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Short Article

Type III Secretion Effectors of the IpaH Family Are E3 Ubiquitin Ligases

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SUMMARY

Many bacteria pathogenic for plants or animals, including *Shigella* spp., which is responsible for shigellosis in humans, use a type III secretion apparatus to inject effector proteins into host cells. Effectors alter cell signaling and host responses induced upon infection; however, their precise biochemical activities have been elucidated in very few cases. Utilizing *Saccharomyces cerevisiae* as a surrogate host, we show that the *Shigella* effector IpaH9.8 interrupts pheromone response signaling by promoting the proteasome-dependent destruction of the MAPKK Ste7. In vitro, IpaH9.8 displayed ubiquitin ligase activity toward ubiquitin and Ste7. Replacement of a Cys residue that is invariant among IpaH homologs of plant and animal pathogens abolished the ubiquitin ligase activity of IpaH9.8. We also present evidence that the IpaH homolog SspH1 from *Salmonella enterica* can ubiquitinate ubiquitin and PKN1, a previously identified SspH1 interaction partner. This study assigns a function for IpaH family members as E3 ubiquitin ligases.

INTRODUCTION

Invading pathogens are sensed by host cells through surveillance systems that initiate signaling cascades alerting the immune system to the presence of pathogens. These signaling cascades include both MAPK and nuclear factor-κB (NF-κB) programs that induce cytokine production and ultimately result in inflammation (Inohara et al., 2005). The regulated destruction of proteins via the ubiquitin proteasome pathway governs many cellular processes, including cell-cycle progression and signal transduction pathways, such as the NF-κB pathway. Ubiquitination involves one ubiquitin-activating enzyme (E1), a limited number of ubiquitin-conjugating enzymes (E2s), and a large number of ubiquitin-ligating enzymes (E3s). The C-terminal Gly residue of ubiquitin is charged via a thioesther linkage onto a Cys residue of E1 and transferred to a Cys residue of E2s. E3s recruit ubiquitinated E2s to specific substrates that are ubiquitinated on Lys residues by an amide linkage. RING and U box E3s promote the transfer of ubiquitin from E2s to targets, whereas HECT domain E3s transfer ubiquitin onto one of their Cys residues and then to targets (Ardley and Robinson, 2005; Liu, 2004). The ubiquitin moiety of ubiquitinated targets can then be ubiquitinated on Lys residues 48 or 63 to produce polyubiquitinated targets. Ubiquitin chains constructed by Lys-48 linkages target proteins for destruction by the proteasome, whereas those constructed by Lys-63 linkages leads to altered protein function, such as the activation of kinases (Liu, 2004).

Many gram-negative bacteria pathogenic for plants or animals utilize a type III secretion (T3S) apparatus to inject effector proteins into host cells (Galan and Cossart, 2005). Bacteria of *Shigella* spp. cause shigellosis in humans by invading the colonic mucosa. Their virulence is dependent upon a 200 kb plasmid encoding a type III secretion (T3S) system (Parsot, 2005). *Shigella* effector proteins that promote bacterial entry are produced and stored within the bacterium at 37°C and transit through the T3S apparatus upon contact with epithelial cells (Menard et al., 1994). A second wave of effectors, whose functions are unknown, are produced only after contact with host cells (Demers et al., 1998). These latter effectors include nine closely related IpaH proteins that are the effectors most abundantly produced by *Shigella* (Demers et al., 1998). Expression of these effectors is dependent upon an AraC family member, MxiE, which activates transcription in response to the activation of the T3S apparatus (Mavris et al., 2002; Penno et al., 2005).

Defining the activity of T3S effectors is key to understanding pathogenesis; however, many effectors share little sequence similarity with proteins of known function. As T3S effectors are injected into cells, their targets are intracellular. Yeasts have many proteins and processes well conserved in higher eukaryotes and have been used to gain clues to the role of effectors (reviewed in Valdivia, 2004). Recently, studies in yeast helped to elucidate the function of the *Shigella* effectors IpgB1 and IpgB2 that act as G protein mimics (Alto et al., 2006). To gain insight into IpaH activity, we utilized *Saccharomyces cerevisiae* as

a surrogate model. We found that expression of IpaH9.8 disrupts signaling through the pheromone response MAPK pathway by promoting the proteasome-dependent degradation of the MAPKK Ste7. In vitro assays were used to demonstrate that effectors of the IpaH family, including IpaH9.8 from *Shigella* and SspH1 from *Salmonella*, constitute a novel class of E3 ubiquitin ligases.

RESULTS AND DISCUSSION

IpaH9.8 Inhibits the Pheromone Response MAPK Pathway

To gain insight to IpaH9.8 activity, we utilized *Saccharomyces cerevisiae* as a surrogate model. Yeasts producing FLAG-tagged IpaH9.8 under the control of the *GAL* promoter were not impaired in their ability to grow at elevated temperatures, in the presence of a variety of ions, or under high and low osmotic stresses. Detection of mating pheromone by a G protein-coupled receptor activates an archetypal MAPK signaling cascade, inducing both arrest of the cell cycle and transcription of mating genes (Figure 1A). The pheromone α factor diffusing from a disk causes cell-cycle arrest in *MATa* cells, resulting in a halo of inhibited growth (Hoffman et al., 2002). Upon exposure to α factor, wild-type yeast producing IpaH9.8 failed to form a halo (Figure 1B) and to induce expression of a pheromone-responsive *FUS1-lacZ* reporter gene (Figure S1A in the Supplemental Data available with this article online), indicating that IpaH9.8 interferes with the pheromone response pathway and acts on or upstream of the MAPK Fus3.

To identify the target of IpaH9.8, we used yeast strains altered in the signaling cascade. Overproduction of the G protein β subunit Ste4 activates the signaling pathway and promotes growth arrest (Cole et al., 1990); production of IpaH9.8 rescued this phenotype, i.e., allowed growth (Figure S1B), indicating that IpaH9.8 acts downstream of Ste4. The constitutively active variant of the MAPKKK Ste11 encoded by the allele *STE11-4* promotes elevated transcription of pheromone-responsive genes, even in the absence of pheromone (Stevenson et al., 1992). Growth of the strain SY2625 harboring a *FUS1-HIS3* pheromone-inducible reporter is dependent on signaling through the pheromone response pathway on a medium lacking histidine and containing 3-amino triazole (Evangelista et al., 1997). SY2625 containing a plasmid encoding Ste11-4, but not those containing the vector, were His^+ (Figure 1C), consistent with activation of the pathway by Ste11-4 and transcription of *FUS1-HIS3*. In contrast, yeasts containing plasmids encoding Ste11-4 and IpaH9.8 were His^- (Figure 1C), indicating that IpaH9.8 interrupts signaling at or downstream of Ste11, on either the MAPK Fus3 or the MAPKK Ste7 (Figure 1A). Immunoblot analysis indicated that the amount of Ste7, but not of Ste11 and Fus3, was drastically reduced in wild-type yeast producing IpaH9.8, regardless of stimulation by α factor (Figure 1D and data not shown). Upon phosphorylation by Ste11, Ste7 is ubiquitinated and, following removal of ubiquitin chains by the specific deubiquitinase Ubp3, is degraded by the proteasome (Wang et al., 2003). In both *ubp3Δ* and *ste11Δ* cells, production of IpaH9.8 still resulted in the disappearance of Ste7 (Figure 1D and Figure S1C), indicating that IpaH9.8-mediated disappearance of Ste7 is independent of the known Ste7 degradation pathway.

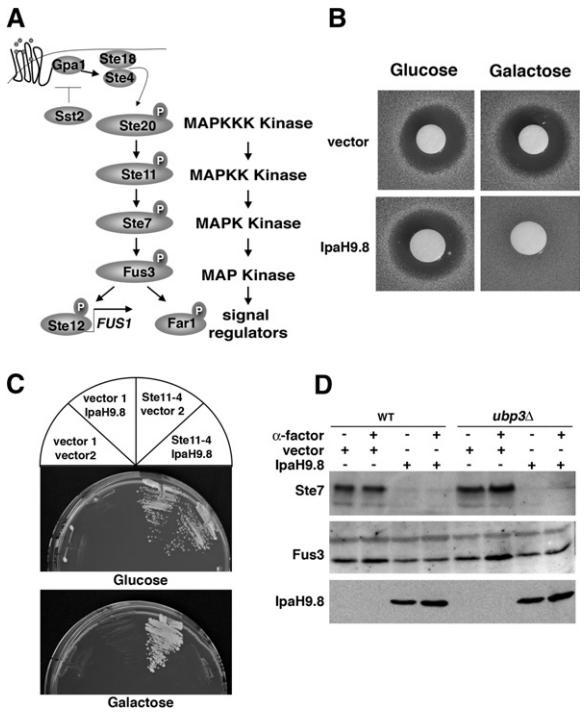

Figure 1. IpaH9.8 Interrupts the Pheromone Response Pathway by Targeting Ste7

(A) The pheromone response pathway and protein activities.

(B) Halo assays on glucose- or galactose-containing plates of wild-type *MATa* yeast harboring either a vector or a plasmid encoding IpaH9.8; the disk was impregnated with α factor.

(C) Growth on plates lacking His and containing 4 mM 3-amino triazole and glucose or galactose of SY2625 yeast (*FUS1-HIS3*) harboring two plasmids encoding (or not encoding, indicated as vector 1 and vector 2) Ste11-4 and IpaH9.8.

(D) Analysis using anti-Ste7, anti-Fus3, and anti-FLAG antibodies of extracts of wild-type (WT) and *ubp3Δ* yeast producing IpaH9.8-FLAG and exposed to α factor.

Structure and Function of IpaH Family Members

Blockage of signaling downstream of Ste11 suggested that IpaH9.8 should rescue *sst2Δ* cells defective for the GTPase-activating protein encoded by *SST2* (Figure 1A); these cells are unable to dampen signaling and can not grow in the presence of pheromone (Dohlman et al., 1996). Indeed, production of IpaH9.8 allowed *sst2Δ* cells to grow in the presence of pheromone (Figures 2A and 2B). IpaH7.8, another IpaH family member from *Shigella*, also rescued the pheromone-induced growth arrest of *sst2Δ* cells (Figure 2A), indicating that IpaH9.8 and IpaH7.8 have similar activities in yeast.

The strong phenotype of *sst2Δ* cells producing IpaH proteins was used to perform a functional analysis of IpaH domains. The nine different IpaHs encoded by the virulence plasmid and the chromosome (Yang et al., 2005)

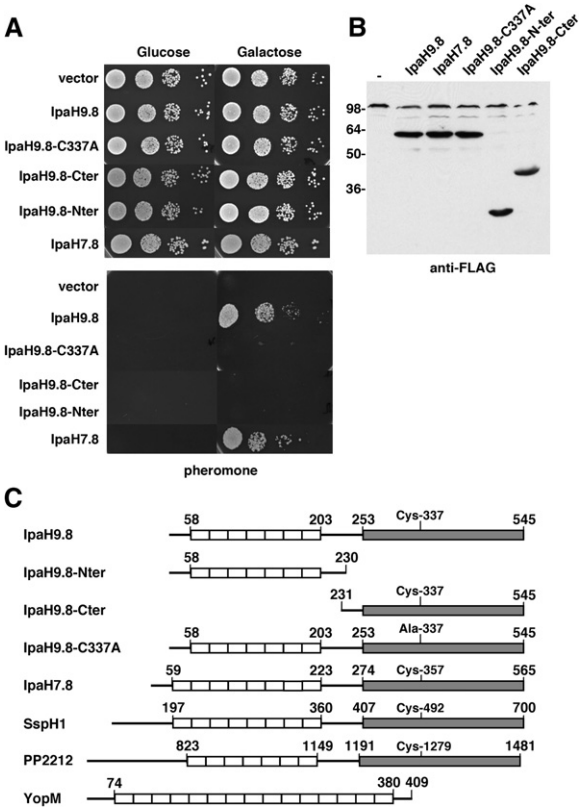

Figure 2. Functional Analysis of IpaH Proteins in *sst2Δ* Yeast and Domain Organization of IpaHs and Homologous Proteins

(A) Growth on glucose- or galactose-containing plates of serial dilutions of *sst2Δ* yeast harboring plasmids encoding indicated proteins; the lower panel shows plates containing α factor.

(B) Immunoblot analysis using anti-FLAG antibodies of extracts of *sst2Δ* yeast producing indicated FLAG-tagged IpaH proteins.

(C) Schematic representation (not to scale) of *S. flexneri* IpaH9.8 and IpaH7.8, *S. enterica* SspH1, *P. putida* PP2212, and *Yersinia* spp. YopM. LRRs and conserved C-terminal domains are shown by open and solid boxes, respectively, and residue positions are indicated by numbers.

consist of an ~250 residue variable N-terminal domain containing six to eight 20 residue leucine-rich repeats (LRR) and an ~300 residue conserved C-terminal domain. Production of neither IpaH9.8-Nter nor IpaH9.8-Cter (Figure 2C) rescued growth of *sst2Δ* cells exposed to pheromone (Figure 2A), indicating that both domains of IpaH9.8 are required for the function in yeast.

Sequence comparisons revealed that the IpaH C-terminal domain shares 25%–40% identity with two groups of proteins of bacteria that contain a T3S system and are pathogens of plants, fish, and mammals (Figure S2). One group includes 18 ~600 residue proteins from *Shigella* spp., *Yersinia pestis* (and *Y. pseudotuberculosis*), *Salmonella enterica*, *Edwardsiella ictaluri*, *Bradyrhizobium japonica*, and *Rhizobium* sp. strain NGR234, and the other includes 15 ~1500 residue proteins from *Pseudomonas putida*, *P. entomophila*, *P. fluorescens*, and *P. syringae*. In both groups, the conserved domain is C terminal and preceded by LRRs (Figure 2C).

The presence of one Cys residue among the nine residues that are identical in all members of the IpaH family suggested that its thiol group might be involved in catalysis. To test this hypothesis, Cys-337 of IpaH9.8 was replaced by Ala in IpaH9.8-C337A. Although IpaH9.8-C337A was produced in similar amounts to IpaH9.8 (Figure 2B), it did not allow *sst2Δ* cells to grow in the presence of pheromone (Figure 2A). Circular dichroism measurements in the far-UV and near-UV regions on purified GST-IpaH9.8 and GST-IpaH9.8-C337A showed that these two proteins have similar secondary and tertiary structure contents (Figure S3), suggesting that the Cys residue conserved in all IpaH homologs is involved in function rather than in structure.

IpaH-Mediated Disappearance of Ste7 Is Proteasome Dependent

To test if the IpaH-mediated disappearance of Ste7 required proteasome function, we used a yeast strain carrying the *cim5-1* allele encoding a component of the 26-S proteasome that is functional at 25°C but not at 37°C (Ghislain et al., 1993). Ste7 was present in *cim5-1* yeast producing IpaH9.8-C337A at both temperatures and IpaH9.8 at 37°C but was not present in yeast producing IpaH9.8 at 25°C (Figure 3A). Moreover, the proteasome inhibitor MG132 prevented the disappearance of Ste7 provoked by IpaH9.8 in a MG132-permeable *erg6Δ* mutant (Lee and Goldberg, 1998) (data not shown). Thus, the disappearance of Ste7 promoted by IpaH9.8 is proteasome dependent.

IpaH9.8 Is an E3 Ubiquitin Ligase for Ste7

The observation that the degradation of Ste7 promoted by IpaH9.8 is proteasome dependent led us to test in vitro if IpaH9.8 might be involved in an ubiquitination pathway. We found that the E2 enzyme UbcH5B was apparently not ubiquitinated by E1 in the presence of GST-IpaH9.8 (Figure 3B). Furthermore, after ubiquitination of UbcH5B, addition of GST-IpaH9.8 to the reaction mixture promoted the removal of ubiquitin from UbcH5B (Figure 3B). This activity was not observed when GST-IpaH9.8-C337A was added to the reaction mixture (Figure 3B), indicating that it required the Cys residue of IpaH9.8.

The amide linkage, but not the thioesther linkage, of ubiquitin to ubiquitinated proteins is resistant to dithiothreitol (DTT). In reactions containing IpaH9.8, but not in those containing IpaH9.8-C337A or lacking UbcH5B, we detected a DTT-resistant ubiquitinated protein of the size of GST-IpaH9.8 (Figure S4). Since some E3 ubiquitin ligases possess an autoubiquitination activity (Beaudenon et al., 2005), these results suggested that IpaH9.8 might be an E3 ubiquitin ligase.

Ubiquitin biotinylated on Lys residues can not support polyubiquitination reactions. To test if IpaH9.8 could polyubiquitinate proteins, reactions were performed using HA-tagged ubiquitin, instead of biotinylated ubiquitin. Anti-HA antibodies detected a ladder of ubiquitinated proteins, from 24 to >200 kDa, in reactions performed in the presence of GST-IpaH9.8, but not in the presence of

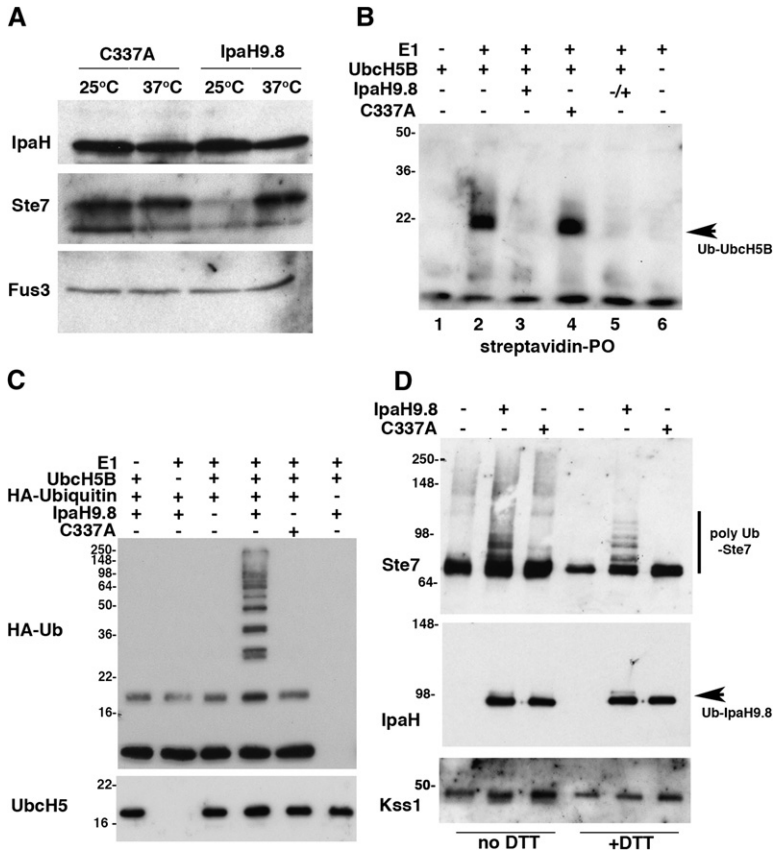

Figure 3. IpaH9.8 Is an E3 Ubiquitin Ligase

(A) Immunoblot analysis using anti-FLAG, anti-Ste7, and anti-Fus3 antibodies of extracts of *cim5-1* yeast producing FLAG-tagged IpaH9.8-C337A (C337A) or IpaH9.8 at 25°C or 37°C.

(B) Immunoblot analysis using streptavidin-peroxidase of reactions performed in the presence of biotinylated ubiquitin, E1, UbcH5B, and either GST-IpaH9.8 (IpaH9.8) or GST-IpaH9.8-C337A (C337A). For the sample loaded in lane 5, GST-IpaH9.8 was added after UbcH5B ubiquitination, and the reaction mixture was further incubated for 30 min.

(C) Immunoblot analysis using anti-HA and anti-UbcH5 of reactions performed in the presence of HA-ubiquitin, E1, UbcH5B, and either GST-IpaH9.8 (IpaH9.8) or GST-IpaH9.8-C337A (C337A).

(D) Immunoblot analysis using anti-Ste7, anti-IpaH, and anti-Kss1 antibodies of reactions performed in the presence of ubiquitin, E1, UbcH5B, GST-IpaH9.8 (IpaH9.8), or GST-IpaH9.8-C337A (C337A), as indicated, and a complex containing Ste7, Ste11-4, and Kss1. Samples were treated or not with DTT prior to loading.

GST-IpaH9.8-C337A (Figure 3C). Anti-UbcH5B antibodies detected a single species corresponding to UbcH5B (18 kDa), indicating that UbcH5B was not polyubiquitinated (Figure 3C). The sizes of species detected by anti-HA antibodies were multiples of the size of HA-ubiquitin (9 kDa), indicating that the molecule that was polyubiquitinated is ubiquitin. Using K48R and K63R ubiquitin variants, we found that IpaH9.8 catalyzed the formation of polyubiquitin chains on Lys-48, but not Lys-63 (data not shown). We did not detect polyubiquitinated proteins using the E2 UbcH7 (data not shown). These results demonstrated that IpaH9.8 is endowed with ubiquitin ligase activity toward ubiquitin and uses UbcH5B, but not UbcH7, as an E2.

To test whether IpaH9.8 might ubiquitinate Ste7, purified active MAPK complexes containing Ste7, as well as Ste11-4 and the MAPK Kss1, were incubated with native ubiquitin, E1, UbcH5B, and GST-IpaH9.8 or GST-IpaH9.8-C337A. Both the non- and monoubiquitinated forms of IpaH9.8 were detected using anti-IpaH antibodies (Figure 3D), confirming the autoubiquitination activity of IpaH9.8. In addition to Ste7, larger species that formed only in the presence of IpaH9.8 were detected by anti-Ste7 antibodies (Figure 3D). Thus, IpaH9.8 is an E3 ubiquitin ligase for Ste7; the proteasome-dependent disappearance of Ste7 in yeast was likely due to the degradation of Ste7 following its polyubiquitination by IpaH9.8 and IpaH7.8.

The IpaH Homolog SspH1 Is an E3 Ubiquitin Ligase for PKN1

SspH1, one of the *Salmonella enterica* Typhimurium homologs of IpaH, has been shown to interact with the mammalian protein kinase PKN1 (Haraga and Miller, 2006). To test whether SspH1 shares activities with IpaH9.8, we purified a GST-SspH1 recombinant protein. As described above for IpaH9.8, SspH1 was endowed with the activities (1) to remove ubiquitin from ubiquitinated UbcH5B, (2) to autoubiquitinate, and (3) to polyubiquitinate HA-tagged ubiquitin (Figure 4). We also tested ubiquitination of PKN1 by SspH1; SspH1, IpaH9.8, or IpaH9.8-C337A was incubated with E1, native ubiquitin, UbcH5B, and GST-PKN1. When the reaction was performed in the presence of SspH1, anti-PKN1 antibodies detected an additional species migrating at a size >250 kDa (Figure 4C). These results demonstrated that SspH1 is an E3 ubiquitin ligase that can use both ubiquitin and PKN1 as substrates.

Concluding Remarks

We presented evidence that IpaH9.8 and SspH1 are E3 ubiquitin ligases, and we identified, in addition to ubiquitin, a yeast substrate for IpaH9.8 (Ste7) and a mammalian substrate for SspH1 (PKN1). Ubiquitin was apparently removed from ubiquitinated UbcH5B upon incubation with IpaH9.8 or SspH1; this latter activity corresponding to the hydrolysis of the thioester bond linking ubiquitin to

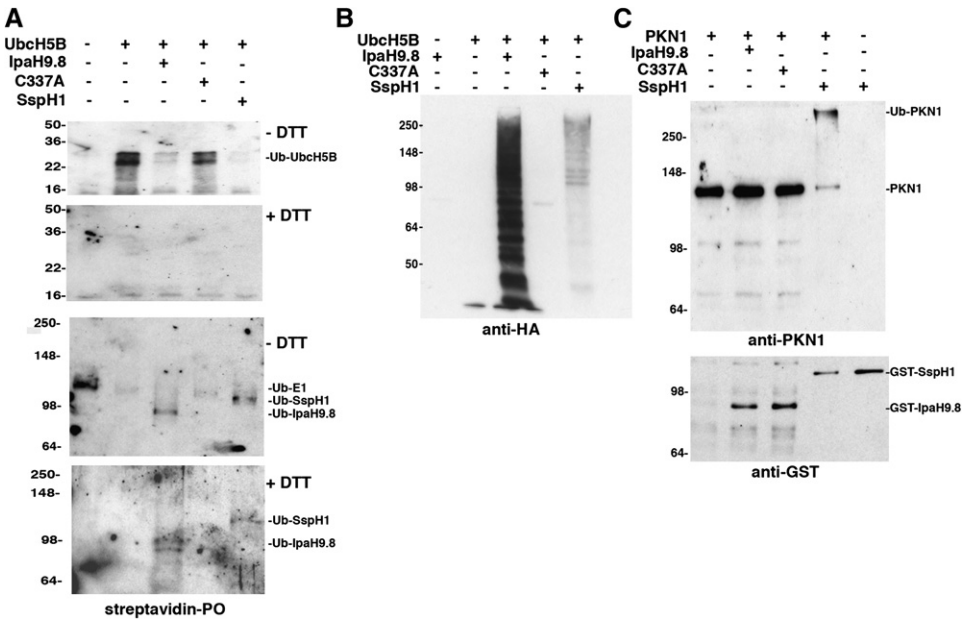

Figure 4. SspH1 Is an E3 Ubiqitin Ligase

(A) Immunoblot analysis using streptavidin-peroxidase (streptavidin-PO) of reactions performed in the presence of biotinylated ubiquitin, E1, UbcH5B, and either GST-IpaH9.8 (IpaH9.8), GST-IpaH9.8-C337A (C337A), or GST-SspH1 (SspH1). The two upper panels correspond to 15% SDS-PAGE, and the two lower panels correspond to 8% SDS-PAGE. Samples were treated or not with DTT prior to loading, as indicated.

(B) Immunoblot analysis using anti-HA antibodies of reactions performed in the presence of HA-ubiquitin, E1, UbcH5B, and either GST-IpaH9.8 (IpaH9.8), GST-IpaH9.8-C337A (C337A), or GST-SspH1 (SspH1).

(C) Immunoblot analysis using anti-PKN1 and anti-GST antibodies of reactions performed in the presence of ubiquitin, E1, UbcH5B, GST-PKN1 (PKN1), and either GST-IpaH9.8 (IpaH9.8), GST-IpaH9.8-C337A (C337A), or GST-SspH1 (SspH1).

the E2 is not equivalent to the activity of deubiquitinating enzymes hydrolyzing the amide bond linking ubiquitin to target proteins (Amerik and Hochstrasser, 2004). It might correspond either to the total consumption of the ubiquitinated E2 by the polyubiquitination activity of IpaH9.8 and SspH1 toward ubiquitin or to the transfer of ubiquitin onto IpaH9.8 and SspH1 prior to its transfer onto the substrate, as described for HECT domain E3s (Ardley and Robinson, 2005; Scheffner et al., 1995). Although the latter hypothesis is consistent with the observation that the Cys residue conserved in all IpaH family members is required for IpaH9.8 activities both in yeast and in vitro, we could not detect ubiquitin-IpaH9.8 and ubiquitin-SspH1 intermediates. The HECT domain of E3s and the C-terminal domain of IpaH proteins do not share sequence similarities; furthermore, residues surrounding the catalytic Cys residue in HECT domain E3s and the conserved Cys residue in IpaH proteins are different. Accordingly, IpaH family members constitute a novel class of E3 ubiquitin ligases.

Co-opting the ubiquitination pathway, either to promote or prevent ubiquitination of host proteins, is emerging as a common strategy employed by pathogens using T3S systems to downregulate host responses. The effector AvrPtoB from the tomato pathogen *P. syringae* is structurally similar to U box and RING finger E3s, possesses autoubiquitination and presumably ubiquitin ligase activities toward host proteins, and blocks signaling cascades that limit infection by activating the cell death program (Abramovitch et al., 2006; Janjusevic et al., 2006). Through an unknown mechanism, the *P. syringae* effector HopM1 promotes the proteasome-dependent degradation of the *Arabidopsis* protein AtMIN7 and inhibits vesicle trafficking required to mount a cell-wall-based defense to infection (Nomura et al., 2006). The *Salmonella* effector SopA has recently been shown to be a HECT-like E3 endowed with an autoubiquitination activity (Zhang et al., 2006). The *Shigella* effector OspG, encoded in the same operon as *ipaH9.8*, is a kinase that binds ubiquitinated E2s, prevents ubiquitination of phospho-IκBα, and dampens inflammation in the host (Kim et al., 2005). As shown here, the *Salmonella* effector SspH1 is an E3 ubiquitin ligase for PKN1, a protein kinase involved in the NF-κB pathway and activated upon cell infection (Haraga and Miller, 2006). The *S. flexneri* chromosomally encoded IpaH proteins have been reported to play a role in dampening inflammation (Ashida et al., 2007). The LRR-containing N-terminal domain of IpaHs is likely involved in protein-protein interactions and substrate recognition; the *Yersinia* effector YopM containing only LRRs related to those of IpaHs (Figure 2C) can act as a scaffolding protein that brings host kinases together (McDonald et al., 2003); and the LRR domain of SspH1 interacts with PKN1 (Haraga and Miller, 2006). In HeLa cells infected by *S. flexneri* for 90 min, we did not observe a significant decrease in the amount of the MAPKK Mek1, Mek3, and IKKα (Figure S5), suggesting that these proteins are not degraded upon

invasion of epithelial cells. The substrates of IpaH proteins in human cells remain to be identified.

The demonstration that IpaH9.8 and SspH1 are E3 ubiquitin ligases paves the way toward understanding the function of these proteins and their homologs during infection by identifying their target(s), possibly protein kinases, in host cells. *Shigella* produces multiple IpaHs that differ in their LRR domain, and likewise, some other pathogens contain several genes encoding IpaH homologs. This diversity suggests that each of these pathogens uses a repertoire of E3 ubiquitin ligases to promote degradation of several host proteins.

EXPERIMENTAL PROCEDURES

Materials

Plasmids encoding FLAG-tagged IpaH9.8, IpaH9.8-C337A, IpaH9.8-Cter, IpaH9.8-Nter, and IpaH7.8 were derivatives of the vector pFL38CII/pGal1 containing the *GAL* promoter (Badis et al., 2004). YCp50-STE11-4 carrying *STE11-4* under the control of its own promoter and pRS316-GAL-STE4 carrying *STE4* under the control of the *GAL* promoter have been described (Dohlman et al., 1995; Stevenson et al., 1992). Yeast strains are described in Table S1. UbcH7, E1, ubiquitin, HA-ubiquitin, biotinylated ubiquitin, ubiquitin-K48R and -K63R, horseradish peroxidase-coupled avidin, MG132, and anti-UbcH5 antibodies were purchased from Boston Biochem. Anti-IκBα, -ubiquitin, -Ste7, -Fus3, -Mek3, -IKKα, -Kss1, and -PKN1 antibodies were purchased from Santa Cruz Biotechnology. Purified GST-PKN1 was purchased from Invitrogen. Anti-Mek1 antibodies and purified active Mek1 were purchased from Upstate Cell Signaling Solutions. The mating pheromone α factor was purchased from Sigma. His-tagged UbcH5B was prepared as described (Kim et al., 2005). GST-IpaH9.8, GST-IpaH9.8-C337A, and GST-SspH1 were prepared as described (Mavris et al., 2002). Complexes containing Ste11-4, Ste7, and Kss1 were prepared as described (Breitkreutz et al., 2001) and eluted from FLAG M2-agarose affinity gel (Sigma) using a FLAG peptide.

In Vitro Assays

Ubiquitination of UbcH5B-His by E1 was performed in a 40 μl reaction mixture containing buffer A (25 mM Tris·HCl [pH 7.5], 50 mM NaCl, 5 mM ATP, 10 mM $MgCl_2$, 0.1 mM DTT), 2 μg of biotinylated ubiquitin, 0.5 μg of E1, and 2 μg of E2 in the presence or absence of 1 μg of GST-IpaH9.8, GST-IpaH9.8-C337A, or GST-SspH1. Reactions were incubated at 37°C for 1 hr and stopped by the addition of an equal volume of Laemmli sample buffer (62.5 mM Tris-Hcl [pH 6.8], 10% glycerol, 2% SDS, 0.0005% bromophenol blue) containing or not containing 100 mM DTT. Ubiquitination reactions were carried out in the same manner except that 2 μg of HA-tagged ubiquitin, ubiquitin, or ubiquitin-48R or -3R were used instead of biotinylated ubiquitin. Approximately 1 μg of Ste11-4:Ste7:Kss1 complexes, or 0.4 μg of GST-PKN1, was incubated in buffer A with 5 μg of ubiquitin, 0.5 μg of E1, and 2 μg of E2 in the presence or absence of 0.6 μg of GST-IpaH9.8, GST-IpaH9.8-C337A, or GST-SspH1. Reaction mixtures were separated by SDS/PAGE, transferred onto a nitrocellulose membrane, and probed with specific antibodies or peroxidase-coupled streptavidin when biotinylated ubiquitin was used.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and one supplemental table and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/1/1/77/DC1/.

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