Identification of Residues in the N-terminal Domain of the \textit{Yersinia} Tyrosine Phosphatase That Are Critical for Substrate Recognition* 

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YopH is a 468-amino acid protein-tyrosine phosphatase that is produced by pathogenic \textit{Yersinia} species. YopH is translocated into host mammalian cells via a type III protein secretion system. Translocation of YopH into human epithelial cells results in dephosphorylation of p130\textsuperscript{Cas} and paxillin, disruption of focal adhesions, and inhibition of integrin-mediated bacterial phagocytosis. Previous studies have shown that the N-terminal 129 amino acids of YopH comprise a bifunctional domain. This domain binds to the SycH chaperone in \textit{Yersinia} to orchestrate translocation and to tyrosine-phosphorylated target proteins in host cells to mediate substrate recognition. We used random mutagenesis in combination with the yeast two-hybrid system to identify residues in the YopH N-terminal domain that are involved in substrate-binding activity. Four single codon changes (Q11R, V31G, A33D, and N34D) were identified that interfered with binding of the YopH N-terminal domain to tyrosine-phosphorylated p130\textsuperscript{Cas} but not to SycH. These mutations did not impair YopH translocation into HeLa cells infected with \textit{Yersinia pseudotuberculosis}. Introduction of the V31G substitution into catalytically inactive (substrate-trapping) forms of YopH interfered with the ability of these proteins to bind to p130\textsuperscript{Cas} and to localize to focal adhesions in HeLa cells. In addition, the V31G substitution reduced the ability of catalytically active YopH to dephosphorylate target proteins in HeLa cells. These data indicate that the substrate- and SycH-binding activities of the YopH N-terminal domain can be separated and that the former activity is important for recognition and dephosphorylation of substrates by YopH \textit{in vivo}.

Three \textit{Yersinia} species (\textit{Yersinia pestis}, \textit{Yersinia pseudotuberculosis}, and \textit{Yersinia enterocolitica}) are highly pathogenic for humans. All three harbor a related 70-kilobase pair plasmid that is essential for virulence (1). Encoded on this plasmid is a type III protein secretion system that is produced by pathogenic \textit{Yersinia} species. YopH is a protein-tyrosine phosphatase that dephosphorylates multiple focal adhesion proteins (10–14). The 468-amino acid YopH protein appears to be composed of two distinct modular domains. Residues 206–468 comprise the C-terminal PTP catalytic domain (9). The PTP activity of this domain is essential for the antiphagocytic function of YopH and \textit{Yersinia} virulence (15, 16). Residues 405–410 form a phosphate-binding loop (P-loop) within the active site (17). Substitution of the nucleophilic Cys at position 403 with either Ser (C403S) or Ala (C403A) has been shown to inactivate the enzyme (9). Catalytically inactive forms of YopH can form stable complexes with substrates \textit{in vivo} (“substrate trapping”) (18) and localize to focal adhesion complexes in infected cultured cells (10, 12). The focal adhesion proteins p130\textsuperscript{Cas} (Cas) (12) (10), focal adhesion kinase (12), and paxillin (11) have been identified as substrates of YopH in cultured human epithelial cells. A region within the PTP domain (residues 223–226) has been shown to be important for targeting of YopH to focal adhesion complexes (19). The N-terminal 129 residues of YopH comprise a second modular domain that is bifunctional. This domain binds to the SycH chaperone in \textit{Yersinia} to orchestrate type III-mediated translocation of YopH into host cells (20). A binding site for SycH has been localized between residues 20 and 69 (20). The N-terminal domain also binds to Cas and paxillin \textit{in vitro} in a phosphotyrosine-dependent manner (11). The efficiency of substrate dephosphorylation by YopH \textit{in vitro} is diminished by removal of the N-terminal domain, suggesting that it is important for substrate recognition (11). As a first step toward elucidating the mechanism of substrate recognition mediated by the YopH N-terminal domain, we have identified several residues that are critical for this activity.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—** The plasmid pLP15 (21) contains a DNA fragment coding for YopH fused to a C-terminal M45 epitope tag inserted between the BamHI and EcoRI sites of pGEX-2T. Here, pLP15 is designated pGEX2T-YopHM45. A NdeI site overlaps the initiation codon of the YopH reading frame, and an XbaI site is present at the point of fusion between the YopH and M45 sequences. The plasmid pGEX-YopH1–129M45 was constructed as follows: A DNA fragment encoding the first 129 residues of YopH flanked by 5' and 3' restriction sites was synthesized by PCR. The PCR was performed using pYopH (22) as template and PTP18 (11) and PTP23

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**The abbreviations used are:** PTP, protein-tyrosine phosphatase; P-loop, phosphate-binding loop; Cas, p130\textsuperscript{Cas}; pTyr, phosphotyrosine; TRITC, tetramethyl rhodamine isothiocyanate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TBST, Tris-buffered saline containing 0.05% Tween 20; PCR, polymerase chain reaction; AD, activation domain.
The N-terminal Domain of YopH Mediates Substrate Recognition

(5′-GATCCCGGAGCCCTCGTCGTCGACCTTTG-3′) as forward and reverse primers, respectively. The primers were designed to incorporate BamHI and Ndel restriction sites into the 5′ end of the product and an XbaI site into the 3′ end. The PCR product was digested with BamHI and XbaI. The pGEX2T-YopHM45 vector was digested with BamHI and XbaI and pGAD424 was digested for cloning purposes by the insertion of new polylinker regions. The polylinker regions of pGEB9 or pGAD424 were removed by digestion with EcoRI and Sall. Two complementary oligonucleotides, MsiteF (5′-AATTGGTAGCTCCCCGGAATTTGGCGCCGC- CGG-3′) and MsiteR (5′-CCCTAGGGCTTACAGCTCGCGGAGC- AGCTC-3′), were annealed and inserted between the EcoRI and Sall sites in pGBT9 and pGAD424. The polylinker regions of pBDM1 and pGAD424 were confirmed by sequencing. A segment of DNA coding for YopH1–129M45 was removed and replaced with the PCR product encoding YopH1–129. This resulted in a translational fusion between the codons specifying residues 1–129 of YopH and the 12-amino acid C-terminal epitope tag. The structure of pGEX-YopH1–129M45 was verified by sequencing.

The plasmids pBDM1 and pGAD424 were derived from the yeast two-hybrid plasmids pBD9 and pGAD424 (27). PTP26 (5′-GATCCCGGAGCCCTCGTCGTCGACCTTTG-3′) and PTP27 (5′-GATCCCGGAGCCCTCGTCGTCGACCTTTG-3′) were designed to incorporate Ndel and EcoRI sites of pBDM1, yielding plpBD-YopH1–129M45. The plasmid pAD-Src was constructed from pADMI by the insertion of a DNA fragment that encodes a mutant form of c-Src (Y416F/Y527F) (23). The DNA fragment coding for c-Src was removed from pGEMT16G and inserted into the unique BglII site in pADMI. A DNA fragment encoding Cas was removed from pEG-P130Cas (24) by digestion with BamHI and NotI and inserted between the BamHI and NotI sites of pADMI or pAD-Src, yielding pAD-Cas and pAD-Cas + Src, respectively.

Codon substitutions isolated in the two-hybrid assay (see below) were introduced into the full-length YopHM45 reading frame by restriction fragment subcloning. DNA fragments containing the various codon substitutions were removed from pBD-YopH1–129M45 by digestion with Ndel1 and SnaBI and substituted for the corresponding Ndel1-SnaBI fragment of pGEX2T-YopHM45. A standard restriction fragment subcloning procedure was used to combine the V31G mutation with either the C403Y or the R409A substitutions. DNA fragments coding for full-length YopH proteins with the various codon substitutions were removed from pGEX-2T backbone by digestion with Ndel1 and EcoRI and inserted between the Ndel1 and EcoRI sites in the plasmid pPROH for expression in Y. pseudotuberculosis. The plasmid pPROH was generated by the insertion of the yopH promoter region into the polylinker region of pMMB66HE (25). The yopH promoter region was amplified by PCR using DNA from yeast cells containing plasmid pMMB66HE and the primers PTP26 (5′-GGCGTCCGGCGCGTTGATGTACTGACGCTC-3′) and PTP27 (5′-TTACATTAGGAATTCATATGTCCCTCCTTATAAATACATCC-3′). The primers were designed to incorporate a BamHI site into the 5′ end of the product and Ndel1 and EcoRI sites into the 3′ end. The PCR product was digested with BamHI and EcoRI, inserted between the BamHI and EcoRI sites of pMMB66HE, and verifified by sequencing.

Identification of Codon Changes in the N-terminal Domain of YopH That Interfere with Binding to Cas in the Two-hybrid System—The DNA sequence coding for the first 129 residues of YopH was subjected to random mutagenesis using a PCR under suboptimal conditions (26). The PCR was performed using pBD-YopH1–129M45 as template and the oligonucleotides 5′-GATCCCGGAGCCCTCGTCGTCGACCTTTG-3′ and 3′-GATCCCGGAGCCCTCGTCGTCGACCTTTG-3′ as forward and reverse primers, respectively. The resultant PCR product contained the YopH1–129M45 coding region flanked on either side by ~100 base pairs of sequence derived from pBDM1. The PCR product was introduced into pBDMI via homologous recombination in yeast cells. For this purpose, pBDM1 was digested with BamHI, and the linear plasmid was mixed with the PCR product and supercoiled pAD-Cas + Src. The mixture was used to transform the yeast two-hybrid reporter strain Y153 to Trp and Leu prototrophy on synthetic dextrose media lacking Trp and Leu (SD-Trp-Leu) (27). The resulting plasmids were isolated from Leu colonies of MH4 and digested with BamHI and EcoRI to remove the YopH1–129M45 coding regions. The regions coding for mutant YopH1–129M45 proteins were inserted between the BamHI and EcoRI sites of pBDM1, and the resulting plasmids were introduced into Y153 along with the pAD-Cas + Src plasmid. Colonies of Leu + Trp + transformants on filters were tested for expression of β-galactosidase as described above. This procedure was repeated using pAD-SycH in place of pAD-Cas + Src to identify mutations that selectively interfered with binding of the YopH N-terminal domain to Cas. The regions coding for YopH1–129M45 in purified pBD-YopH1–129M45 plasmids were sequenced to identify mutations that resulted in reduced binding to Cas. The G41S and D106G codon substitutions were separated by a standard restriction fragment subcloning procedure.

β-galactosidase activity in Y153 strains containing mutant pBD-YopH1–129M45 plasmids was quantitated using a colorimetric liquid assay as described previously (27). Cultures of Y153 strains containing mutant pBDM1-YopH1–129M45 plasmids and pAD-Cas + Src were grown in selective media (SD-Trp-Leu) to an A600 value of ~1.0. Yeast cells in 5 ml of culture were assayed for β-galactosidase activity using chlorophenol red-β-naphthol as substrate (27). Reactions were incubated for 35 min. Measurement of β-galactosidase activity in yeast cells containing mutant plasmids pBDM1-YopH1–129M45 and pAD-SycH was performed using cells derived from 0.1 ml of culture and the substrate O-nitrophenyl-β-naphthol (27). Reactions were incubated for 15 min. Units of β-galactosidase activity were calculated as described (27).

Antibodies—Hybridoma supernatant containing monoclonal anti-M45 antibody was provided by Dr. P. Hearing (State University of New York at Stony Brook). Anti-M45 recognizes the 12-amino acid epitope SRDRLPPFETET. Anti-M45 was purified from the supernatant using protein A-Sepharose as described (28). Anti-M45 was used at a dilution of 1:1,000 for immunoblotting and at a final concentration of 0.5 μg/ml for immunoprecipitations. Monoclonal anti-phospho-tyrosine (pTyr) antibody 4G10 was purchased from Upstate Biotechnology. The anti-pTyr antibody was used at a dilution of 1:1,000 for immunoblotting and at final concentration of 5 μg/ml for immunofluorescence labeling. Monoclonal anti-Cas (P27820) was purchased from Transduction Laboratories and used at a dilution of 1:1,000 for immunoblotting. Immunoprecipitations were performed with 1.0 μg of anti-Cas per sample. The rabbit anti-YopH antibody SB360 was prepared in a commercial facility (Calico Biologicals, Inc.) using a purified glutathione S-transferase-YopH fusion protein as antigen. SB360 was used at a dilution of 1:1,000 for immunoblotting. Anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Sigma and used at dilutions of 1:1,000 or 1:15,000, respectively. Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated F(ab′)2 goat anti-mouse secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. and used at a dilution of 1:250.

Bacterial and HeLa Cell Cultures and Infection Conditions—The Y. pseudotuberculosis serogroup III strains YP17 (ypopHyopE) and YP19 (ypopHyopEypopB) and their growth conditions have been described previously (21). Both strains carry a naturally occurring deletion in the yopT gene and are devoid of YopT activity. Expression plasmids derived from pPROH that produce wild-type or mutant YopH proteins were introduced into these strains from E. coli by conjugation (10). For infection assays, bacteria were grown overnight at 26°C with shaking in Luria broth containing 100 μg/ml ampicillin. Bacteria were harvested in the early exponential phase (100–1,000 colony-forming units/ml) and suspended in 2.5 mM CaCl2 to an A600 value of 0.1. Cultures were shaken at 37°C for 2 h. Bacteria were pelleted by centrifugation and resuspended in warm 37°C Hanks’ balanced salt solution to an A600 value of 1.0 (~1 × 10⁸ colony-forming units/ml).

J. Zitzler and J. Bliska, unpublished results.
HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) and 1 mM sodium pyruvate in a 5% CO2 humidified incubator at 37 °C. For the immunofluorescence experiments, 1 × 106 HeLa cells were seeded in 1 ml of media onto sterile glass slides placed in a 24-well plate and incubated at 37 °C overnight. For the translocation, immunoprecipitation, and in vivo dephosphorylation assays, 2 × 106 HeLa cells in 10 ml of media were seeded into 100-mm tissue culture dishes and cultured overnight. HeLa cells were overlaid with fresh media 30 min prior to bacterial infection. Cells were left uninfected or infected with bacteria grown as described above at a multiplicity of infection of 50:1 at 37 °C in a 5% CO2 incubator.

**Immunofluorescence Assays**—All steps following a 2-h infection (see above) were performed at room temperature. Coverslips were washed twice with phosphate-buffered saline (PBS) containing 1 mM Na3VO4, fixed with 4% parafomaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 10 min. Coverslips were washed twice with PBS containing 1% bovine serum albumin (BSA) and then incubated for 1 h with primary antibody (anti-pTyr or anti-M45) diluted in PBS containing 3% BSA. Coverslips were washed with PBS and then incubated for 1 h with TRITC-conjugated secondary antibody diluted in PBS containing 3% BSA. Coverslips were washed well with PBS before mounting on slides in 10% Airvol (Air Products, Inc.), 100 mM Tris, pH 8.0, 1% Nonidet P-40, 1 mM Na3VO4, and 10 mM NaF for 15 min on ice with occasional rocking. Cells were scraped into microcentrifuge tubes and centrifuged at 10,000 × g at 4 °C for 10 min. The supernatants were transferred to new tubes, and protein concentrations were determined using the Bio-Rad protein assay. Samples of 20 µl containing 5 µg of total cell protein each were separated on 10% SDS polyacrylamide gels and analyzed by immunoblotting using anti-M45.

**Immunoprecipitation Assay**—After 2 h of infection the dishes were placed on ice and washed twice with 10 ml of ice-cold PBS. Cells were lysed in 0.5 ml of a 50% suspension of protein A-Sepharose beads (Amersham Pharmacia Biotech) was added to each lysate sample, and the tubes were incubated for 30 min at 4 °C with rotation as a pre-clearing step. After the protein A-Sepharose beads were removed by centrifugation, the supernatants were transferred to new tubes and mixed with anti-M45. The tubes were incubated for 3 h at 4 °C with rotation. Immune complexes were recovered by addition of 50 µl of 50% protein A-Sepharose, followed by incubation for 3 h at 4 °C with rotation. The beads were pelleted by centrifugation, washed twice with 1 ml of 4 °C lysis buffer, resuspended in 60 µl of 1× Laemmli sample buffer, and boiled for 5 min. Immunoprecipitated proteins were separated on 7.5% SDS polyacrylamide gels under reducing conditions and analyzed by immunoblotting with anti-Cas, anti-pTyr, or anti-YopH antibodies as described below.

**In Vivo Dephosphorylation Assay**—HeLa cells were left uninfected or infected for 15, 30, 60, or 120 min. Dishes were placed on ice and washed twice with 10 ml of ice-cold PBS containing 1 mM Na3VO4. Cells were lysed in 0.5 ml of a 50% suspension of protein A-Sepharose, followed by incubation for 3 h at 4 °C with rotation. Cells were scraped into microcentrifuge tubes and centrifuged at 10,000 × g at 4 °C. The supernatants were transferred to new tubes, and protein concentrations were determined using the Bio-Rad protein assay. Supernatant samples containing 50 µg of protein in a volume of 20 µl were mixed with an equal volume of 2× Laemmli sample buffer and boiled for 3 min. The resulting samples were separated on 7.5% SDS polyacrylamide gels under reducing conditions and analyzed by immunoblotting with anti-pTyr or anti-YopH as described below.

**Immunoblotting**—Proteins separated in SDS polyacrylamide gels were electrothermally transferred to nitrocellulose filters (Schleicher & Schüll). Unless indicated all subsequent steps were performed at room temperature. The nitrocellulose filters were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 1% BSA for 1 h. Filters were washed four times in TBST and then incubated for 1 h with the appropriate secondary antibody diluted (1:1000 for anti-mouse and 1:15,000 for anti-rabbit) in TBST. The filters were washed four times in TBST and developed using the Renaissance (PerkinElmer Life Sciences) chemiluminescence system. In some cases, the blots were stripped of bound antibodies by incubation in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol at 50–55 °C for 30 min. After the filter was extensively washed in TBST, the immunoblotting procedure was then repeated starting with the blocking step.

**RESULTS**

**Interaction of the YopH N-terminal Domain with Tyrosine-phosphorylated Cas in the Yeast Two-hybrid System**—A modified yeast two-hybrid system (23) was used to detect association of the YopH N-terminal domain with tyrosine-phosphorylated Cas. A yeast strain carrying a lacZ reporter gene under control of the Gal4p transcription factor was transformed with two expression vectors. One vector produced the DNA binding domain of Gal4p fused to residues 1 to 129 of YopH adjacent to a C-terminal Myc epitope tag (BD-YopH1–129M45). The other vector produced a modified form of the Src tyrosine kinase and the activation domain of Gal4p fused to Cas (AD-Cas). Based on previous studies (23), ectopic expression of Src was expected to result in tyrosine phosphorylation of the AD-Cas fusion protein in yeast cells. β-galactosidase activity was detected when both fusion proteins and Src were produced in the yeast reporter strain (Fig. 1A). In contrast, β-galactosidase activity was not detected when both fusion proteins were expressed in yeast cells in the absence of Src (data not shown). Thus, a specific two-hybrid interaction between the N-terminal domain of YopH and tyrosine-phosphorylated Cas was detected.

**Identification of Amino Acid Substitutions in the YopH N-terminal Domain That Interfere with Binding to Tyrosine-phosphorylated Cas**—Random mutagenesis was used to identify amino acid substitutions in the YopH N-terminal domain that interfere with binding to tyrosine-phosphorylated Cas in the two-hybrid system. The DNA sequence encoding the first 129 residues of YopH was amplified using a PCR and Taq DNA polymerase under suboptimal conditions (26). The resulting DNA product was inserted into a Gal4p binding domain fusion vector in a yeast reporter strain using in vivo recombination. Approximately 1500 yeast colonies were screened on filters for reduced β-galactosidase activity. 43 colonies that showed reduced β-galactosidase activity were chosen for further characterization (see “Experimental Procedures”). Five of these colonies produced intact BD-YopH1–129M45 protein that interacted weakly with tyrosine-phosphorylated Cas in the two-hybrid system. The DNA binding domain vectors were isolated from these colonies, and the regions coding for residues 1–129 of YopH were sequenced. Four plasmids contained single codon changes in the YopH coding region (corresponding to Q11R, V31G, A33D, and N34D). One plasmid contained two codon changes (G41S and D106G) that were subsequently separated by restriction fragment subcloning. As shown in Fig. 1A, five of these single amino acid substitutions (the exception being D106G) resulted in reduced levels of β-galactosidase activity in the two-hybrid assay.

We tested each of the mutant BD-YopH1–129 proteins by two-hybrid assay for interaction with an AD-SycH fusion protein to determine whether any of the amino acid substitutions interfered with binding to the chaperone. With the exception of the Q11R mutation, all of the substitutions resulted in higher levels of β-galactosidase activity as compared with the wild-type protein and the wild-type AD-YopH1–129M45.
Amino Acid Substitutions in the N-terminal Domain That Interfere with Binding to Cas Do Not Interfere with YopH Translocation—

We next examined the effect of the substitutions in the N-terminal domain on translocation of YopH into HeLa cells. The Q11R, V31G, A33D, and N34D codon changes were inserted into the full-length yopH gene carried on a bacterial expression vector (pPYopHM45), and the resulting plasmids were introduced into a catalytically inactive form of YopHM45 (YopHC403SM45) to determine whether the N-terminal domain is important for substrate trapping in vivo. The V31G mutation was selected for this purpose, because it strongly interfered with substrate-binding activity in the two-hybrid system (Fig. 1A). A plasmid encoding the mutant protein was introduced into Y. pseudotuberculosis strain (YP17), and the resulting strain was used to infect HeLa cells. Initially, the HeLa cells were processed for immunofluorescence microscopy using the anti-pTyr antibody (Fig. 5A). Infected HeLa cells were also labeled with an anti-pTyr antibody to demonstrate that focal adhesions were not disrupted under the conditions of the infection (Fig. 4G).

Detergent lysates of the infected HeLa cells were prepared and subjected to immunoprecipitation with M45 antibody to determine the amount of Cas that was directly bound to the mutant YopH proteins. The immune complexes were analyzed by immunoblotting with either anti-Cas antibody (Fig. 5A) or anti-pTyr antibody (Fig. 5B). The results obtained with the
In addition, Arg-409 plays an important role in substrate binding (29), and therefore the R409A substitution was predicted to interfere with binding of tyrosine-phosphorylated Cas to the P-loop. The R409A substitution by itself did not interfere with localization of YopH to focal adhesions (Fig. 4D) or with binding of YopH to Cas (Fig. 5B, lane 4), indicating that the N-terminal domain is the major determinant of substrate binding in vivo. When the V31G and the R409A substitutions were combined, localization of YopH to focal adhesions was abolished (Fig. 4F) and Cas-binding activity was further decreased but not eliminated (Fig. 5B, lane 5). These results indicated that the P-loop containing the C403S substitution does bind tyrosine-phosphorylated Cas in vivo but that this substrate-binding activity is substantially weaker than that of the N-terminal domain.

The Substrate-binding Activity of the YopH N-terminal Domain Is Required for Efficient Substrate Dephosphorylation in Vivo—We next examined the effect of the V31G substitution on dephosphorylation of substrates by YopH in infected HeLa cells. Lysates prepared from HeLa cells infected for 15, 30, 60, or 120 min with YP17 strains producing either YopH or YopHV31G were analyzed by immunoblotting with anti-pTyr antibody. Two heavily phosphorylated protein bands, of 130 kDa and 68 kDa, were detected in lysates of uninfected HeLa cells. Lysates prepared from HeLa cells infected for 15, 30, 60, or 120 min with YP17 strains producing either YopH or YopHV31G were analyzed by immunoblotting with anti-pTyr antibody. Two heavily phosphorylated protein bands, of 130 kDa and 68 kDa, were detected in lysates of uninfected HeLa cells. Lysates prepared from HeLa cells infected for 15, 30, 60, or 120 min with YP17 strains producing either YopH or YopHV31G were analyzed by immunoblotting with anti-pTyr antibody. Two heavily phosphorylated protein bands, of 130 kDa and 68 kDa, were detected in lysates of uninfected HeLa cells. Lysates prepared from HeLa cells infected for 15, 30, 60, or 120 min with YP17 strains producing either YopH or YopHV31G were analyzed by immunoblotting with anti-pTyr antibody. Two heavily phosphorylated protein bands, of 130 kDa and 68 kDa, were detected in lysates of uninfected HeLa cells. Lysates prepared from HeLa cells infected for 15, 30, 60, or 120 min with YP17 strains producing either YopH or YopHV31G were analyzed by immunoblotting with anti-pTyr antibody. Two heavily phosphorylated protein bands, of 130 kDa and 68 kDa, were detected in lysates of uninfected HeLa cells.
FIG. 6. The N-terminal domain of YopH is required for efficient dephosphorylation of substrates in vivo. HeLa cells were left uninfected (lanes 1 and 6) or were infected for the indicated time (in min) with YP17 (pPYopHM45) (lanes 2–5) or YP17 (pPYopHV31GM45) (lanes 7–10). Samples of detergent lysates prepared from the infected cells were separated by SDS polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-pTyr (A). The filter analyzed in A was stripped of anti-pTyr and analyzed by immunoblotting with anti-YopH (B) to control for differences in expression levels of YopHM45 and YopHV31GM45.

FIG. 7. The N-terminal domain of YopH is required for efficient dephosphorylation of Cas in vivo. Cas was immunoprecipitated from HeLa cell lysates and analyzed by immunoblotting with anti-pTyr (A). The filter analyzed in A was stripped of anti-pTyr and analyzed by immunoblotting with anti-Cas (B) to control for recovery of Cas during the immunoprecipitation.

that the substrate-binding activity of the YopH N-terminal domain is required for efficient dephosphorylation of substrates in vivo.

DISCUSSION

The goal of this study was to identify residues in the YopH N-terminal domain that are important for substrate-binding activity but not SycH-binding activity. We used random mutagenesis to isolate single amino acid changes in the N-terminal domain that interfere with binding to tyrosine-phosphorylated Cas in a yeast two-hybrid system. Four single codon changes (Q11R, V31G, A33D, and N34D) and one double codon change (G41S and D106G) were identified in the screen. The double codon change introduced a single-stranded b-strand 1 and b-strand 2. This region is rich in positively charged residues (e.g., K26, R28, K35, and R49) and is flanked on either side by pockets, which may together form a shallow phosphotyrosine-binding cleft.

The three-dimensional structure of the YopH N-terminal domain has recently been determined to a resolution of 2.2 Å.3 It is a single, highly compact domain composed of 4 a-helices sandwiched between one two-stranded b-sheet and one three-stranded b-sheet. The fold of the YopH N-terminal domain is unlike that of other known phosphotyrosine binding domains such as the SH2 domain of Src (34) or the phosphotyrosine binding domain of insulin receptor substrate-1 (35). Q11, V31, A33, N34, and G41 are located on the surface along a loop connecting b-strand 1 and b-strand 2. This region is rich in positively charged residues (e.g., K26, R28, K35, and R49) and is flanked on either side by pockets, which may together form a shallow phosphotyrosine-binding cleft.

The role of the N-terminal domain in substrate recognition in vivo was addressed by the introduction of the V31G substitution into the full-length YopH reading frame. Initially, the V31G substitution was introduced into two different catalytically inactive forms of YopH, one in which the P-loop in the PTP domain retained the capacity to bind substrate (C403S) and the other in which binding of substrate to the P-loop is blocked (R409A). This strategy allowed us to independently assess the contributions of the N-terminal domain and the P-loop to the formation of a stable enzyme-substrate complex. Substrate-binding activity was scored by the ability of the mutant proteins to localize to focal adhesions in infected HeLa cells and to coprecipitate with Cas in detergent lysates of infected HeLa cells. These results of both assays indicated that the N-terminal domain is the major determinant of substrate-binding activity in YopH but that the P-loop also contributes, albeit weakly, to substrate trapping. The ability of Cas to coprecipitate with YopH was not completely eliminated by the presence of the V31G and R409A substitutions, suggesting that an additional substrate-binding interface may be present elsewhere in the protein. Persson et al. (19) have reported that part of a surface-exposed loop in the PTP domain (residues 229–228) is except for Q11R, fell within this putative binding site for SycH. This may indicate that tyrosine-phosphorylated proteins and SycH bind to an overlapping region of the YopH N-terminal domain.
involved in targeting YopH to focal complexes. However, their data argue that this region functions as a localization sequence and is not involved in substrate recognition (19).

The V31G substitution was introduced into catalytically active YopH to determine whether the N-terminal domain is important for efficient dephosphorylation of substrates by YopH in vivo. The presence of the V31G substitution resulted in a dramatic decrease in the efficiency of Cas and paxillin dephosphorylation by YopH in infected HeLa cells. These results support our original proposal that the N-terminal domain allows YopH to act processively during the dephosphorylation of multiply phosphorylated substrates such as Cas and paxillin.

It is becoming apparent that translocated proteins of other type III secretion systems may be arranged in a modular fashion with at least two, and possible more, distinct effector domains (36–38). In addition, a single modular domain may in fact be multifunctional, as is the case for the YopH N-terminal domain. This raises the possibility that the N-terminal domain of other translocated type III proteins will perform multiple functions.

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