symptoms and/or pathogen growth on susceptible hosts (Innes, 2001). Determining how Avr proteins contribute to virulence on susceptible hosts and avirulence on resistant hosts represents a major goal in plant pathology.

Our interest in the mechanisms underlying bacterial pathogenesis led to the unexpected finding of amino acid sequence identity between the *Yersinia* YopT effector and the *P. syringae* AvrPphB avirulence protein. Extending our initial sequence analysis of YopT and AvrPphB, we identified a total of 19 open reading frames with similarity to YopT that are found in a large number of bacteria that are pathogenic to animals and plants. This observation suggested that this family of proteins might share similar functions in bacterial pathogenesis on a wide variety of hosts. YopT has recently been shown to induce a cytotoxic effect in mammalian cells when delivered by the *Yersinia* type III secretion system (Iriarte and Cornelis, 1998). This cytotoxicity is characterized by the disruption of the actin cytoskeleton and rounding up of the cells (Iriarte and Cornelis, 1998). Furthermore, YopT is conserved in all three pathogenic *Yersinia* species, suggesting that it plays an important role in pathogenesis. However, the biochemical function of YopT is poorly understood. Infection of host cells with a mutant *Yersinia* strain secreting only YopT causes an isoelectric point shift of RhoA, a small GTPase known to regulate the actin cytoskeleton (Zumbihl et al., 1999). In addition, Sorg et al. (2001) recently demonstrated that YopT can cause the release of RhoA from cell membranes or artificial vesicles. Even less is known about the biochemical function of AvrPphB. *P. syringae* strains expressing the *AvrPphB* gene elicit an HR in *Arabidopsis* plants that carry the *RPS5* resistance gene, but the

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mechanism is unknown (Simonich and Innes, 1995). Likewise, the function of AvrPphB in pathogenesis on susceptible host plants remains undefined.

In this report we show that both YopT and AvrPphB are cysteine proteases. YopT recognizes the posttranslationally modified Rho GTPases and carries out a proteolytic cleavage near their carboxyl termini. This results in the loss of a C-terminal lipid modification on these GTPases, leading to their release from the membrane. The cleavage that liberates RhoA from the membrane is dependent upon amino acids C139, H258, and D274 of YopT. These residues are also essential for the disruption of the actin cytoskeleton that occurs when mammalian cells are transfected with YopT. In addition, the corresponding C/H/D residues are also present in AvrPphB. We show here that AvrPphB undergoes autoproteolytic cleavage that requires the C/H/D residues. Furthermore, the C/H/D residues are essential for eliciting the HR in resistant plant hosts. Therefore, the known biological effects of YopT and AvrPphB are dependent upon the C/H/D residues that are also invariant in the entire YopT family. Our results suggest that all 19 open reading frames corresponding to the Yop T family are cysteine proteases. These observations provide important new insights that should lead to a better understanding of animal and plant pathogenesis.

Results

Sequence Analysis Reveals that YopT Belongs to a Family of Proteins Involved in Bacterial Pathogenesis

We carried out a BLAST search and identified five open reading frames with sequence similarity to YopT (the first five genes shown in Figure 1). Each of these five open reading frames was subjected to multiple PSI-BLAST iterations (Altschul et al., 1997), identifying a total of 19 sequences with significant regions of similarity. The BLOSUM matrix was used to generate the alignment shown in Figure 1. The overall amino acid sequence identity among the YopT family members is not extensive, yet every member of the family shows several invariant residues, including C139, H258, and D274 (numbered from YopT sequence) (Figure 1). In addition, the predicted secondary structure of each YopT family member (http://jura.ebi.ac.uk:8888/jnet/) is similar, with the highest level of structural identity surrounding the conserved C139, H258, and D274 residues. All YopT family members are from bacteria that infect animals and plants, including two bacterial species considered to be plant symbionts. None of the YopT family members have a known biochemical function.

C139, H258, and D274 Are Essential for YopT Cytotoxicity

Previous studies have shown that infection of HeLa cells with a *Yersinia* strain secreting only YopT leads to a cytotoxic effect (Iriarte and Cornelis, 1998; Zumbihl et al., 1999). We transiently transfected EGFP-YopT fusion constructs into HeLa, HEK293T, and COS7 cells. HeLa cells transfected with YopT rounded up, and their actin stress fibers were disrupted as indicated by the rhodamine-phalloidin staining (Figure 2A). This phenotype is similar to the phenotype observed in the YopT infection model noted above. However, cells expressing any of the EGFP-YopT mutants (C139S, H258A, and D174A) showed normal morphology and intact actin stress fibers that were indistinguishable from the control cells (data not shown for D274A; Figure 2A). Similar results were also observed with EGFP and YopT cotransfection (data not shown). These results demonstrate that the invariant C/H/D residues are required for YopT to disrupt the actin filamentous structure in mammalian cells.

In an effort to identify the physiological targets of YopT, we attempted to perform a yeast two-hybrid screen. However, we were unable to obtain any yeast transformants, suggesting YopT is cytotoxic to *S. cerevisiae*. To test this hypothesis, we expressed YopT driven by the galactose-inducible promoter *GAL10*. Yeast strains harboring this YopT expression plasmid grew normally when YopT expression was suppressed by glucose in the medium (Figure 2B). However, when YopT expression was induced by shifting to the galactose-containing medium, no growth was apparent (Figure 2B). *S. cerevisiae* can serve as a powerful system to study the function of microbial virulence factors (Lesser and Miller, 2001). We decided to test whether the invariant C/H/D residues are also required for the toxic phenotype induced by YopT in yeast. We found that none of the C/H/D mutants (C139S, H258A, and D274A) were toxic when assayed under the same condition (Figure 2B). All the nontoxic mutant proteins were expressed at similar levels (data not shown). This observation was consistent with the results we observed in HeLa cells. Interestingly, replacement of another conserved residue (W146A) resulted in the loss of YopT cytotoxicity, while nonconserved substitutions such as R165A, E279A, and S300A displayed no detectable effect on yeast toxicity (Figure 2B).

Yeast Cdc42 Is a Multicopy Suppressor for YopT

The toxicity of YopT in yeast suggested to us that a multicopy suppressor screen might lead to the identification of the physiological target(s) of YopT. The yeast multicopy suppressor screen resulted in the isolation of the genomic clone shown in Figure 3A. This clone suppressed YopT cytotoxicity when expressed in yeast (Figure 3B, pYep13-Cdc42). One of the open reading frames included in this genomic clone encodes the yeast Cdc42 protein, a member of the Rho family of small GTPases. We focused on the Cdc42 gene for the following reasons. First, small GTP binding proteins are common targets of bacterial toxins (Aktories et al., 2000; Boquet, 2000); second, Rho GTPases including Cdc42 play a critical role in regulating the actin cytoskeleton (Hall, 1998); and third, previous studies have shown that infection of a YopT-expressing strain of *Yersinia* could induce an isoelectric point shift in RhoA (Zumbihl et al., 1999). In order to test our hypothesis that Cdc42 was able to suppress the cytotoxic effects of YopT, the yeast Cdc42 gene was expressed using the glyceraldehyde 3-phosphate dehydrogenase (GPD) promoter in YopT-expressing cells. As can be seen in Figure 3B, Cdc42 alone was capable of suppressing the cytotoxic effects of YopT, indicating that small GTP binding proteins like Cdc42 are potential targets of YopT.

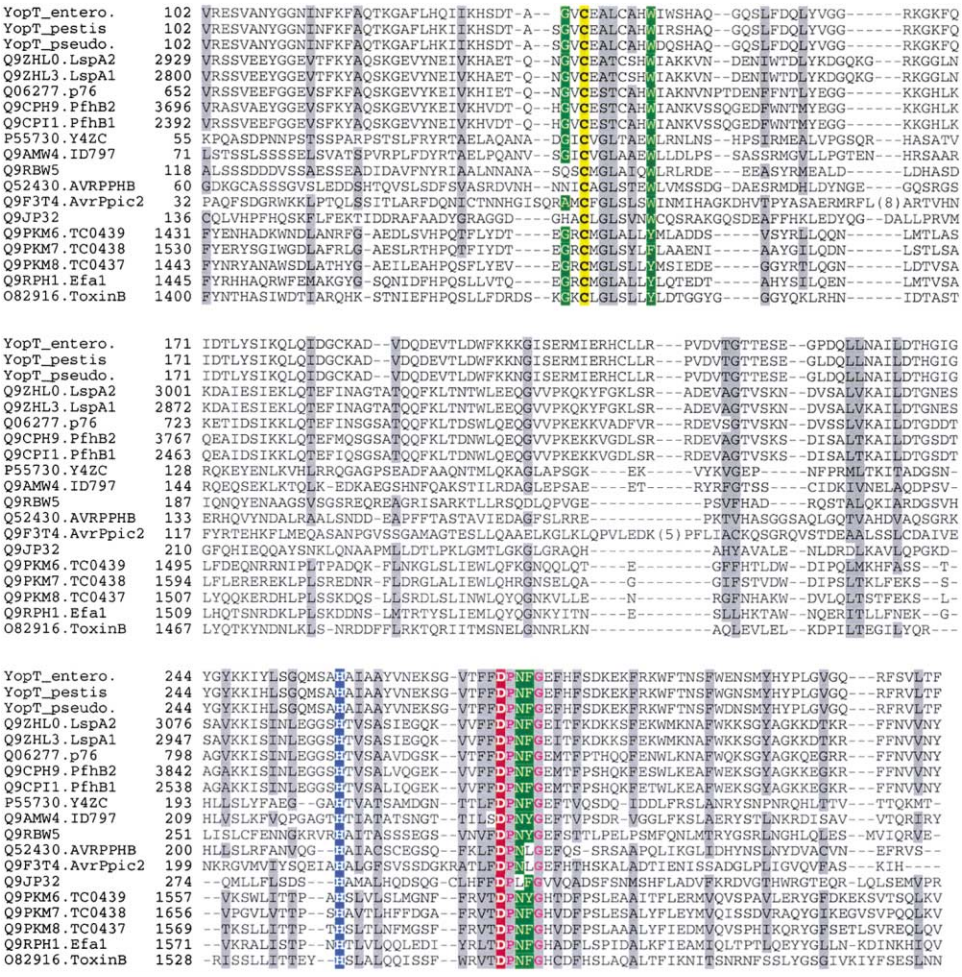

Figure 1. Multiple Sequence Alignment of the YopT Family

The YopT family was identified by PSI-BLAST searches and aligned by the MacVector program. The YopT family contains the YopT gene from *Yersinia pestis, Yersinia entocolitica*, and *Yersinia pseudotuberculosis*. Other members are listed by SWISS-PROT or TrEMBL identification numbers followed by the known gene names. These include Q9ZHL0 and Q9ZHL3 from *Haemophilus ducreyi*, Q06277 from *Haemophilus somnus*, Q9CPH9 and Q9CPI1 from *Pasteurella multocida*, P55730 from *Rhizobium*, Q9AMW4 from *Bradyrhizobium*, Q9RBW5 and Q52430 (AvrPphB) from *Pseudomonas syringae pv. phaseolicola*, Q9F3T4 from *Pseudomonas syringae pv. pisi*, Q9JP32 from *Pseudomonas syringae* pv. *tomato*, Q9PKM6, Q9PKM7, and Q9PKM8 from *Chlamydia muridarum*, Q9RPH1 from *Escherichia coli*, and O82916 from *Escherichia coli O157:H7*. The putative catalytic cysteine, histidine, and aspartic acid are highlighted in yellow, blue, and red, respectively. Other invariant and conserved residues are colored in pink and green, respectively. Residues with similar chemical properties are in gray. The entire YopT family members fall into two groups: YopT from *Yersinia* and orfs from *Pseudomonas* and *(Brady)Rhizobium* encode small proteins with the size of 30–40 kDa, while the deduced molecular weights of the remainders (except p76) are greater than 300 kDa.

YopT Directly Binds to RhoA/Rac/Cdc42 in a Posttranslational Modification-Dependent Manner

Our finding that the yeast Cdc42 gene is a suppressor of YopT cytotoxicity, as well as the previous report that the isoelectric point of RhoA was modified in host cells following *Yersinia* infection (Zumbihl et al., 1999), prompted us to determine if RhoA, Rac, and Cdc42 could all directly interact with YopT. To address this question, we carried out a two-hybrid assay using YopT (C139S) and RhoAL63, a constitutively active mutant form of RhoA. The selective growth on the His-deficient plates (YC-WHULK) shown in Figure 3C clearly suggests that there is an interaction between these two proteins. The interaction with wild-type RhoA, as opposed to the RhoAL63 mutant, was much weaker (data not shown). Unfortunately, we were not able to test the interaction with Rac and Cdc42 because both constitutively active forms of Rac and Cdc42 are toxic to yeast under the assay conditions.

To confirm the interaction with RhoA and to avoid the yeast toxicity seen with Rac and Cdc42, we used a GST pull-down assay. GST-tagged forms of constitutively active RhoAL63, RacL61, and Cdc42L61, but not GST alone, coprecipitated with FLAG-tagged YopT (C139S) when the respective plasmids were coexpressed in HEK293T cells and the cell lysates were subjected to GST pull-down (Figure 3D). In the reciprocal assay, re-

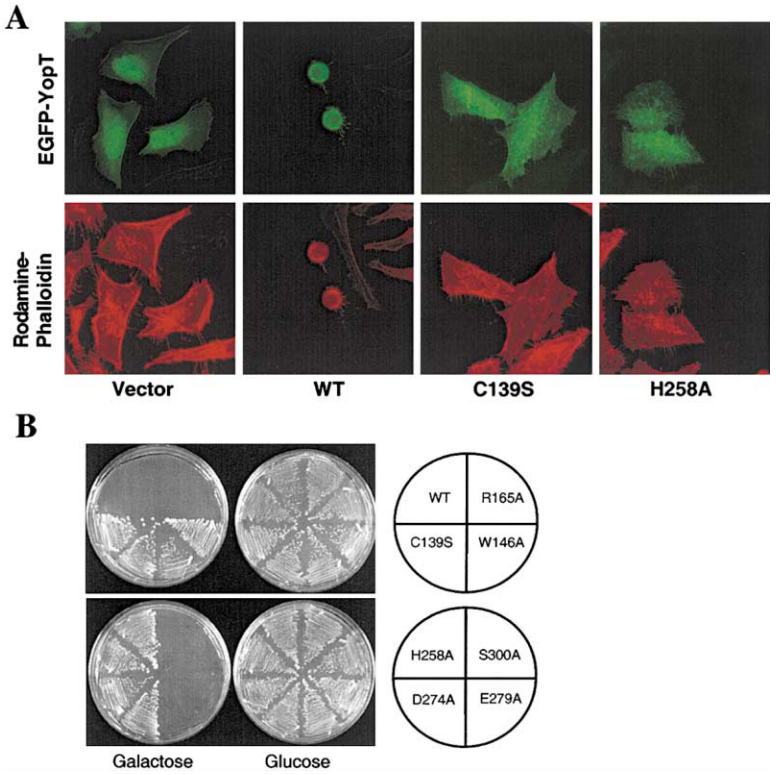

Figure 2. C139, H258, and D274 Are Essential for YopT Cytotoxicity

(A) Phenotypic assay of YopT mutants in HeLa cells. HeLa cells were transfected with pEGFP vector or indicated EGFP-YopT fusion constructs. Cell transfection, YopT expression, and cell morphology were visualized by EGFP staining (top). Actin filaments were visualized by rhodamine phalloidin staining (bottom). Expression of wild-type YopT, but neither of the C139S, H258A mutants, led to the disruption of actin cytoskeleton and subsequent rounding up of HeLa cells.

(B) The invariant C/H/D residues are required for YopT toxicity in yeast. YopT and mutants were expressed under galactose-inducible promoter (p413Gal) in *S. cerevisiae* strain W303a. The yeast strains were grown in either glucose or galactose medium. YopT is cytotoxic to yeast shown by inhibited growth in galactose medium. Mutants of YopT in the conserved residues including C139S, W146A, H258A, and D274A are not cytotoxic, while nonconserved mutations (R165A, S300A, and E279A) remain cytotoxic.

combinant GST-YopT (C139S) immobilized on glutathione beads was able to pull down RhoAL63, RacL61, and Cdc42L61 from the HEK293T cell lysates when these GTPases were overexpressed (data not shown). These results indicate that Rho family GTPases including RhoA, Rac, and Cdc42 are all potential direct targets of YopT.

We were unable to detect any interaction between YopT and RhoAL63, RacL61, and Cdc42L61 produced in bacteria. Since bacteria are not capable of posttranslationally modifying the GTPases, this suggested to us that the interaction of YopT with Rho GTPases could be mediated via posttranslational modification. RhoA, Rac, and Cdc42 are all known to undergo sequential posttranslational modifications at their carboxy-terminal CAAX box (C, cysteine; A, aliphatic residue; X, any residue) (Zhang and Casey, 1996). The CAAX box provides the recognition elements for prenylation (geranylgeranylation) of the cysteine, followed by proteolysis of the AAX tripeptide and methyl esterification of the cysteine. The lipid modification allows for the membrane anchorage of the GTPases (Zhang and Casey, 1996). We decided to test the hypothesis that removal of the CAAX box from the GTPases would affect the interaction between YopT (C139S) and Rho GTPases. Deletion of the CAAX box abrogates the two-hybrid interaction between YopT (C139S) and RhoAL63 (Figure 3C). In contrast to the full-length GTPases, CAAX deletion mutants of RhoAL63, RacL61, and Cdc42L61 were unable to coprecipitate with the YopT (C139S) mutant when coexpressed in HEK293T cells (Figure 3D). In addition, we placed the CAAX box of RhoA onto the carboxyl terminus of GFP and assayed the two-hybrid interaction with the YopT C139S mutant. GFP-CAAX has been shown to undergo the same posttranslational modifications as Rho GTPases when it is expressed in yeast (Figueroa et al., 2001). We observed a weak interaction between YopT (C139S) and GFP-CAAX in the yeast two-hybrid assay, while there is no detectable interaction with GFP alone (Figure 3C). Taken together, these results demonstrate that the direct interaction between YopT and Rho GTPase relies on the CAAX-dependent modification of RhoA, Rac, and Cdc42.

The Enzymatic Activity of YopT Leads to the Loss of the Prenyl Group from RhoA/Rac/Cdc42

Collectively, our data pointed to an interaction between the C-terminal-modified forms of RhoA, Rac, and Cdc42 and YopT (C139S). To explore the nature of this interaction, we employed the established Triton X-114 partitioning assay, which partitions lipid-modified proteins in the detergent phase and nonmodified proteins in the aqueous phase (Hancock, 1995). Overexpressing GST-RhoAL63 in HEK293T cells resulted in almost equal amounts of RhoAL63 protein partitioning in the detergent phase and aqueous phase (Figure 4A). Cotransfection of wild-type YopT with RhoAL63 resulted in an almost complete loss of RhoAL63 from the detergent phase. In contrast, transfection of the YopT mutants (C139S and H258A) shows substantial amounts of RhoAL63 in the detergent phase (Figure 4A). α-FLAG Western blots showed that YopT and the corresponding mutants were all expressed as anticipated (Figure 4A). Similar results were obtained for both RacL61 and Cdc42L61 (data not shown). Although the expression levels of GTPases are slightly different between YopT-

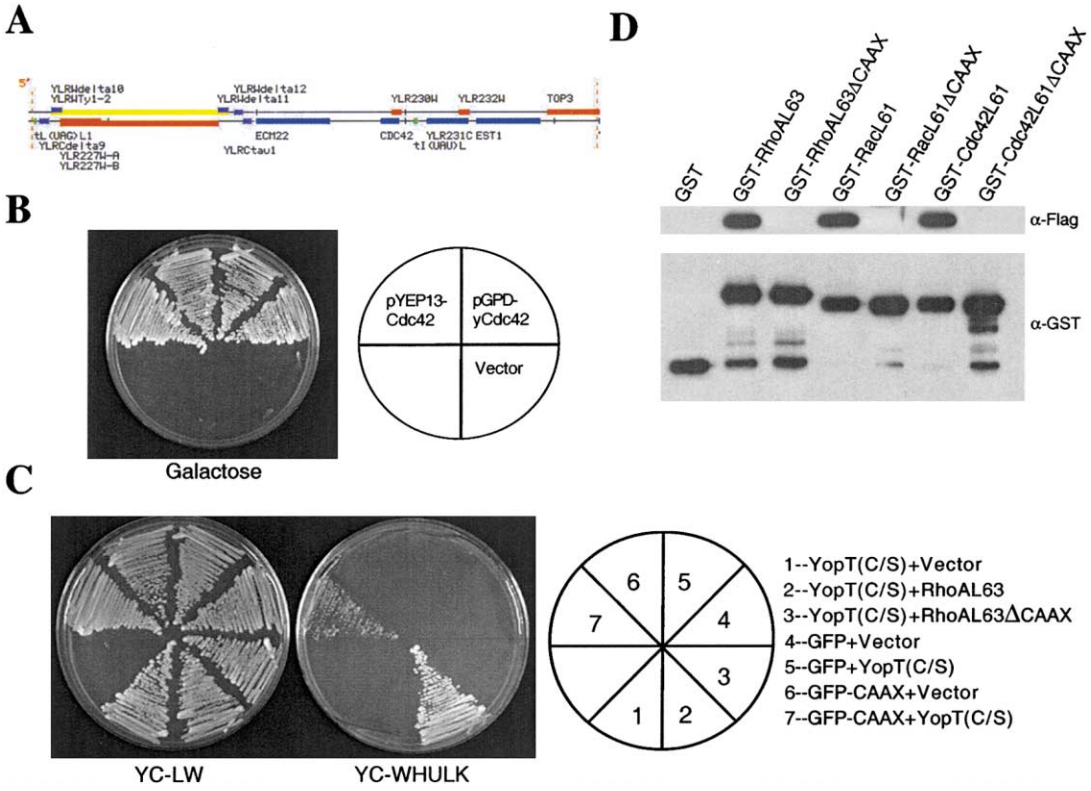

Figure 3. The Yeast Cdc42 Is a Suppressor for YopT Cytotoxicity and YopT Interacts with RhoA/Rac/Cdc42 in a Posttranslational Modification-Dependent Manner

(A) The genomic clone obtained from the suppressor screen contains the yeast Cdc42 gene. This schematic was downloaded from the *Saccharomyces* genome database (http://genome-www.stanford.edu/*Saccharomyces*/).

(B) Overexpression of yeast Cdc42 can suppress YopT cytoxicity in yeast. The genomic clone (pYEP13-Cdc42) listed in (A) and the yeast Cdc42 expression plasmid driven by GPD promoter (p415GPD) were capable of overcoming YopT cytotoxicity as assayed by growth in galactose-containing medium.

(C) Yeast two-hybrid assay of YopT and CAAX-modified RhoA. The yeast L40 strain was transformed with the various plasmid combinations, and the activation of the *HIS3* reporter was assessed by growth on YC-WHULK plates. To test the interaction between YopT and RhoA, the C139S mutant of YopT was fused with the LexA DNA binding domain, and RhoAL63 or RhoAL63ΔCAAX that lacks the CAAX box were fused to the VP16 activation domain. GFP-CAAX is a fusion protein between the LexA DNA binding domain and GFP containing the carboxy-terminal RhoA tetrapeptide CLVL. GFP vector corresponds to the GFP-LexA fusion protein. The YopT C139S mutant was expressed as a fusion protein with the VP16 activation domain.

(D) GST pull-down assay of the prenylation-dependent interaction between YopT and Rho GTPases. GST or GST fusion proteins of the indicated GTPases were purified from lysates prepared from HEK293T cells cotransfected with FLAG-YopT (C139S) and GST-tagged GTPases using glutathione agarose. The pull-downs were analyzed by Western blotting with anti-FLAG antibodies (top) or anti-GST antibody (bottom). The sample volumes loaded in the bottom gel are 1/15th of those of the top gel. ΔCAAX indicates a deletion of the carboxy-terminal CAAX box.

and vector-transfected cells, a comparison of wild-type and mutant YopT-transfected cells clearly suggests that YopT leads to the loss of the prenyl group of RhoA, Rac, and Cdc42.

Since the previous experiments were carried out in vivo, we could not distinguish between the possibility that YopT inhibits the prenylation of the GTPases and the possibility that YopT actually removes the isoprenoid moiety of the prenylated GTPases once they are anchored to the membrane. To address this question, we assayed the loss of the prenyl group under in vitro conditions. Cell lysates prepared from HEK293T cells transfected with RhoAL63, RacL61, and Cdc42L61 were incubated with recombinant wild-type or mutant GST-YopT. Following a 30 min incubation at 37°C, each lysate was subjected to a GST-RhoGDI (the guanine nucleotide dissociation inhibitor for Rho family of GTPases) pull-down assay. The prenyl group is known to be essential for the interaction between RhoGDI and Rho GTPases (Hancock and Hall, 1993; Hoffman et al., 2000). As shown in Figure 4B, recombinant GST-RhoGDI immobilized on glutathione beads did not capture detectable amounts of RhoAL63, RacL61, and Cdc42L61 from the corresponding lysate incubated with wild-type (wt) YopT. In contrast, incubation of the same lysate with any of the YopT mutants results in RhoGDI pull-down of RhoA, Rac, and Cdc42 (Figure 4B). GST beads alone did not bind to any GTPases under the same assay conditions (data not shown). This result suggests that YopT could remove the prenyl group from Rho GTPases after they are posttranslationally modified. Sorg et al. (2001) recently reported that YopT treatment of RhoA results in

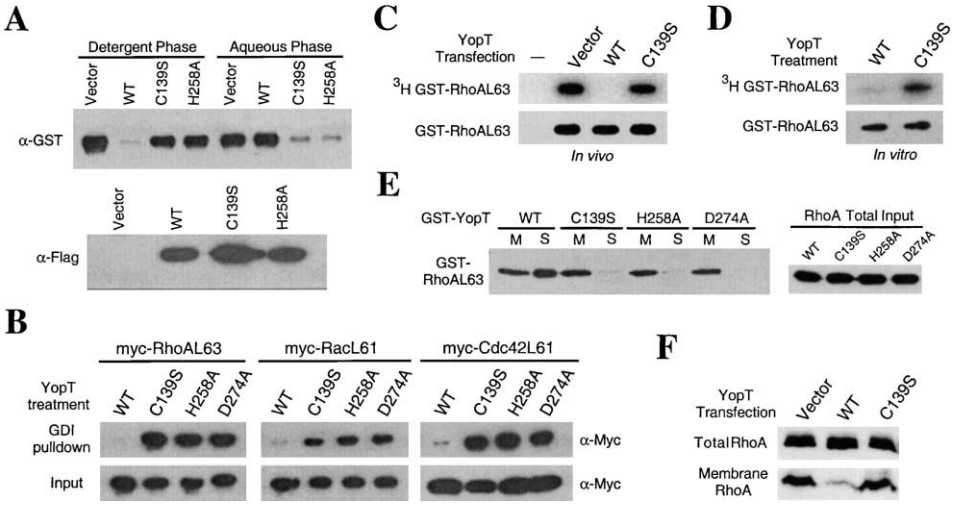

Figure 4. YopT Leads to the Loss of the Prenyl Group of RhoA, Rac, and Cdc42

(A) Triton partition assay of the effect of YopT expression on the lipid modification of RhoA. HEK293T cells were cotransfected with GST-RhoAL63 and indicated YopT constructs. The cell lysates were subjected to Triton X-114 partitioning and GST pull-down as described in Experimental Procedures. Equal amounts of the pull-downs were analyzed by α-GST Western blotting (top). Expression of YopT was shown in the bottom gel.

(B) GDI pull-down assay of the effect of YopT on the lipid modification of Rho GTPases. HEK293T cells were transfected with myc-tagged GTPases as indicated. 200 μl of cleared lysates were incubated with wt or mutant recombinant YopT. Input corresponds to 5 μl of the 200 μl lysate postincubation. The lysates were then subjected to GST-RhoGDI pull-down. Samples eluted from the GST beads (top) as well as the input samples (bottom) were analyzed by immunoblotting with anti-myc antibody. RhoA, Rac, and Cdc42 incubated with wild-type YopT, as opposed to incubation with the YopT mutants, were incapable of binding to RhoGDI.

(C) In vivo lipid labeling assay of the removal of the prenyl group from RhoA by YopT. HEK293T cells transfected with GST-RhoAL63 construct and either pSFFV vector or pSFFV-FLAG-YopT (wt or C139S) were labeled with ^{3}H-mevalonic acid as described in Experimental Procedures. The amount of radiolabeled GST-RhoAL63 was analyzed by autoradiography (top), and the total amount of GST-RhoAL63 was shown by anti-RhoA Western blotting (bottom).

(D) Removal of the tritium-labeled prenyl group from RhoA by recombinant YopT in vitro. GST-RhoAL63 with tritium-labeled prenyl group was prepared by metabolic labeling and immobilized onto the GST beads. The beads were incubated with recombinant wild-type or mutant YopT. The remaining amounts of RhoA and tritiated RhoA on the beads were analyzed by anti-RhoA Western blotting (bottom) and autoradiography (top), respectively.

(E) YopT can release RhoA from membranes in vitro. Membranes were isolated from HEK293T cells overexpressing GST-RhoAL63. The membranes were then incubated with recombinant YopT or YopT mutants as indicated. The amount of RhoA in the membrane fractions (M) and the corresponding soluble factions (S) were analyzed by anti-RhoA Western blotting. The gel on the right shows the total input of RhoA used in each assay.

(F) YopT leads to the membrane detachment of the endogenous RhoA in vivo. HEK293T cells were transfected with either vector or wild-type or C139S mutant YopT. Equal amounts of total lysates (top) or membrane fractions (bottom) were subjected to anti-RhoA immunoblotting analysis.

an increase in RhoGDI binding. The difference between our experiment and that of Sorg et al. is that we monitored the GTP bound form of RhoA while Sorg and her colleagues assayed for the endogenous RhoA. Our experimental design avoids the interchange between the two populations (GTP and GDP bound forms) of RhoA, which differ dramatically in their affinity to bind to RhoGDI (Sasaki et al., 1993). Furthermore, our results are consistent with the membrane detachment of RhoA induced by YopT as was noted by Zumbihl et al. (1999) as well as our other data described below demonstrating that YopT leads to the loss of the prenyl group of RhoA.

To directly assess loss of the prenyl group of Rho GTPases, we expressed GST-RhoAL63 in HEK293T cells in the presence of YopT or YopT (C139S) and metabolically labeled the cells with ^{3}H-mevalonic acid, which is incorporated into the prenyl group of RhoA. As shown in Figure 4C, coexpression of wild-type YopT, but not YopT (C139S), resulted in a complete loss of the prenyl group from GST-RhoAL63, although the total GST-RhoAL63 remained at a similar level. To confirm that the loss of the prenyl group is a direct effect of YopT enzymatic activity, we performed the cleavage reaction in vitro using recombinant YopT or YopT mutant (C139S) and tritiated GST-RhoAL63. The tritium-labeled GST-RhoAL63 was purified from HEK293T cells labeled with ^{3}H-mevalonic acid and immobilized onto the GST beads. As shown in Figure 4D, incubation with wild-type YopT, but not YopT (C139S), resulted in a dramatic loss of the prenyl group from GST-RhoAL63. Taken together, our data suggest that YopT harbors an enzymatic activity toward Rho GTPases that can lead to the loss of their prenyl groups. To address whether YopT cleaves Rho GTPases in a GTP bound-dependent manner, we carried out the in vitro cleavage assay described above using both RhoAL63 (GTP bound form) and RhoAN19, the nucleotide-free form of RhoA. Preliminary results indicate that both the GTP bound and nucleotide-free forms of

RhoA are equally good substrates for recombinant YopT in vitro (data not shown). These data suggest that recognition by YopT is independent of the nucleotide bound to the GTPases.

Loss of the Prenyl Groups of Rho GTPases Leads to Their Membrane Detachment

As a consequence of the removal of the prenyl group, recombinant YopT should be capable of releasing Rho GTPases from membranes into the soluble fraction in vitro as recently demonstrated (Sorg et al., 2001). We employed a similar assay to test whether the invariant C/H/D residues are required for YopT to release Rho GTPases from the membranes. As expected, a significant amount of RhoAL63 was released from the membrane into the soluble fraction upon incubation with wild-type YopT (Figure 4E). Incubation with the same amounts of the mutant forms of YopT (C139S, H258A, and D274A) produced no soluble GST-RhoAL63 (Figure 4E). Similar results were observed for Rac and Cdc42 (data not shown), confirming that YopT affects all the representative members of Rho family GTPases. We have shown that YopT can cause the cytoskeleton disruption phenotype in mammalian cells, and this depends on the invariant C/H/D residues (Figure 2A). To test whether this phenotype is the consequence of the membrane detachment of Rho GTPases induced by YopT as we observed in vitro, we monitored the membrane distribution of the endogenous RhoA in the presence of YopT. The results are shown in Figure 4F. Upon transfection of wild-type, but not the mutant YopT (C139S), the endogenous RhoA dissociated from the membranes. Levels of total RhoA expression are similar in the three experiments shown in Figure 4F. These data suggest that the cytotoxicity of YopT arises from its enzymatic activity, resulting in the removal of the lipid modification of the endogenous Rho GTPases and their consequent membrane detachment.

YopT Is a Cysteine Protease Cleaving Near the Carboxyl Termini of Rho GTPases

The three invariant residues (C/H/D) required for YopT function are also residues that compose the catalytic triad of many cysteine proteases (Rawlings and Barrett, 1994). Traditionally, cysteine proteases are classified into approximately 40 different families based on sequence similarity (Barrett and Rawlings, 2001; Rawlings and Barrett, 1994). The YopT family lacks apparent sequence identity to the known families of cysteine proteases listed in the MEROPS protease database (http://www.merops.co.uk), suggesting that YopT might define a new family of cysteine proteases. Barrett and Rawlings (2001) have classified all families of cysteine proteases into seven clans, each with a different evolutionary history. The cysteine proteases within each clan share common secondary structural features in spite of the lack of overt sequence similarity between some families within the same clan (Barrett and Rawlings, 2001). We noticed that the predicted secondary structural profiles of the YopT family members exhibit a number of common characteristics defining the so-called CA clan of cysteine proteases (Figure 5A). These structural characteristics include (1) the N-terminal half of the protease domain is predicted to be largely made up of α helix (shown in red), while the majority of the C terminus is composed of β strands (green); (2) the order of the C/H/D (or N) residues making up the catalytic triad are similar in the CA clan and the YopT family; and (3) the catalytic C is located at the end of a coil and the beginning of a major helix, the H follows a coil and is succeeded by a β strand, and the D (N) is at the end of a β strand (Figure 5A).

In view of these similarities, we wanted to test the possibility that YopT defines a new family of the cysteine proteases belonging to the CA clan. We carried out the membrane release assay described above in the presence of class-specific protease inhibitors. Release of RhoAL63 from membranes upon YopT treatment was unaffected by the aspartyl protease inhibitor pepstatin (Figure 5B), the serine protease inhibitor PMSF, and metal chelator EDTA (data not shown). However, membrane release of RhoAL63 was terminated by the thiol-blocking reagent N-ethylmaleimide and significantly inhibited by the clan CA selective cysteine protease inhibitor E64 (Figure 5B; Barrett and Rawlings, 2001). To further confirm the effect of protease inhibitors, we also performed the in vitro cleavage assay as described in Figure 4D using recombinant YopT preincubated with either E64 or N-ethylmaleimide. Consistently, E64 and N-ethylmaleimide blocked the release of prenyl moiety from RhoA upon incubation with YopT (Figure 5C). These data support our conclusion that YopT is a cysteine protease belonging to the CA clan. To assess the proteolytic activity of YopT directly, we decided to monitor the fate of the prenylated cysteine. GST-RhoAL63, labeled with either tritiated mevalonate or ^{35}S cysteine, was isolated from HEK293T cells and then incubated with wild-type or mutant YopT. After incubation, the reaction solution was subjected to chloroform extraction to isolate the lipid components. Figure 5D shows the radioactivity present in the chloroform phase. Treatment of ^{35}S-Cys- or ^{3}H-mevalonate-labeled GST-RhoA with wild-type YopT resulted in a 4-fold increase in radioactivity in the chloroform phase when compared with treatment of the same labeled substrates with C/S mutant of YopT. This result clearly demonstrated that YopT functions as a cysteine protease to remove the prenylated cysteine from the carboxyl terminus of RhoA. The cleavage site of YopT is near the COOH-terminal modified cysteine, since there are no major changes seen in the size of the GTPases on SDS-PAGE (data not shown).

AvrPphB, a Member of the YopT Family, Is also a Cysteine Protease

Although the majority of the sequences shown in Figure 1 have no known biological functions, AvrPphB (originally named AvrPph3) is an avirulence protein from *P. syringae* pv. *phaseolicola,* which causes halo-blight disease in its bean host (Jenner et al., 1991). Puri and his colleagues (Puri et al., 1997) have shown that AvrPphB is synthesized in *Pseudomonas* as a precursor of a 35 kDa protein that is subsequently processed into a 28 kDa protein. AvrPphB contains the invariant C/H/D residues common to all members of the YopT family. We wanted to test the possibility that the 35 kDa AvrPphB protein

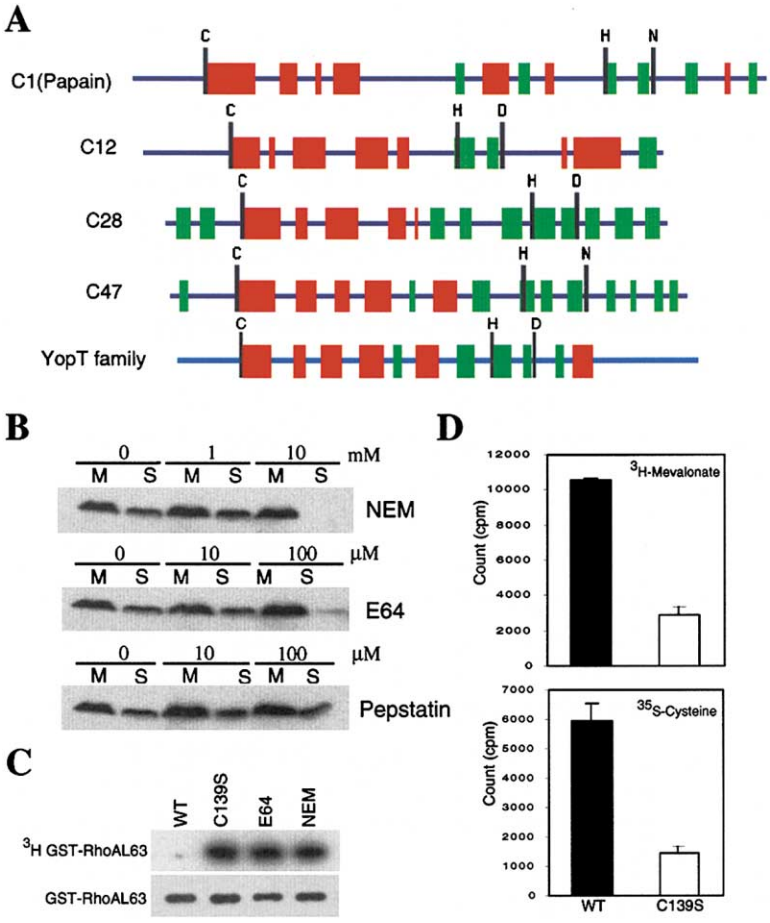

Figure 5. YopT Is a Cysteine Protease Belonging to the CA Clan

(A) The YopT family harbors structural characteristics defining the CA clan of cysteine proteases. The predicted secondary structural profile of the YopT family is similar to the CA clan cysteine proteases with known structures (C1, C12, C28, and C47 family). The order of the invariant C/H/D residues within the YopT family is similar to that of the catalytic triad [C/H/D(N)] of the CA clan cysteine proteases. The secondary structures surrounding the invariant C/H/D residues of the YopT family and the catalytic triad of the CA clan cysteine protease are almost identical. The red and green boxes designate the α helix and the β strand, respectively. The diagrams of the secondary structures of CA clan cysteine protease were downloaded from the MEROPS protease database (htttp://www.merops.co.uk).

(B) The effects of protease inhibitors on YopT assayed by membrane release. Membrane release assay of RhoA was performed in the presence of indicated protease inhibitors. The membrane release activity of YopT was blocked by thiol blocking agent N-ethylmaleimide (NEM) and CA clan-specific inhibitor E64 in a concentration-dependent manner. Aspartyl protease inhibitor pepstatin has no effect on YopT activity at 100 μM. M corresponds to the membrane fractions and S is the soluble fractions.

(C) The effects of protease inhibitors on YopT assayed by the removal of the tritiated prenyl group from RhoA in vitro. The experiment was the same as shown in Figure 4D except that the recombinant wild-type YopT was preincubated with 10 mM N-ethylmaleimide (NEM) or 100 μM E64 at 30°C for 30 min.

(D) YopT is a protease that removes the prenylated cysteine from RhoA. GST-RhoAL63 labeled with either tritiated lipids or [^{35}S]cysteine were incubated with recombinant YopT. After incubation, the lipids were separated from the reaction solution by chloroform extraction. The amounts of the tritiated prenyl group (top) and the ^{35}S-labeled prenylaed cysteine (bottom) present in the chloroform phase were measured by liquid scintillation counting.

might undergo autocatalytic processing to produce the corresponding 28 kDa protein. The first gel in Figure 6A shows the time course for the conversion of the wild-type protein from the 35 kDa form to the 28 kDa form in *E. coli*. When any of the invariant residues were mutated (C98S, H212A, and D227A) in AvrPphB, no conversion of the 35 kDa protein to the 28 kDa protein was observed (Figure 6A). These data suggest that the conversion is autocatalytic and dependent upon the invariant C/H/D residues, supporting the idea that AvrPphB is most likely a protease. If the cleavage event is indeed autocatalytic, then the processing event should be independent of the type of cells employed to produce the protein. We therefore generated AvrPphB in a rabbit reticulocyte cell-free system and again observed the 35 kDa to 28 kDa conversion (Figure 6B). Similar to what was seen in *E. coli*, mutations in the three invariant residues precluded conversion of the 35 kDa to the 28 kDa protein in the rabbit reticulolysate system (Figure 6B). The dependence of the autocatalytic processing of AvrPphB upon the C/H/D residues conserved in the YopT family suggests that AvrPphB is also a cysteine protease. This raises the intriguing possibility that the YopT family represents a large and diverse family of cysteine proteases functioning in both plant and animal pathogens.

Proteolytic Inactive Mutants of AvrPphB Are Not Capable of Inducing a Plant Hypersensitive Response

P. syringae strains carrying *avrPphB* induce an HR when inoculated into *Arabidopsis* plants carrying the cognate resistance gene *RPS5* (Simonich and Innes, 1995). *AvrPphB* also induces an HR when transiently expressed in tobacco leaves (see below). To determine if proteolytic activity is required for the avirulence activity of AvrPphB, we injected *Arabidopsis* leaves with bacteria containing the wild-type and the proteolytically inactive mutants of AvrPphB and assayed for the induction of the HR as assessed by visible leaf collapse. The results are shown in Figure 7A. An HR was induced in 95% of the leaves inoculated with the strain carrying the wild-type AvrPphB. Mutations in the AvrPphB catalytic triad (C98S, H212A, and D227A) reduced the number of leaves expressing a visible HR to the level of the negative control (10% or less). As expected, a mutation in a non-

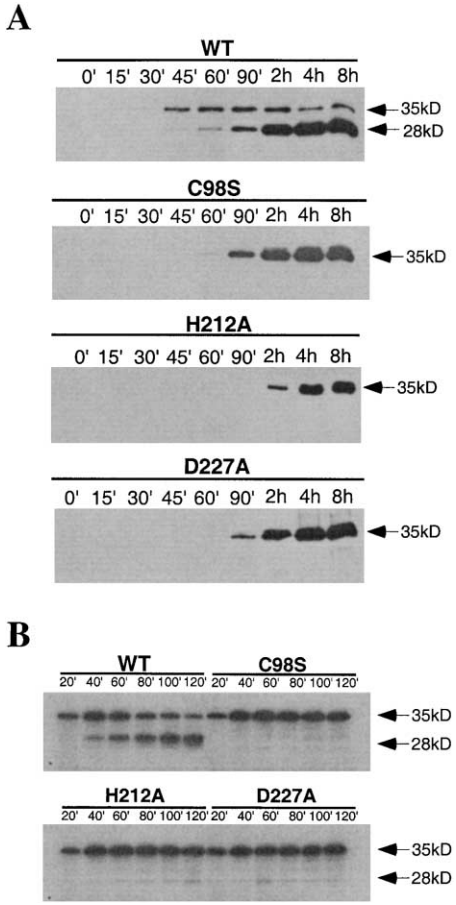

Figure 6. AvrPphB Can Undergo Autoproteolytic Processing

(A) Autoproteolytic processing of AvrPphB in bacteria. Full-length AvrPphB (wt and indicated mutants) with a C-terminal polyhistidine tag were expressed in *E. coli* BL21 (DE3). Equal amounts of bacterial culture taken at the designated time points after induction were subjected to α-His Western blotting analysis. The wild-type AvrPphB protein was synthesized as a 35 kDa precursor and converted to a 28 kDa form. All three mutants are deficient in the conversion from the 35 kDa to the 28 kDa protein.

(B) Autoproteolytic processing of AvrPphB in rabbit reticulolysate. AvrPphB was in vitro transcribed/translated in the reticulolysate system, labeled with [^{35}S]methionine. Aliquots of the reaction removed at the time points indicated were stopped by addition of SDS sample buffer. The samples were then separated by SDS-PAGE and the protein was visualized by autoradiography. The wild-type AvrPphB underwent the conversion from the 35 kDa precursor to the 28 kDa form. All the C/H/D mutants remained in the 35 kDa form.

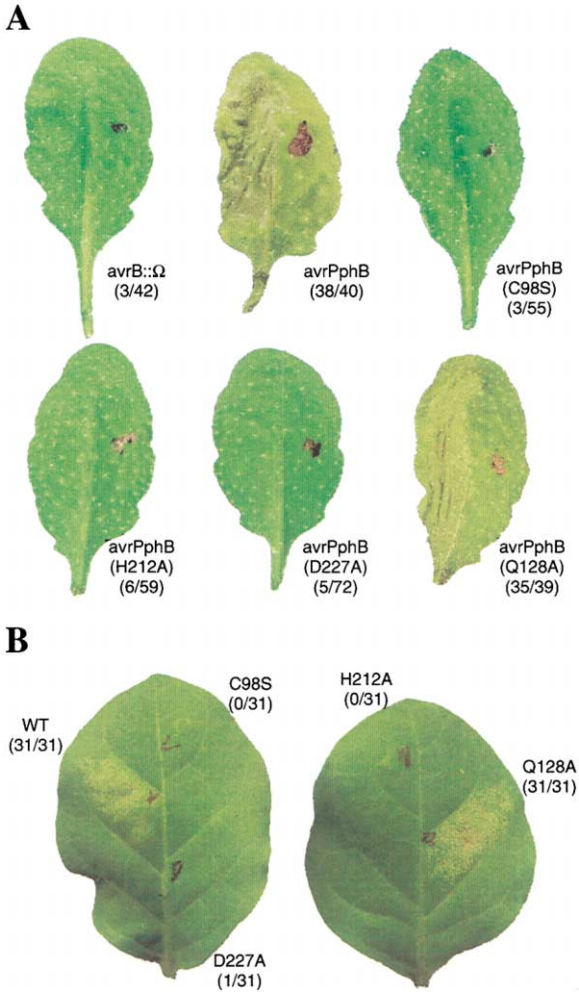

Figure 7. Proteolytic Inactive AvrPphB Is Unable to Elicit the Plant HR

(A) Assessment of the HR-inducing activity of AvrPphB mutants delivered by *P. syringae*. Leaves of 5-week-old *Arabidopsis* plants were injected with *P. syringae* strain DC3000 expressing wild-type and mutant forms of AvrPphB. C98S, H212A, and D227A indicate mutations in the putative catalytic residues of AvrPphB, while Q128A is a mutation in a residue not conserved among YopT family members. As a negative control, leaves were injected with a DC3000 strain carrying an inactivated *Avr* gene (AvrB::Ω) in the same vector. Photographs were taken 22–24 hr after inoculation. The black dots mark the side of the leaf that was not injected, and the numbers represent the leaves expressing an HR over the total leaves injected. Mutations in the AvrPphB catalytic triad reduce the HR to the level of the negative control (AvrB::Ω), while the Q128A mutant has no effect on the induction of the HR.

(B) Assessment of the HR-inducing activity of AvrPphB mutants expressed directly in tobacco leaves using *Agrobacterium*-mediated transient transformation. Leaves of 5-week-old tobacco plants were injected with *Agrobacterium* strains carrying wt and mutant forms of AvrPphB under the control of a steroid-inducible promoter. Plants were sprayed with dexamethasone 48 hr after injection to induce expression of AvrPphB. Leaves were scored for the presence of an HR 24 hr after hormone application. AvrPphB catalytic mutants failed to elicit an HR, while the Q128A mutation had no effect on HR expression.

conserved residue (Q128A) had no effect on the ability of AvrPphB to induce the HR.

The results described above suggest that proteolytic activity is required for AvrPphB to induce an HR. However, we could not conclude that AvrPphB functions as a protease to induce the HR in plants if the autocatalytic processing event is a prerequisite for AvrPphB function. An *Agrobacterium* transient expression assay was carried out in *Nicotiana tabacum* leaves using a construct encoding the processed 28 kDa version of AvrPphB (either wild-type or catalytic inactive mutants). Similar

to the results observed with *P. syringae* infection assays, the C/H/D mutants of the 28 kDa AvrPphB were completely inactive and failed to induce the HR, in contrast to wild-type and the Q128A mutant (Figure 7B). These data demonstrate that the proteolytic triad of AvrPphB is required not only for its autocatalytic processing but also for inducing the HR in plants.

Discussion

Proteolytic Activity of YopT and AvrPphB Represents a Widely Used Mechanism in Bacteria-Host Interactions

Seventeen members of the YopT family are found in pathogenic bacteria. These include the animal pathogens *Yersinia*, *Haemophilus ducreyi* (LspA1 and LspA2), *Haemophilus somnus* (p76), *Pasteurella multocida* (PfhB1 and PfhB2), *Chlamydia muridarum* (TC0437, TC0438, and TC0439), and pathogenic *E. coli* (Efa1 and ToxinB), as well as the plant pathogen *Pseudomonas syringae* (AvrPphB, AvrPpic2, Q9RBW5, and Q9JP32). The other two members within this family are from plant symbionts *Rhizobium sp*. NGR234 (Y4ZC) and *Bradyrhizobium japonicum* (ID797). The entire YopT family has the invariant C/H/D residues, suggesting that all members of the family are likely cysteine proteases. The proteolytic activities shown with YopT and AvrPphB likely define a widely used mechanism employed in bacteria-host interactions.

The YopT family members can be divided into two groups. One group comprises members from *Yersinia*, *Pseudomonas*, and *(Brady)Rhizobium*. These proteins have a size of approximately 30–40 kDa. Proteins within this group have either been shown to be or are likely to be secreted via the type III secretion system. Members within a second group of the YopT family are greater than 300 kDa and have functional domains in addition to the YopT-like protease domain (p76 is an exception to this observation). For example, Efa1 from pathogenic *E. coli* contains a glycosyltransferase domain in addition to the YopT-like protease domain (Nicholls et al., 2000). Very little is known about how these proteins function in pathogenesis and how they are exported from the bacteria.

Proteolytic Activity of YopT in *Yersinia* Pathogenesis

Six Yop effectors have been identified and all of them (except YopM, which has no known function) have catalytic activity (Juris et al., 2002). YopH, a potent protein tyrosine phosphatase (Guan and Dixon, 1990), and YopE, a RhoGAP for Rho family GTPases (Black and Bliska, 2000; Von Pawel-Rammingen et al., 2000), have been shown to play important roles in antagonizing phagocytosis during *Yersinia* infection (Rosqvist et al., 1988, 1990). YpkA is a protein serine/threonine kinase and also believed to have a potential function in inhibiting phagocytosis, since it can induce cytoskeletal rearrangements in cultured cells (Galyov et al., 1993; Juris et al., 2000). YopJ is required for downregulation of multiple signaling pathways in host cells and can induce macrophage apoptosis (Orth, 2002). YopJ also contributes to the inhibition of the inflammatory response of macrophage upon *Yersinia* infection (Palmer et al., 1998) and shows sequence similarity to the ubiquitin-like protein proteases (Orth et al., 2000). Extensive studies have shown that activation of Rho family GTPases is a pivotal event in the rearrangement of the actin cytoskeleton during macrophage phagocytosis and integrin-mediated bacteria internalization in nonphagocytic cells (Chimini and Chavrier, 2000). Proteolytic inactivation of Rho GTPases by YopT would likely impair the ability of host cells to execute phagocytosis and internalization. An emerging question is why *Yersinia* has two effectors (YopT and YopE), both inactivating Rho family GTPases and potentially contributing to antiphagocytosis. There are several possible ways to explain this redundancy. First, the mechanisms of YopT and YopE function are completely different. Accelerated GTP hydrolysis of Rho GTPases by YopE can be reversed by the guanine nucleotide exchange factors (GEF) within host cells, whereas the proteolytic cleavage of the carboxyl termini of Rho GTPases by YopT results in irreversible inactivation of Rho GTPases. Second, it has been demonstrated that phagocytosis mediated by immunoglobin and complement receptor requires selective activation of Rac/Cdc42 and RhoA, respectively (Caron and Hall, 1998). Therefore, it is possible that YopT and YopE may specifically inactivate different members of Rho GTPases in the course of *Yersinia* infection. Third, YopT and YopE might be selectively secreted into different types of host cells or into the same host cells at different concentrations.

A Mechanism of Bacterial Toxin Targeting to the Small GTPases

For the purposes of altering the host cytoskeleton or inhibiting phagocytosis, bacterial pathogens have produced a variety of toxins that act on the small GTP binding proteins via a wide array of mechanisms. These toxins either function to regulate the cycling of the nucleotides bound to the GTPases by mimicking eukaryotic GAP or GEF proteins or function to covalently modify the GTPases at specific residues, resulting in constitutive GTP or GDP bound forms. ADP-ribosylation, UDP-glycosylation, and glutamine deamination of small GTP binding proteins by a number of bacterial toxins have been reported (Boquet, 2000; Lerm et al., 2000). Here, we describe the first example of proteolytic cleavage of the small GTP binding proteins by a bacterial toxin. Different from the mechanisms noted above, inactivation of the GTPases by YopT does not affect the nucleotide bound by these GTPases.

Potential Cleavage Site of Rho GTPases by YopT

We were unable to distinguish the cleaved Rho GTPases from the uncleaved GTPases by SDS-PAGE. This observation is consistent with the report of Zumbihl et al. (1999), who observed a change in the isoelectric point but not the molecular weight of RhoA. Our labeling experiments suggest that the cysteine along with its prenyl group is removed by a proteolytic cleavage. Although the exact cleavage site has not been determined, it must occur very near the C terminus of the proteins. Experiments designed to identify the cleavage products by mass spectrophotometry are currently in progress.

Substrate Specificity of YopT and Putative YopT Family Proteases

We have demonstrated that YopT recognizes prenylated Rho GTPases and executes a proteolytic cleavage near their C termini. It will be interesting to know whether all the prenylated proteins could serve as a substrate for YopT. Preliminary experiments with Ras indicate that YopT does not cleave Ras although it is also prenylated (F.S. and J.E.D., unpublished data). In a more general sense, it will also be of interest to gain insights into the substrates of other YopT family members. Examination of the sequence similarities among YopT family members revealed a potential subfamily containing LspA1 and LspA2 from *Haemophilus ducreyi*, p76 from *Haemophilus somnus*, and PfhB1 and PfhB2 from *Pasteurella multocida* as well as YopT from *Yersinia*. The sequence identities within this subfamily are much higher than those outside of this subfamily (Figure 1). Hence, it is tempting to speculate that these putative virulence proteases may utilize similar substrates as YopT. The sequence diversity among the other YopT family members might be interpreted to suggest that they will recognize a diverse array of substrates. For example, expression of AvrPphB in mammalian cells does not lead to the cytotoxic phenotype as seen with YopT, suggesting AvrPphB may not utilize Rho GTPases as a substrate (F.S. and J.E.D., unpublished data). Additional insights into substrates for AvrPphB may be obtained from noting that AvrPphB undergoes autoproteolytic cleavage after the lysine residue in the sequence GK↓GCASSSG. This cleavage exposes a potential N-terminal myristoylation site. Mutation of the glycine residue at the cleavage site to an alanine causes AvrPphB to localize to the soluble fraction rather than the membrane fraction (Nimchuk et al., 2000). Other members of the YopT family from plant pathogens also have potential myristoylation sites within their N termini that could be generated by autoproteolytic processing, indicating that posttranslational modification of the plant YopT family members may serve as a mechanism for directing them to a subcellular location where they can encounter their respective plant substrates.

Implications Relative to Plant Pathogen Recognition

Recognition of AvrPphB by *Arabidopsis* is mediated by the *R* gene *RPS5* (Warren et al., 1999), which belongs to the nucleotide binding site leucine-rich repeat (NBS-LRR) class of plant *R* genes. The NBS-LRR class is the largest class of *R* genes in plants, with the *Arabidopsis* genome encoding 128 such genes (Initiative, 2000). How NBS-LRR proteins mediate recognition of pathogen Avr proteins is poorly understood and is currently the subject of much debate and speculation (Bonas and Lahaye, 2002; Dangl and Jones, 2001). The simplest and most commonly invoked model for this process is a receptor-ligand model in which the LRR portion of the *R* gene product physically binds to the Avr protein. Direct evidence for the receptor-ligand model comes from work on the AVR-Pita protein from the rice blast fungus, which has been shown to interact with the *Pi-ta R* gene product of rice in both a yeast two-hybrid assay and in an in vitro filter binding assay (Jia et al., 2000). However, no such interactions have been shown between other Avr proteins and plant NBS-LRR proteins, despite extensive attempts by several laboratories. In addition, the AvrD protein of *P. syringae* is known to be recognized indirectly, as it encodes an enzyme that catalyzes the synthesis of a low molecular weight compound (syringolide), which is itself detected in an *R* gene-specific manner (Smith et al., 1993).

Because the proteolytic activity of AvrPphB appears to be required for its HR-inducing activity, we propose that RPS5 recognizes AvrPphB indirectly by binding to a peptide or protein released from a target of AvrPphB proteolysis. A potential candidate for such a target is the PBS1 kinase, as deletion of the *PBS1* gene from *Arabidopsis* specifically blocks recognition of AvrPphB by RPS5 (Swiderski and Innes, 2001). It is tempting to speculate that most NBS-LRR proteins may detect pathogen effector molecules by similar indirect means. Using such a mechanism would enable plants to detect a very large number of potential pathogen effector molecules by detecting a more limited number of potential target modifications. Even the AVR-Pita:Pi-ta interaction described above could represent an indirect detection mechanism, as *AVR-Pita* encodes a putative metalloprotease, and mutations in the protease consensus motif of AVR-Pita eliminate its avirulence activity (Orbach et al., 2000). A third example consistent with this model is the AvrRxv/AvrBsT family of AVR proteins, which belong to the YopJ family of cysteine proteases (Barrett and Rawlings, 2001; Orth et al., 2000). As with the AvrPphB and AVR-Pita, mutation of the putative catalytic residues of AvrBsT eliminates avirulence activity, suggesting that a product of AvrBsT-mediated proteolysis may be the direct activator of resistance. To test the above "enzymatic product" model for Avr protein recognition, it will be necessary to identify the targets and/or products of AvrPphB proteolytic activity. Identification of targets and products would also provide significant insights into the virulence function(s) of AvrPphB. In a similar vein, it would be quite informative to identify all of the targets of YopT in mammalian cells.

Experimental Procedures

Plasmids

The YopT gene with flanking XbaI and NotI sites was amplified from *Y. pestis* plasmid pYV019 (accession number T43601). The sequence encoding the FLAG epitope tag was included in the primer, resulting in a N-terminal-tagged protein. For expression in mammalian cells, the PCR fragment was then cloned into pSFFV (Orth et al., 1999). This construct was used as the PCR template for subcloning. The EGFP-YopT fusion constructs were generated by inserting the PCR fragment of YopT into the SacI/BamHI sites of the pEGFP(C1) vector (Clontech). For expression in yeast, the galactose-inducible expression plasmid (*Gal10* promoter) for YopT was constructed by inserting a PCR fragment encoding YopT into the BamHI/SalI sites of the p413GAL vector (provided by Dr. Dennis J. Thiele, University of Michigan). YopT was subcloned in-frame into the BamHI/SalI sites of the pLexAde vector and the BamHI/EcoRI sites of the pVP16 vector (Vojtek and Hollenberg, 1995) in order to carry out the yeast two-hybrid assay. The bacterial GST-YopT expression plasmid was obtained by inserting YopT coding sequence into pGEX-KG via the BamHI/EcoRI sites (Guan and Dixon, 1991). Full-length AvrPphB was expressed in *P. syringae* in a broad-host range vector pVSP61 (Simonich and Innes, 1995). For dexamethasone-inducible expression, the 28 kDa processed form of AvrPphB was cloned into the vector pTA7002 (Swiderski and Innes, 2001). pET21a-AvrPphB-His6 was generated by amplification of AvrPphB from pVSP61-AvrPphB

and subsequent cloning into the NdeI/SalI sites of pET21a (Novagen). pRK5-myc-RhoGTPases constructs (RhoA, RhoAL63, Rac, RacL61, Cdc42, and Cdc42L61) were generously provided by Dr. Kun-Liang Guan (University of Michigan) and used as the PCR template for subcloning. Mammalian GST fusion constructs for RhoA, Rac, and Cdc42 were generated by cloning the corresponding PCR products in-frame into the BamHI/ClaI sites of the pEBG-3X vector. pVP16-RhoA and RhoAL63 were prepared by subcloning the corresponding PCR fragments into the BamHI/EcoRI sites of the pVP16 vector. pLex-GFP was a gift from Dr. Anne B. Vojtek (University of Michigan). All the CAAX constructs were generated using PCR strategies. p415GPD-yCdc42 was produced by PCR amplification of the yeast Cdc42 gene from the genomic clone pYEP13-Cdc42 and subsequent cloning into the SpeI/SalI restriction sites of p415GPD. The bacterial GST fusion expression construct for RhoGDI was produced by amplification of the GDI2 gene from pcDNA-GS-RhoGDI (Invitrogen) and subsequent subcloning into the BamHI/EcoRI sites of pGEX-KG. All the point mutation constructs were generated by QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). All the plasmids were verified by DNA sequencing.

Protein Expression and Purification

GST-YopT was expressed in *E. coli* strain TG1, and GST-RhoGDI was expressed in the DH5α strain. *E. coli* strains harboring the corresponding plasmids were grown in LB medium containing 100 μg/ml ampicillin to a density of 0.6 (A_{600}). Protein expression was induced overnight at room temperature with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were lysed in the PBS (phosphate-buffered saline) lysis buffer supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM β-mercaptoethanol. The GST fusion proteins were purified by affinity chromatography using glutathione agarose beads (Sigma). GST-YopT was eluted off the glutathione beads with 10 mM glutathione in 50 mM Tris-HCl (pH 7.4). Protein concentrations were estimated by Coomassie blue staining of SDS-PAGE gels using BSA standards.

Yeast Manipulation

Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose). Yeast transformations were carried out by using the lithium acetate method (Gietz et al., 1992). Two-hybrid assays were performed following the procedure of Vojtek and Hollenberg (1995). Expression of YopT driven by the *GAL10* promoter was induced by using 2% galactose as the carbon source in the medium. The *S. cerevisiae* genomic library in the Yep13 vector (ATCC) was used in the suppressor screen. The library was transformed into the W303a strain (*MAT* a *ade2-1 trp1-1 leu2-3, 112 his3-11, 15 ura3 can1-100* [psi+]) harboring p413GAL-YopT. Transformants were selected on 2% galactose medium for survival. Positive clones were isolated and reassayed for complementation. The yeast Cdc42 gene was expressed under the GPD promoter.

Cell Culture, Transfection, and Fluorescence Microscopy

HEK293T and HeLa cells were maintained in DMEM containing 10% (v/v) FBS, 2 mM glutamine, and 100 μg/ml penicillin/streptomycin (GIBCO) at 37°C in a 5% CO_2 incubator. Transfection was performed by using the FuGENE 6 transfection kit (Roche Molecular Biochemicals) as recommended by the manufacturer. To assess cell morphology, HeLa cells cultured in 6-well plates were transfected with 1.5 μg of pEGFP-YopT constructs. The cell morphology and the actin stress fibers were stained and visualized as described before (Juris et al., 2000).

GST Pull-Down Assay

HEK293T cells (6×10^6) cotransfected with 2 μg of FLAG-tagged YopT (C139S) and 5 μg of the specified GST-RhoGTPases (or GST alone) were lysed in 1 ml of the buffer containing 10 mM HEPES (pH 7.5), 50 mM NaCl, 1% Triton, 2 mM EDTA, and a protease inhibitor mixture (Roche Molecular Biochemicals). Precleared lysates were subjected to the standard GST pull-down procedure, and beads were eluted in SDS sample buffer. The eluted samples were resolved on SDS-PAGE and analyzed by Western blotting with FLAG M2 antibody (Sigma) or GST antibody (Santa Cruz). For the RhoGDI pull-down assay, 6×10^6 HEK293T cells were transfected with 4 μg pRK5-myc-RhoAL63 or RacL61 or Cdc42L61, and the cells were harvested in 1 ml of the buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA. The cells were lysed by sonication, and 200 μl of the clear lysates were then supplemented with 5 mM dithiotreitol and incubated with 2 μg of recombinant YopT at 37°C for 40 min. A volume of 5 μl of the lysates were used as the input sample. The lysates were further incubated with 10 μg of the bacterial-expressed GST-RhoGDI prebound to the GST agarose beads at 4°C for 1 hr. The beads were washed extensively and eluted with SDS sample buffer. The samples were separated by SDS-PAGE and analyzed by Western blotting with anti-myc antibody (9E10, BABCO).

Triton Partitioning

HEK293T cells (6×10^6) cotransfected with 4 μg of the expression plasmids for YopT (or mutants) and 4 μg of GST-RhoAL63 were lysed in 1 ml of the buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-114, and a protease inhibitor mixture. The soluble cell lysates with equal amounts of total proteins were partitioned into the aqueous phase and the detergent phase and further subjected to GST pull-down (Hancock, 1995). The pull-downs were resolved on SDS-PAGE followed by Western blotting with GST antibody.

Membrane Release Assay and Membrane Fractionation

HEK293T (6×10^6) cells transfected with either 5 μg of GST- or myc-tagged RhoAL63 or RacL61 or Cdc42L61 were harvested in 1 ml of the buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, and a protease inhibitor mixture. The cells were lysed by sonication and the membranes were isolated as described by Sorg et al. (2001). The membranes were incubated with 10 μg recombinant GST-YopT or mutants in the reaction buffer (50 mM Tris-HCl, 50 mM NaCl, and 4 mM dithiotreitol) for 40 min at 37°C. After incubation, the membranes and supernatants were separated again by ultracentrifugation and further subjected to SDS-PAGE followed by Western blotting using anti-RhoA antibody (26C4, Santa Cruz). To test the effect of the protease inhibitors, various concentrations of protease inhibitors N-ethylmaleimide (Sigma), E64 (Calbiochem), EDTA (Sigma), PMSF, and Pepstatin (Roche Molecular Biochemicals) were added in the reaction.

To look at the endogenous RhoA, 6×10^6 HEK293T cells were transfected with 8 μg of YopT or YopT (C139S) constructs. Cells were lysed as described above with the exception of using 200 μl lysis buffer. An aliquot of 20 μl was removed out as the total RhoA. The lysates were then subjected to membrane separation. The amounts of total RhoA and RhoA in the membrane fraction were detected by anti-RhoA Western blotting.

Metabolic Labeling

HEK293T cells cultured in 60 mm dish were cotransfected with 1.5 μg of GST-RhoAL63 construct, 1.5 μg of pMEV plasmid (ATCC) encoding a mevalonate transporter. To test the activity of YopT in vivo, 2 μg of either pSFFV vector or pSFFV-Flag-YopT (wt or C139S) was also included in the transfection. Twelve hours after transfection, cells were treated with 30 μM Lovastatin (Calbiochem) for 1.5 hr to inhibit the endogenous cholesterol biosynthesis. Cells were then labeled with 150 μCi of ^{3}H-mevalonic acid (NEN) per dish in the presence of 20 μM Lovastatin for 10 hr. To label the cysteine, the same amounts of cells were transfected with 4 μg of GST-RhoAL63 constructs. Twelve hours after transfection, cells were labeled with 0.06 mCi/ml ^{35}S-Cys and ^{35}S-Met labeling mix (NEN) in the cysteine-free medium. Cells were lysed and the lysates were subjected to GST pull-down as described above. The amount of ^{3}H-GST-RhoAL63 was detected by autoradiography, and the total amount of GST-RhoAL63 was measured by Western blotting using anti-RhoA antibody.

In Vitro Cleavage Assay and Lipid Extraction

For the in vitro cleavage assay, the tritium or ^{35}S-labeled GST-RhoA immobilized on the GST beads was washed twice with the lysis buffer and then incubated with 2 μg of YopT or YopT C139S mutant in the buffer (containing 50 mM Tris-HCl, 50 mM NaCl, and 4 mM dithiotreitol) for 45 min at 37°C. After incubation, the remaining

amount of tritiated RhoA on the beads was detected by autoradiography. The reaction solution was then subjected to lipid extraction. Briefly, 5 vol of $CHCl_3$/MeOH/HCl (30/60/1) was added into the solution and mixed well. $CHCl_3$ (1 vol) and 1 vol of H_2O were added subsequently and mixed by vortex. The chloroform and aqueous phases were separated, and the amount of prenylated cysteine present in the chloroform phase was assessed by liquid scintillation counting.

AvrPphB Autoprocessing Experiments

pET21a-AvrPphB-His6 and mutants were expressed in *E. coli* BL21(DE3) at room temperature. Equal amounts of bacterial culture were collected at the indicated time points after induction and lysed in SDS sample buffer. The samples were then subjected to SDS-PAGE followed by Coomassie blue staining and immunoblotting using anti-His antibody (Santa Cruz). AvrPphB and mutants (pET21a-AvrPphB-His6) were in vitro transcribed/translated in the rabbit reticulolysates (Promega), labeled with [^{35}S]methionine (Amersham) according to the manufacturer's instructions. Aliquots (4 μl) of the reaction mixtures (50 μl) were taken at the designated time points, separated by SDS-PAGE, and analyzed by autoradiography.

Plant HR Tests

Mutant pVSP61::*avrPphB* constructs were mated into *P. syringae* strain DC3000. Bacteria were harvested and resuspended to an OD_{600} of 0.075 ($\sim 7.5 \times 10^7$ cfu/ml) in 10 mM $MgCl_2$. The bacterial suspension was injected into the bottom side of fully expanded leaves on 5-week-old *Arabidopsis* plants using a blunt syringe. Enough inoculum was introduced to fill approximately one-half of the leaf. Plants were returned to standard growth conditions (9 hr days), and leaves were scored for HR-associated tissue collapse 22–24 hr post-injection.

Agrobacterium tumefaciens GV3101 strains carrying the various *avrPphB* constructs were prepared for transient transformation of 5-week-old tobacco plants (*Nicotiana tobaccum cultivar xanthi*) as described by Nimchuck et al. (2000) with the following modifications. The *Agrobacterium* induction medium contained 150 μg/ml acetosyringone, and the cells were incubated at 20°C for 5–7 hr. The bacterial suspension was injected into the bottom side of fully expanded tobacco leaves using a blunt syringe. 48 hr postinjection plants were sprayed with 50 μM dexamethasone, covered with a propagation dome, and returned to the growth chamber. Twelve hours after spraying, domes were removed and leaves were scored for HR 24 hr after spraying.

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