Disruption of Signaling by *Yersinia* Effector YopJ, a Ubiquitin-Like Protein Protease

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Homologs of the *Yersinia* virulence effector YopJ are found in both plant and animal bacterial pathogens, as well as plant symbionts. These YopJ family members were shown to act as cysteine proteases. The catalytic triad of the protease was required for inhibition of the mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) signaling in animal cells and for induction of localized cell death in plants. The substrates for YopJ were shown to be highly conserved ubiquitin-like molecules, which are covalently added to numerous regulatory proteins. YopJ family members exert their pathogenic effect on cells by disrupting this posttranslational modification.

Yersinia pestis is the bacterial pathogen that caused the Black Death in the Middle Ages (*1*). *Yersinia* species harbor a 70-kB plasmid that encodes a type III secretion system and several effector proteins known as Yops (*Yersinia* outer proteins). Yops are translocated from the bacteria into the host cell, where they alter crucial signaling machinery to prevent phagocytosis and induction of the host immune response (*1*). One of the effector proteins, YopJ, inhibits the host immune response by preventing activation, via phosphorylation, of the MAPK pathway and the NF-κB pathway, preventing the production of cytokines and activation of the host immune response and antiapoptotic factors (*2*–*7*).

The YopJ protein is a member of a family of effectors regulating diverse bacterial-host cellular interactions (*8*–*11*). Although sequence analysis using Ψ-BLAST failed to identify additional viral or eukaryotic proteins similar to YopJ, structure predictions using CPHmodels and Threader programs both suggested YopJ had a secondary structure similar to that of the known secondary structure of adenovirus protease (AVP) (*12*) (Fig. 1). The alignment of AVP and YopJ demonstrated that critical residues forming the catalytic triad in the AVP cysteine protease were present in all YopJ family members (Fig. 1). However, recombinant protein preparations of YopJ were catalytically inactive when assayed with a variety of radiolabeled or fluorometric peptides (*13*). The lack of catalytic activity observed for YopJ was not unexpected, because AVP is also an inactive recombinant protein that requires a cofactor for activation (a specific 11–amino acid peptide or a nucleic acid) (*14*, *15*).

In the absence of in vitro protease activity, we assessed whether mutations in the predicted catalytic site would affect the ability of YopJ to inhibit specific signaling pathways (Fig. 2). To do so, 293 cells were transfected with or without hemagglutinin (HA) epitope–tagged MAPK (also known as extracellular signal–regulated kinase) (HA-ERK) in the presence or absence of YopJ and YopJ mutants. After stimulation with epidermal growth factor (EGF), cells were analyzed for HA-ERK activity by using an immune kinase complex assay with myelin basic protein as a substrate (*3*). Cells transfected with HA-ERK and the empty vector control displayed ERK activity, whereas cells cotransfected with HA-ERK and wild-type YopJ did not exhibit ERK activity (Fig. 2A). In contrast, cells cotransfected with HA-ERK and YopJ encoding mutants in the catalytic domain [His to Ala at codon 109 or Cys to Ala at codon 172 (H109A or C172A)] were unable to inhibit the MAPK pathway, as demonstrated by the presence of ERK kinase activity (Fig. 2A).

Mutations of catalytic residues in YopJ also blocked its ability to inhibit the NF-κB pathway (Fig. 2B). After 293 cells were cotransfected with or without mitogen-activated protein kinase (or extracellular signal–regulated kinase) kinase kinase 1 (ΔMEKK1) in the presence or absence of wild-type or mutant YopJ, they were tested for the presence of activated NF-κB via a NF-κB gel-shift assay. Nuclear extracts from cells cotransfected with the ΔMEKK1 and an empty control vector were able to cause a mobility shift in the radiolabeled NF-κB consensus oligonucleotide, indicating activation of the NF-κB pathway (Fig. 2B). In contrast, nuclear extracts from cells cotransfected with ΔMEKK1 and wild-type YopJ were unable to shift the radiolabeled NF-κB consensus oligonucleotide, indicating that the activation of the NF-κB pathway was inhibited. Mutation of either the catalytic His (H109A) or the Cys (C172A) residue completely abolished the ability of YopJ to inhibit the NF-κB pathway. Mutation of another residue [Glu to Ala at codon 186 (E186A)] distant from the catalytic site had no effect on the ability of YopJ to inhibit either of these pathways (Fig. 2, A and B).

To further confirm that these putative catalytic residues were biologically relevent for the activity of YopJ, we examined their effect in infection assays. Infection of macrophages with a *Y. pseudotuberculosis* YopJ null strain resulted in induction of tumor necrosis factor–α (TNF-α) production (*4*) (Fig. 3A). However, when the null strain was complemented with a wild-type copy of YopJ, the effector was translocated into the macrophage (Fig. 3A) and production of TNF-α was inhibited (Fig. 3A) (*4*). Although the YopJC172A mutant was expressed and translocated into macrophages (Fig. 3B), the mutant failed to suppress TNF-α expression by infected macrophages (Fig. 3A). Thus, an intact catalytic site is required for in vivo YopJ activity.

Inherent in our hypothesis is that all YopJ family members would use a common catalytic mechanism for pathogenesis. We analyzed similar mutations in the putative catalytic core of AvrBsT, the plant pathogen homolog (*16*). AvrBsT enables *Xanthomonas campestris* (*X.c.*) to induce localized cell death (classically referred to as the hypersensitive response, HR) and plant resistance in *Nicotiana benthamiana* (this work) and pepper plants (*17*). Mutation of the conserved His (H154A), Glu (E173A), or Cys (C222A) found in the catalytic core of AvrBsT completely abolished the ability of *X.c.* pv. *campestris* (*X.c.c.*) to induce localized cell death in the infected plant leaf (Fig. 3C). Leaves infected with *X.c.c.* containing the vector control displayed no symptoms, whereas the Lys to Ala at codon 236 (K236A) mutant protein (mutation distant from the catalytic core) maintained AvrBsT activity. It is noteworthy that both wild-type and mutant AvrBsT proteins were secreted from *X. campestris* pv. *vesicatoria* (*X.c.v.*) via the type III pathway (*18*). Furthermore, plants in which transient expression of wild-type and mutant AvrBsT protein was induced by using *Agrobacterium*-mediated transient transformation exhibited phenotypes similar to that observed for the *X.c.c.* infections (*18*). Thus, mutations in the catalytic core of AvrBsT, as observed with YopJ, were not

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affecting type III delivery of this effector to the plant cell. Our mutational analysis of critical catalytic residues in YopJ family members, which function in both plant and animal hosts, strongly suggests that these proteins are acting as proteases to disrupt universal signaling pathways.

Insight into the potential substrates of YopJ was provided by the observation that AVP shows limited sequence identity to the family of ubiquitin-like protein proteases (*19*) (Fig. 4A). Ubiquitin-like protein proteases function proteolytically to process the COOH-terminus of a ubiquitin-like protein, SUMO-1 (*20*, *21*), so that the resulting product terminating in two glycine residues can be used as a substrate to modify target proteins covalently (*22*). In addition, the protease can cleave the isopeptide bond that links the COOH-terminus of the ubiquitin-like protein to an ε-amine of a lysine residue on the target protein, such as SUMO-1-RanGAP (*19*, *22*). Alignment of the catalytic domains of YopJ (residues 109 to 195), AVP, and the yeast ubiquitin-like protein protease Upl-1 demonstrates that conservation of four residues essential for AVP catalysis are conserved, even

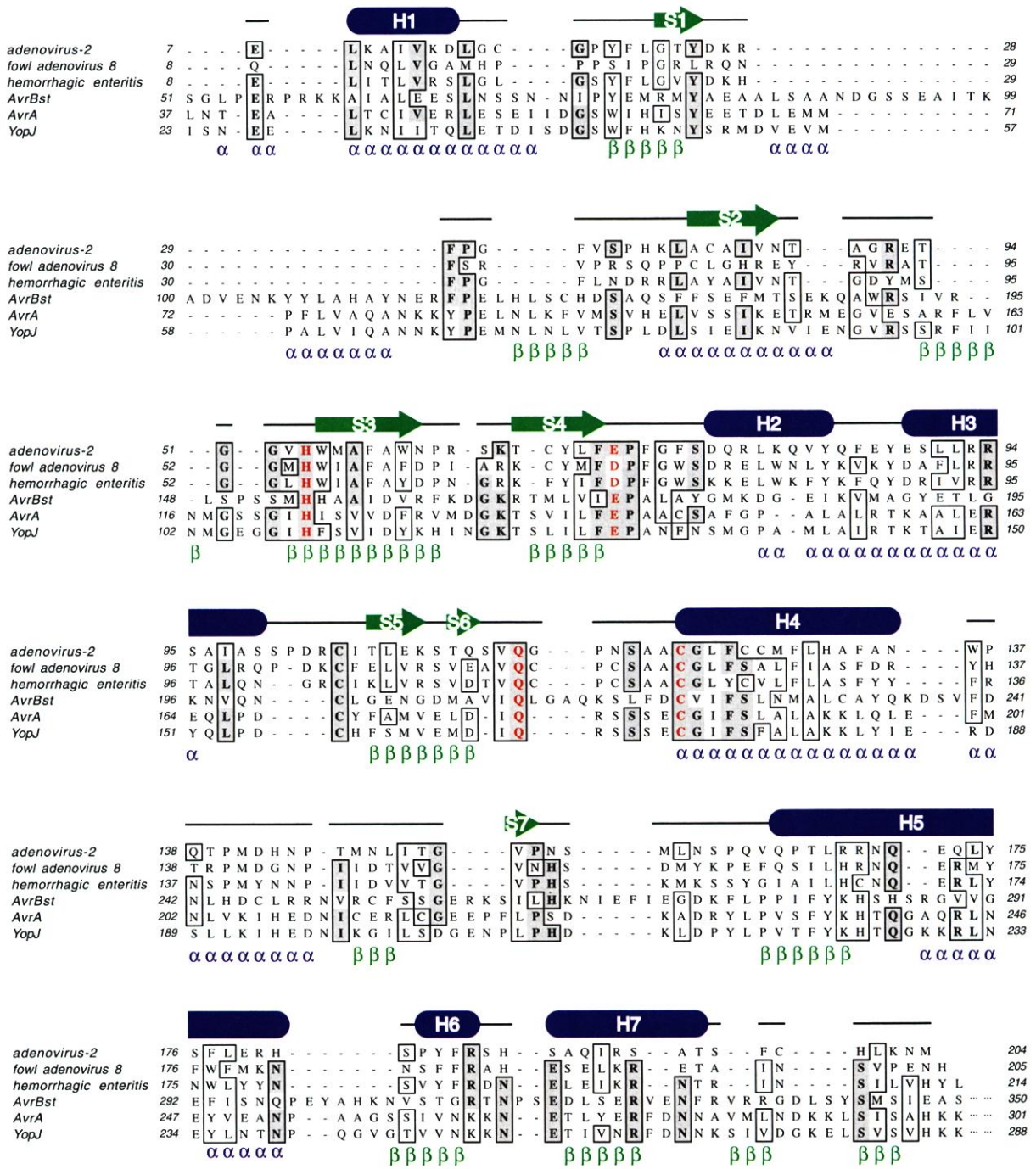

Fig. 1. A sequence alignment of representative members of the adenovirus protease-2 (AVP) family of proteins and the YopJ-family of proteins including the adenovirus-like proteases (human adenovirus type 2, fowl adenovirus 8, *Hemorrhagic enteritis* virus), as well as YopJ-like proteases [*Y. pseudotuberculosis* (YopJ), *Salmonella typhimurium* (AvrA) and *Xanthomonas campestris* pv. *vesicatoria* (AvrBsT)]. The catalytic triad (His, Asp/Glu, and Cys) and the invariant glutamine residue in the oxyanion hole of the active site are colored in red. Secondary structures determined from the structure of AVP are shown above the sequence of AVP. Blue ovals are α helices and green arrows are β strands. Predicted secondary structures based on the primary sequence of YopJ are shown below the YopJ sequence (*28*).

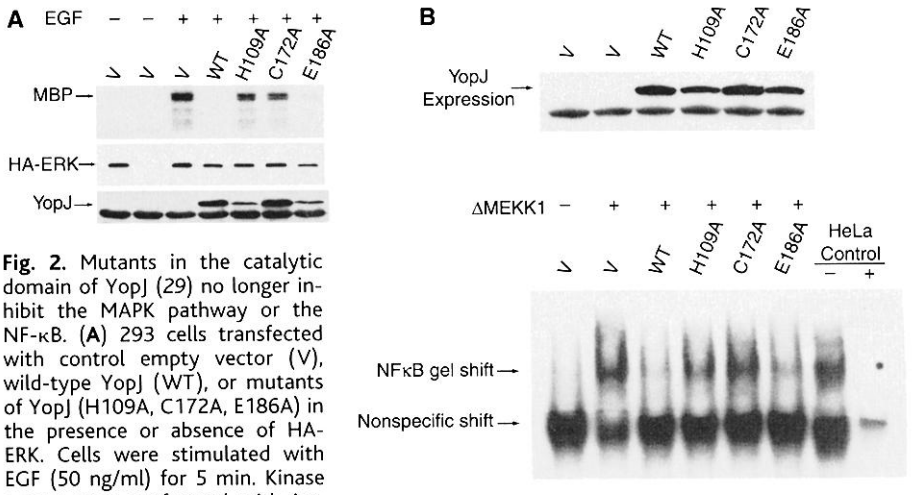

Fig. 2. Mutants in the catalytic domain of YopJ (*29*) no longer inhibit the MAPK pathway or the NF-κB. (**A**) 293 cells transfected with control empty vector (V), wild-type YopJ (WT), or mutants of YopJ (H109A, C172A, E186A) in the presence or absence of HA-ERK. Cells were stimulated with EGF (50 ng/ml) for 5 min. Kinase assays were performed with immunoprecipitated HA-ERK by using myelin basic protein as a substrate, as previously described (*3*). Expression of YopJ was confirmed by immunoblot analysis using the monoclonal FLAG antibody (Sigma). (**B**) 293 cells, transfected in the presence or absence of the ΔMEKK1 with either control empty vector (V), wild-type YopJ (WT), or mutants of YopJ (H109A, C172A, E186A). Nuclear extracts (~4 μg) were incubated with radiolabeled NF-κB consensus oligonucleotide, and complexes were assayed for a gel shift by separation in a nondenaturing 4% acrylamide gel (Promega, Madison, WI, gel-shift assay system). Control HeLa nuclear extract was incubated in the presence (+) or absence (–) of excess NF-κB consensus oligonucleotide that was not radiolabeled. Expression of YopJ was confirmed by immunoblot analysis with the use of the monoclonal FLAG antibody (Sigma).

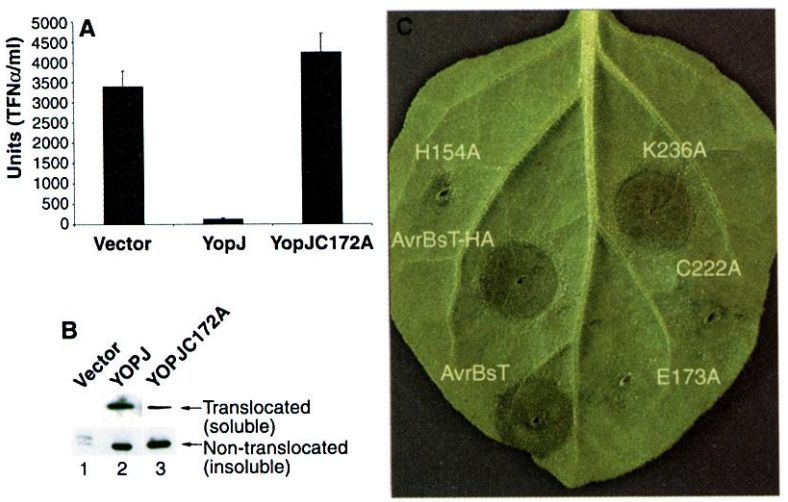

Fig. 3. Mutation of catalytic residues in YopJ and a YopJ homolog from *X.c.*, AvrBsT, disrupt in vivo infection activity. (**A**) Wild-type YopJ is required for inhibition of macrophage TNF-α production. Macrophages were infected with *Y. pseudotuberculosis* YopJ null strain harboring a control, wild-type YopJ, or YopJC172A vector. Infection studies were carried out as previously described (*4*). (**B**) Both wild-type YopJ and YopJC172A are translocated from *Y. pseudotuberculosis* into macrophages during infection. Expression of YopJ was confirmed by immunoblot analysis with monoclonal M45 antibody as previously described (*4*). (**C**) Mutations in the predicted catalytic core of AvrBsT inhibit AvrBsT-induced cell death in plants. *N. benthamiana* leaves were inoculated with a 1×10^9 cells/ml suspension of *X. campestris* pv. *campestris* expressing AvrBsT, AvrBsT-HA, AvrBsT(H154A)-HA, AvrBsT(E173A)-HA, AvrBsT(C222A)-HA, or AvrBsT(K236A)-HA. Symptoms were photographed 1.5 days after inoculation.

though the three proteins share only limited identity (Fig. 4A).

Based on the identity of YopJ with the catalytic core of Ulp-1, we predicted that the in vivo substrate for YopJ could be either SUMO-1 or SUMO-1–conjugated proteins. Thus, we attempted to ascertain in vivo whether YopJ had an effect on the profile of SUMO-1–conjugated proteins (Fig. 4B). To mimic an infected state, signaling pathways were activated in 293 cells by transfecting cells with glutathione *S*-transferase–B-Raf (GST-BRaf), which exhibits elevated Raf basal kinase activity (*23*, *24*). These cells were also transfected with NH_2-terminally HA-tagged SUMO-1 (HA–SUMO-1) in the presence or absence of YopJ. Immunoblot analysis with an antibody to the HA epitope demonstrated the presence of free HA–SUMO-1 and multiple SUMO-1–conjugated proteins in the vector control lane (Fig. 4B). By contrast, when cells were cotransfected with HA–SUMO-1 and wild-type YopJ, a reduction in the total amount of free HA–SUMO-1 and HA–SUMO-1–conjugated proteins was observed. Furthermore, when cells were cotransfected with HA–SUMO-1 and YopJ-C172A, an inactivating mutant of YopJ, no effect was observed on the level of free HA–SUMO-1 or SUMO-1–conjugated proteins. Thus, YopJ decreases the cellular concentration of SUMO-1–conjugated proteins. In addition, the levels of free HA–SUMO-1 were decreased. This observation is in contrast to overexpression experiments with a human Ulp-1 homolog, in which a decrease in HA-SUMO conjugates is coincident with an increase in free HA–SUMO-1 (*25*).

We envision that YopJ acts as cysteine proteases in both plants and animals. SUMO-1 and its protein conjugates appear to be substrates for YopJ. SUMO-1 is highly conserved in all eukaryotic species and contains the invariant COOH-terminal cleavage site Gln-XXX-Gly-Gly↓XXX, which is also the consensus sequence recognized for processing of the isopeptide bond in protein conjugates (*21*). We anticipate that part of the substrate recognition by YopJ resides in the cleavage of substrates after the invariant Gly-Gly↓ residues. Ubiquitin and other ubiquitin-like protein conjugates also have two glycine residues at their isopeptide junction, and these protein conjugates could also serve as YopJ substrates. An increasing number of regulatory proteins such as c-Jun and p53 have been seen to be modified by ubiquitin-like proteins (*26*). In a previous study, we demonstrated that YopJ binds to the superfamily of MAPK kinase kinases (MKKs) and blocks their activation via phosphorylation (*3*). Although we have not observed MKK to be modified by SUMO-1, our results suggest that, in addition to the known effects of protein phosphorylation on MKK activity, SUMO-1 conjugation plays a yet-to-be-identified regulatory role in the MAPK signaling cascade (*27*).

In summary, we have identified the molecular mechanism used by the pathogenic protein YopJ to inhibit multiple signaling pathways. It is remarkable that animal and plant pathogens, as well as plant symbionts, have used this universal mechanism of proteolysis to inhibit or modulate a variety of

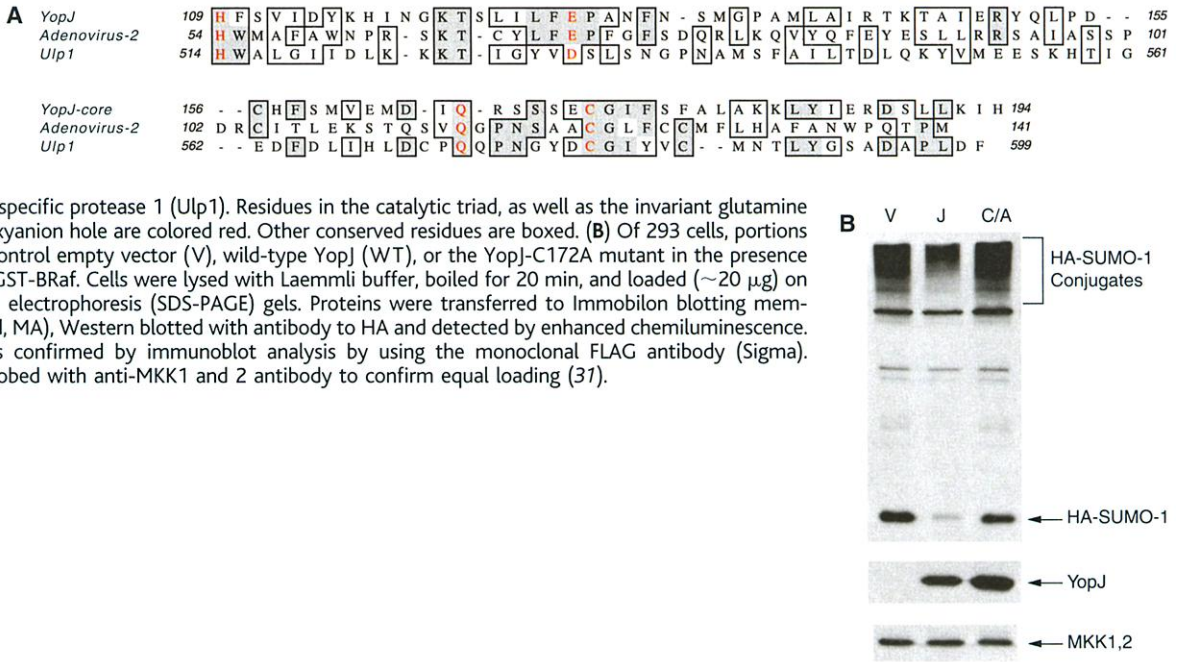

Fig. 4. YopJ inhibits conjugation of SUMO-1 to target proteins in mammalian cells. (**A**) Sequence alignment of the "catalytic core" of a clan of cysteine proteases including YopJ, AVP, and the yeast Ubl-specific protease 1 (Ulp1). Residues in the catalytic triad, as well as the invariant glutamine residue located in the oxyanion hole are colored red. Other conserved residues are boxed. (**B**) Of 293 cells, portions were transfected with control empty vector (V), wild-type YopJ (WT), or the YopJ-C172A mutant in the presence of HA-SUMO (*30*) and GST-BRaf. Cells were lysed with Laemmli buffer, boiled for 20 min, and loaded (~20 μg) on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to Immobilon blotting membrane (Millipore, Bedford, MA), Western blotted with antibody to HA and detected by enhanced chemiluminescence. Expression of YopJ was confirmed by immunoblot analysis by using the monoclonal FLAG antibody (Sigma). Immunoblots were reprobed with anti-MKK1 and 2 antibody to confirm equal loading (*31*).

signaling pathways and that this results in a spectrum of phenotypes. This work also underscores the importance of ubiquitin-like protein conjugation and deconjugation in regulating key signaling pathways within the cell.

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13. K. Orth, W. F. Mangel, J. E. Dixon, unpublished observation. Substrates tested include peptide substrates such as (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine, (Leu-Gly-Arg-NH)$_2$-rhodamine, (Ala-Arg-NH)$_2$-rhodamine, (Ala-Ala-NH)$_2$-rhodamine, H-Gly-Gly-7-aminio-4-chloromethylcoumarin (AMC), H-Gly-Ala-AMC, H-Gly-Ala-AMC, and Z-Leu-Arg-Gly-Gly-AMC, and radiolabeled substrates such as ^{35}S-methionine–labeled HA–SUMO-1 or RanGAP.
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16. The plant pathogen homolog AvrBsT was amplified by polymerase chain reaction from *X.c.v.* DNA and cloned into pDD62 a vector with a broad host range (D. Dahlbeck, B. Staskawicz, unpublished observations), with Bam HI and Xho I restriction enzymes. AvrBsT alanine mutants were generated by overlapping polymerase chain reaction. The NH$_2$-terminus of AvrBsT was amplified with a 5′ primer and a 3′ primer containing the core codon replaced with alanine. Similarly, the COOH-terminus of AvrBsT was amplified with a 5′ primer containing the same sequence with the alanine codon and a terminal 3′ avrBsT primer with the HA sequence. The two amplified products were melted and annealed, providing mutant template for the final amplification using outside 5′ and 3′ AvrBsT primers. Constructs were confirmed by DNA sequence analysis.
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27. In previous studies, we demonstrated that YopJ binds to the superfamily of MKKs and blocks their activation via phosphorylation. Therefore, we next asked whether our previous results were consistent with our present findings. Evidence herein and by others (see below) support our hypothesis that phosphorylation and sumoylation are not mutually exclusive, but can synergize to modulate signaling pathways. We have shown that YopJ and the corresponding mutant C172A attenuate MAPK and NF-κB pathway signaling and TNF-α production in a manner similar to the way SUMO-1 conjugates are modulated. Additionally, a similar activity profile is observed with the wild-type and mutant YopJ plant pathogen homolog, AvrBsT, with respect to plant hypersensitive signaling. We envision that the MKKs could act as escorts or activators of YopJ, delivering it to signaling complexes within the cell and, in turn, resulting in the silencing of critical regulation by SUMO-1 conjugates. Evidence to support this concept has been reported by Bliska and colleagues who demonstrated that, upon kinase activation, YopJ moves into the "ruffled" edges of activated cells (*2*). We propose that SUMO-1 conjugation is likely an important post-translational modification associated with the MAPK signaling complexes and other key regulatory signaling processes.
28. This alignment demonstrated that critical residues forming the catalytic triad in the AVP cysteine protease were present in all YopJ family members. These residues include His^{109} and Cys^{172}, as well as Glu^{128} (or Asp) (YopJ numbering). Stabilization of the tetrahedral intermediate is predicted to occur by the conserved R group of Gln^{166} or by its backbone amide. The α/β fold of AVP and the ordering of the catalytic diad (His followed by Cys) puts YopJ into the new cysteine protease family known as C5. Although the ordering of the catalytic His and Cys is not always the same in all cysteine proteases, the secondary structure surrounding the catalytic diad is conserved in all C5 family members, as well as in papain (i.e., a Cys at the start of an α helix; a His at the beginning of a β strand).
29. Mutants of YopJ were generated using the Stratagene (La Jolla, CA) Quik Change site-directed mutagenesis kit, and mutants were confirmed by DNA sequence analysis.
30. Human SUMO-1 was amplified from the Research Genetics (Huntsville, AL) EST 2578604 and cloned in pcDNA3 with an NH$_2$-terminal HA-epitope tag with Xba I and Apa I restriction ezymes.
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