The EMBO Journal Vol.16 No.10 pp.2730–2744, 1997

Identification of p130^{Cas} as a substrate of *Yersinia* YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions

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A number of pathogenic bacteria utilize type III secretion pathways to translocate virulence proteins into host eukaryotic cells. We identified a host target of YopH, a protein tyrosine phosphatase that is translocated into mammalian cells by *Yersiniae*. A catalytically inactive 'substrate-trapping' mutant, YopHC403S, was used as a probe to determine where YopH substrates localize in eukaryotic cells. Immunofluorescence microscopy demonstrated that YopHC403S localized to focal adhesions in human epithelial cells infected with *Y.pseudotuberculosis*. YopHC403S stabilized focal adhesions, as shown by its dominant-negative effect on focal adhesion disassembly mediated by YopE, a translocated protein which disrupts actin stress fibers. Conversely, YopH destabilized focal adhesions, even in the absence of YopE, as shown by loss of phosphotyrosine staining. Immunoprecipitation revealed that YopHC403S was trapped in a complex with a hyperphosphorylated 125–135 kDa protein, identified by immunoblotting as the focal adhesion protein p130^{Cas}. YopHC403S bound directly to p130^{Cas} in a phosphotyrosine-dependent manner *in vitro*. Translocation of YopH into cells plated on fibronectin resulted in rapid and selective dephosphorylation of p130^{Cas}. These results demonstrate that YopH targets focal adhesions in host cells and that p130^{Cas}, a docking protein for multiple SH2 domains, is a direct substrate of this enzyme *in vivo*.

Keywords: focal adhesion/p130^{Cas}/tyrosine phosphatase/*Yersinia*

Introduction

Signal transduction pathways in eukaryotic cells are subject to modulation by a large number of pathogenic bacteria. For example, *Salmonella*, *Shigella*, enteropathogenic *Escherichia coli* and *Yersinia* target various components of eukaryotic signal transduction pathways in order to attach to or enter into host cells (for reviews, see Bliska *et al.*, 1993b; Rosenshine and Finlay, 1993; Galan, 1994; Galan and Bliska, 1996). These bacteria are capable of translocating effector proteins directly into host cells in order to modulate eukaryotic signal transduction pathways (Rosqvist *et al.*, 1994, 1995; Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995). Translocation of these effectors is mediated by a highly conserved type III protein secretion pathway (reviewed in Galan and Bliska, 1996). Although many of the components of the secretory pathways have been characterized, very little is known about the effector proteins themselves or their host targets.

Yersinia pseudotuberculosis is an opportunistic enteric pathogen of humans and a close relative of *Y.pestis*, the agent of bubonic plague (reviewed in Brubaker, 1991). *Y.pseudotuberculosis* invades and replicates in Peyer's patches, mesenteric lymph nodes and deeper tissues such as the spleen. The infection appears to involve interactions with several different types of host cells, including M (microfold) cells, epithelial cells, platelets and professional phagocytes (Fujimura *et al.*, 1992; Simonet *et al.*, 1990). At late stages of infection, the bacteria preferentially replicate in extracellular niches and suppress cell-mediated immunity in a localized fashion (Brubaker, 1991). *Y.pseudotuberculosis* infects a variety of cultured mammalian cell lines *in vitro*, which has allowed for a dissection of this bacterial–host cell interaction at the molecular level (for reviews, see Bliska *et al.*, 1993b; Forsberg *et al.*, 1994; Isberg and Tran Van Nhieu, 1994). The bacteria initially respond to a physiological temperature of 37°C by activating the transcription of factors important for infection (reviewed in Cornelis *et al.*, 1989; Falkow *et al.*, 1992). The initial interaction with host cells occurs when at least one of several different bacterial surface proteins binds to a host cell receptor (Isberg and Tran Van Nhieu, 1994). The best characterized of these proteins is invasin, which binds with high affinity to a subclass of β1 integrin extracellular matrix receptors (Isberg and Tran Van Nhieu, 1994). Once the bacterium is tightly attached, a type III secretion pathway encoded by the *Yersinia* virulence plasmid is activated, and a set of effectors known as Yops are translocated into the cytoplasm of the host cell at the point of contact (for reviews, see Straley *et al.*, 1993; Forsberg *et al.*, 1994; Galan and Bliska, 1996). Within minutes, cultured mammalian cells lose actin filaments, round up and begin to detach from the extracellular matrix (Portnoy *et al.*, 1981; Rosqvist *et al.*, 1991). In the absence of the virulence plasmid, invasin mediates efficient uptake of the bacteria into host cells by a β1 integrin-mediated phagocytic-like process (Isberg, 1989; Isberg and Tran Van Nhieu, 1994). In contrast, when the bacteria harbor the virulence plasmid, phagocytosis appears to be antagonized by the action of the translocated Yops, a phenomenon referred to as anti-phagocytosis (Forsberg *et al.*, 1994).

Cellular disruption and anti-phagocytosis are mediated primarily by two Yops that are translocated into mammalian cells, YopE and YopH (Yop51) (Rosqvist *et al.*, 1988, 1990; Bliska *et al.*, 1993a; Sory and Cornelis, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995; Ruckdeschel *et al.*, 1996). YopE (25 kDa) and YopH (51 kDa) both contain N-terminal domains important for bacterial secretion and translocation into mammalian cells (Persson *et al.*, 1995; Sory *et al.*, 1995). YopE is homologous to the N-terminal domain of ExoS, a toxin which ADP-ribosylates a number

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of low molecular weight GTP-binding proteins, including Ras (Coburn *et al.*, 1989; Kulich *et al.*, 1994). YopE has a potent cytotoxic effect on host cells and is associated with disruption of actin stress fibers (Rosqvist *et al.*, 1990, 1991). YopH has a weaker cytotoxic effect on host cells that can only be detected in the absence of YopE (Rosqvist *et al.*, 1990; Bliska *et al.*, 1993a). YopH contains a C-terminal protein tyrosine phosphatase (PTPase) domain (Guan and Dixon, 1990) and a central proline-rich region that appears to bind host cell Src homology 3 (SH3) domains (Bliska, 1996). The activity of the PTPase domain has been shown to be essential for the pathogenicity of *Y.pseudotuberculosis* (Bliska *et al.*, 1991). Production of YopH during bacterial infection of cultured mammalian cells is associated with dephosphorylation of proteins in human epithelial cells and murine macrophages (Bliska *et al.*, 1991, 1992; Hartland *et al.*, 1994; Andersson *et al.*, 1996). Although the primary host cell target(s) of this PTPase has not been identified, it may be involved in integrin-mediated signaling, since wild-type YopH promotes detachment of epithelial cells from the extracellular matrix, while a catalytically inactive form of the protein, in which the essential Cys403 in the PTPase domain is changed to Ser (YopHC403S) or Ala (YopHC403A), is defective for cellular detachment (Bliska *et al.*, 1993a). Thus, although significant emphasis has been placed on the role of YopH as an inhibitor of integrin-mediated bacterial internalization, the PTPase could play an equally or more important role as an inhibitor of integrin-mediated cell adhesion.

The first suggestion that a catalytically inactive PTPase could be used to trap substrates *in vivo* was derived from the observation that tyrosine-phosphorylated host proteins co-precipitated with YopHC403A (Bliska *et al.*, 1992). Subsequently, a number of other inactive PTPases including CSW (C583S) (Herbst *et al.*, 1996), SHP-1 (C453S) (Plas *et al.*, 1996) and PTP-PEST (C231S) (Garton *et al.*, 1996) have been used to trap substrates *in vivo*. In some cases, these substrate-trapping mutants also promote substrate hyperphosphorylation *in vivo*, apparently by binding to phosphotyrosyl residues and sequestering these sites from the action of endogenous PTPases (Herbst *et al.*, 1996; Plas *et al.*, 1996). Here we demonstrate that a catalytically inactive PTPase can also be used as a cytological probe to investigate the cellular localization of substrates. Using this approach, we identified the focal adhesion protein p130^{Cas} (Cas) as a direct substrate of YopH in human epithelial cells.

Results

YopHC403S localizes to focal adhesions

The first step of our approach to identify an *in vivo* substrate of YopH was to determine if the substrate localized to a particular subcellular compartment or structure in host cells. We used confocal immunofluorescence microscopy to determine where YopH localizes in host cells, under the assumption that YopH, and in particular its catalytically inactive derivative YopHC403S, could act as substrate-specific probes (Bliska *et al.*, 1992). Human epithelial (HeLa) cells were used as host cells in these studies because their well defined cellular architecture facilitated our microscopic analysis. HeLa cells were

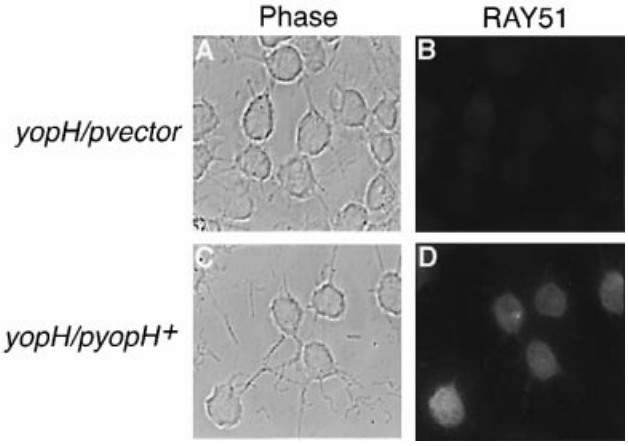

Fig. 1. YopH localizes to the cytoplasmic compartment of HeLa cells infected with *Y.pseudotuberculosis*. HeLa cells were infected for 2 h with IP15/pVector, a *Y.pseudotuberculosis yopH* mutant carrying an empty expression plasmid (**A** and **B**) or IP15/pYOPH, a *yopH* mutant complemented in *trans* with a wild-type *yopH*$^{+}$ gene (**C** and **D**). The infected cells were processed for confocal immunofluorescence microscopy using an affinity-purified rabbit polyclonal anti-Yop51 antibody (RAY51) (see Materials and methods). Corresponding phase (A and C) and rhodamine (B and D) images are shown.

infected with *Y.pseudotuberculosis* at a multiplicity of infection of 50:1. Two hours after infection, the cells were fixed, permeabilized and processed for confocal immunofluorescence microscopy using a polyclonal rabbit anti-Yop51 antibody (RAY51), either alone, or in combination with a monoclonal anti-phosphotyrosine antibody (4G10). Samples were treated gently during processing to avoid detachment of infected HeLa cells. As a control for the specificity of the RAY51 antibody, we examined HeLa cells that were infected with IP15/pVector, a *yopH* deletion mutant carrying an empty expression vector, and observed only a low level of background fluorescence (Figure 1B). When the wild-type *yopH*$^{+}$ gene was introduced in *trans* into the *yopH* mutant (IP15/pYOPH), a strong fluorescent signal was detected in the cytoplasmic compartments of HeLa cells (Figure 1D), demonstrating that RAY51 was specifically recognizing YopH. As shown by the phase images in Figure 1A and C, the HeLa cells infected with either IP15/pVector or IP15/pYOPH were rounded up and partially detached due to the cytotoxic activity of YopE. Therefore, we also carried out infections with a *yopEyopH* double mutant complemented with the *yopH*$^{+}$ gene (IP17/pYOPH) to examine the localization of YopH in the absence of YopE-mediated actin stress fiber disruption. In HeLa cells infected with IP17/pYOPH, some cytotoxicity was still evident, although the cells were significantly less rounded (Figure 2A). YopH also localized to the cytoplasmic compartment, but the RAY51 labeling was distributed more diffusely (Figure 2A). The *yopEyopH* mutant was used in certain experiments described below in order to prevent the extreme disruption of cell morphology that was associated with the activity of YopE.

When the *yopHC403S* gene was introduced in *trans* into the *yopEyopH* mutant (IP17/pYOPHC403S), a very different localization pattern was seen in infected HeLa cells. In this case, YopHC403S was found in punctate structures that co-localized with phosphotyrosine (Figure 2C and D, arrows). A similar localization pattern was evident after a 1 h infection, although the signal was

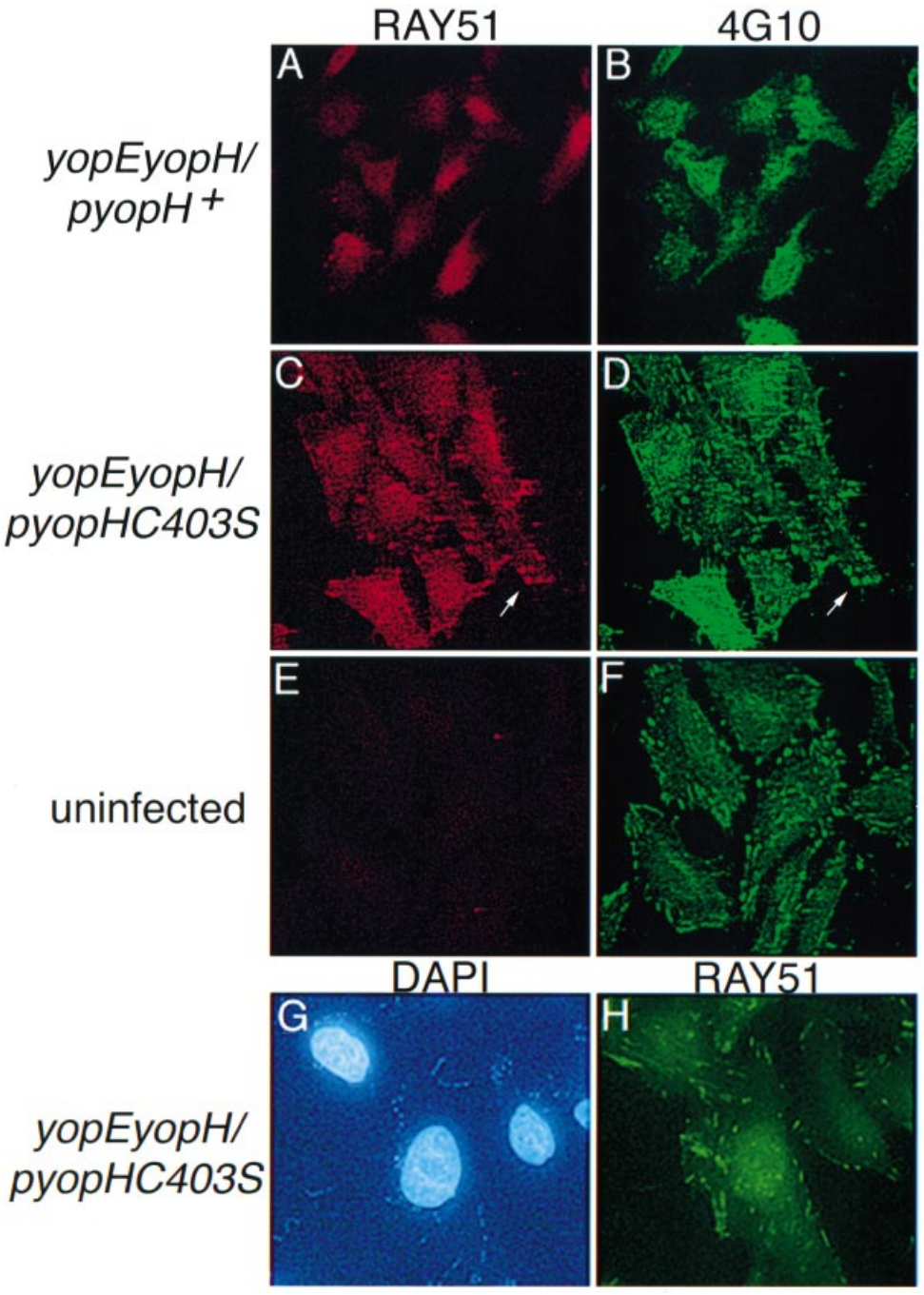

Fig. 2. YopHC403S co-localizes with phosphotyrosine in focal adhesions in infected HeLa cells. HeLa cells infected for 2 h with IP17/pYOPH (**A** and **B**), IP17/pYOPHC403S (**C**, **D**, **G** and **H**) or left uninfected (**E** and **F**) were processed for immunofluorescence microscopy using RAY51 and mouse monoclonal anti-phosphotyrosine antibody (4G10) or DAPI (see Materials and methods). Corresponding rhodamine (A, C and E) or DAPI (G) and fluorescein (B, D, F and H) images are shown. (A–F) show confocal microscopic images. Images in (G) and (H) were obtained by epifluorescence microscopy.

weaker (data not shown). Two hour infections were used in subsequent experiments in order to maximize the detection of YopHC403S in these intracellular structures. Similar punctate structures were seen in uninfected HeLa cells labeled with 4G10 (Figure 2F). Analysis of optical sections demonstrated that these punctate structures were located at the basal surface of HeLa cells (data not shown). These structures were reminiscent of focal adhesions, which are multiprotein complexes that form at the interface between integrin receptors and actin filaments and are enriched in tyrosine-phosphorylated proteins (reviewed in Clark and Brugge, 1995). Parallel samples of IP17/pYOPHC403S-infected cells were processed for epifluorescence microscopy using 4′,6′-diamidino-2-phenylindole (DAPI; a probe for DNA) and RAY51 to determine if the infecting bacteria co-localized with YopHC403S. As shown in Figure 2, most of the punctate labeling of YopHC403S occurred in regions that were devoid of bacteria, although on occasion the signals did overlap (compare Figure 2G and H). Thus, the majority of YopHC403S was localizing in structures that were distant from the site of bacterial–host cell interaction. These

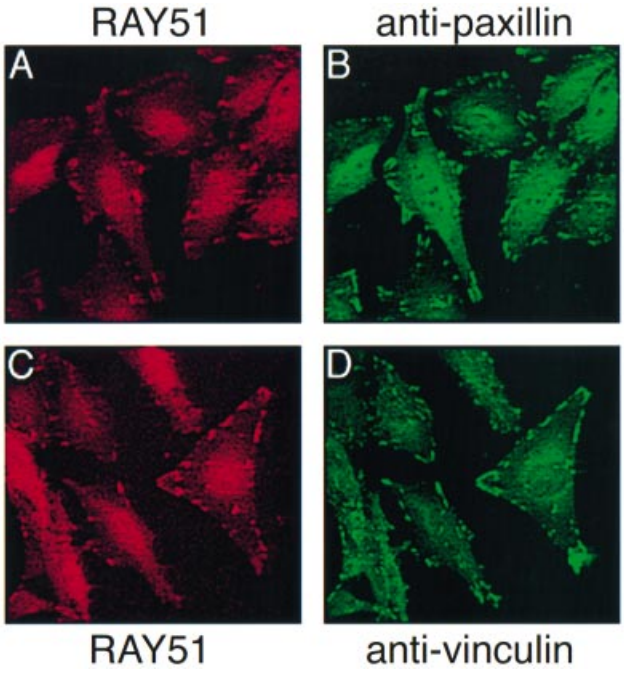

Fig. 3. Co-localization of YopHC403S with paxillin or vinculin in focal adhesions in infected HeLa cells. HeLa cells infected with IP17/pYOPHC403S for 2 h were processed for confocal immunofluorescence microscopy using RAY51 and mouse monoclonal anti-paxillin (**A** and **B**) or mouse monoclonal anti-vinculin (**C** and **D**) antibodies (see Materials and methods). Corresponding rhodamine (A and C) and fluorescein (B and D) images are shown.

data suggested that following translocation, YopHC403S localized to focal adhesions because these structures contained a major substrate of this enzyme. Consistent with the idea that focal adhesions contained a substrate of YopH was the observation that the labeling of these structures with 4G10 decreased in HeLa cells infected with IP17/pYOPH (compare Figure 2B and F). Interestingly, the labeling of cells infected with IP17/pYOPHC403S with 4G10 was 2- to 4-fold brighter than that of uninfected cells (compare Figure 2D and F), indicating that YopHC403S not only localized to focal adhesions but actually increased the level of phosphotyrosine in the cells. At this point, we were unable to determine if this resulted from an increase in the number of focal adhesions or an increase in phosphotyrosine in existing focal adhesions. One explanation for the latter possibility was that YopHC403S was binding to its substrate and protecting phosphotyrosyl residues in the substrate from the action of an endogenous PTPase. After fixation and permeabilization of the HeLa cells, these protected phosphotyrosyl residues were nevertheless accessible to the 4G10 antibody (see Discussion for a possible explanation).

To determine if YopHC403S was localizing to characteristic focal adhesions, we compared the localization pattern of YopHC403S with that of two well characterized focal adhesion proteins, paxillin and vinculin. Paxillin and vinculin concentrated in focal adhesions in uninfected cells as expected (data not shown). In HeLa cells infected with IP17/pYOPHC403S, YopHC403S co-localized with paxillin (Figure 3A and B) and vinculin (Figure 3C and D). These data confirmed that YopHC403S localized to characteristic focal adhesions. Using paxillin and vinculin as indicators of focal adhesions, we found that there were similar numbers of focal adhesions in uninfected cells and cells infected with IP17/pYOPHC403S (data not shown), which suggested that YopHC403S was not actually increasing the number of these structures in the cell.

YopHC403S has a dominant-negative effect on focal adhesion disruption mediated by YopE

When HeLa cells were infected with the *yopE*$^{+}$*yopH* strain IP15/pVector and examined by immunofluorescence microscopy using 4G10, anti-vinculin or anti-paxillin antibodies, no focal adhesions were observed, while focal adhesions were observed in IP17/pVector-infected cells (Figure 4A and E and data not shown). This result suggested that YopE had sufficient activity to disrupt focal adhesions, most likely through its ability to destabilize stress fibers (Rosqvist *et al.*, 1991). The loss of actin stress fibers in IP15/pVector-infected cells was confirmed by microscopic examination of the cells after staining with phalloidin (data not shown). Interestingly, when HeLa cells were infected with IP15/pYOPHC403S and examined by immunofluorescence microscopy using 4G10 and anti-vinculin antibodies, significant numbers of focal adhesion-like structures were detected (Figure 4C and G). The focal adhesions detected in IP15/pYOPHC403S-infected cells contained YopHC403S, as shown by the co-localization of RAY51 and 4G10 antibodies (Figure 4C and D) or RAY51 and anti-vinculin antibodies (Figure 4G and H). This result suggested that the interaction of YopHC403S with a substrate in focal adhesions had a dominant-negative effect on disassembly of these structures in response to destabilization of stress fibers. One interpretation of this finding was that dephosphorylation of the protein bound to YopHC403S was necessary for focal adhesion disassembly.

Translocation of YopHC403S into HeLa cells is associated with the hyperphosphorylation of a 125–135 kDa protein

As a first step toward identification of the focal adhesion protein interacting with YopHC403S, HeLa cells infected with IP17-derived strains were lysed in a relatively harsh detergent mixture (RIPA), separated into soluble and insoluble fractions, and samples of the resulting fractions (equivalent to 5×10^4 and 1×10^6 HeLa cells, respectively) were examined by immunoblotting with 4G10. In the RIPA-soluble fraction of HeLa cells, a broad band of hyperphosphorylated protein that migrated between 125 and 135 kDa was detected in cells infected with IP17/pYOPHC4O3S (Figure 5, lane 4), but not in uninfected cells or in cells infected with IP17/pVector or IP17/pYOPH (lanes 1–3, respectively). In addition, a broad band of hyperphosphorylated protein of similar mobility was present in the RIPA-insoluble fraction of HeLa cells infected with IP17/pYOPHC403S (Figure 5, lane 8, right side arrow). Similar results were obtained when the cells were lysed in the less solubilizing detergent Triton X-100 (data not shown). These results demonstrated that a protein (or proteins), hereafter designated p125-135, was hyperphosphorylated in the presence of YopHC403S. Taken together with the preferential localization of YopHC403S to focal adhesions, p125-135 was likely to

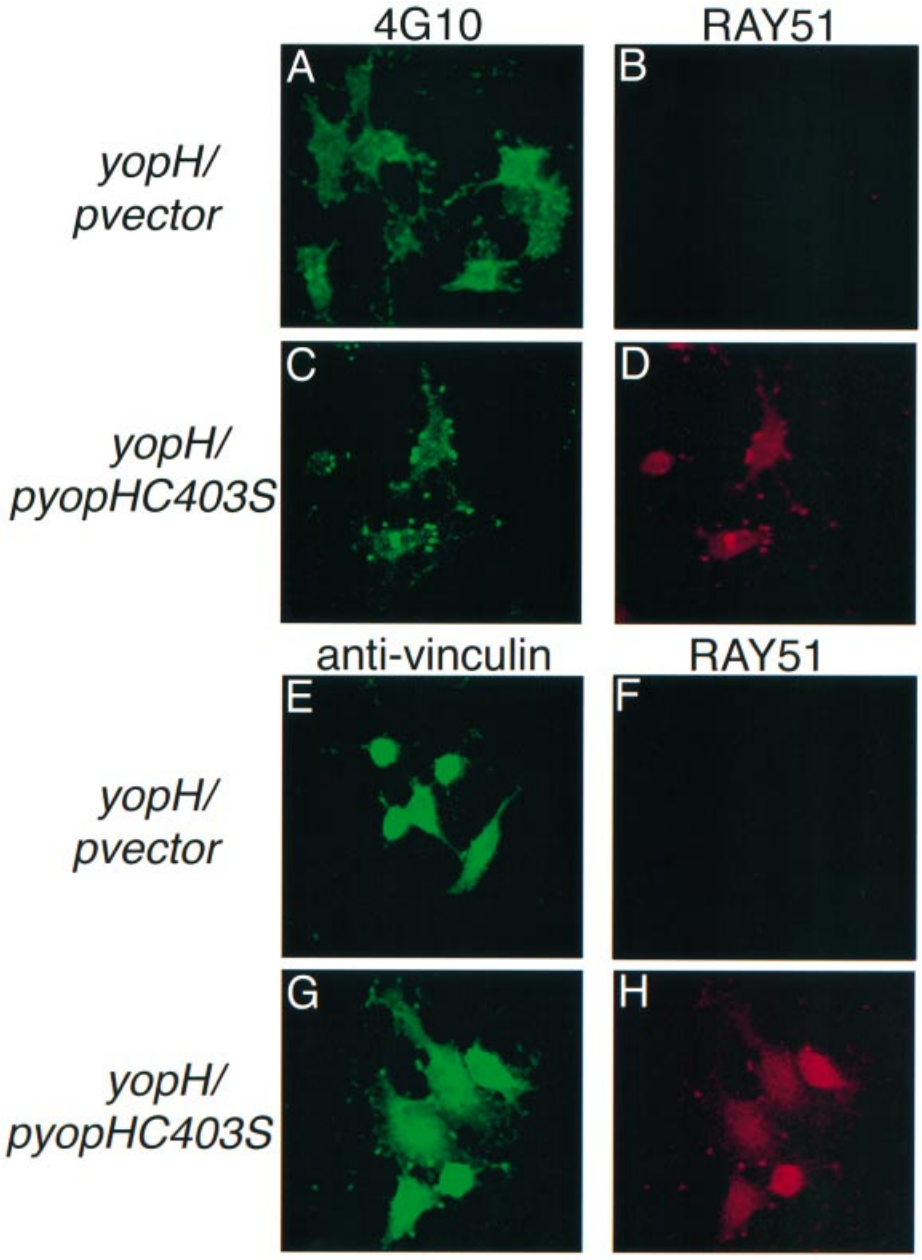

Fig. 4. YopHC403S has a dominant-negative effect on focal adhesion disruption by YopE. HeLa cells infected for 2 h with IP15/pVector or IP15/pYOPHC403S were processed for confocal immunofluorescence microscopy using 4G10 and RAY51 antibodies (**A**–**D**) or mouse monoclonal anti-vinculin and RAY51 antibodies (**E**–**H**) (see Materials and methods). Corresponding fluorescein (A, C, E and G) and rhodamine (B, D, F and H) images are shown.

be associated with these structures. The fact that p125-135 partitioned into a detergent-insoluble fraction of HeLa cells was consistent with this hypothesis, since this cell fraction is known to be enriched in cytoskeleton-associated proteins that are phosphorylated on tyrosine.

Infection of HeLa cells with IP17/pYOPH was associated with decreased phosphorylation of several proteins (Figure 5, lane 3), including a 125 kDa protein (p125) detected as a faint band in lysates of uninfected and IP17/pVector-infected cells (lanes 1 and 2, upper left arrow). Since this protein was dephosphorylated in a YopH-specific manner, it represented a potential candidate for the protein that became hyperphosphorylated in response to expression of YopHC403S. Many proteins, including two more prominent molecules in the 125–135 kDa range, were not dephosphorylated in IP17/pYOPH-infected cells (Figure 5, lane 3), demonstrating that YopH PTPase activity was directed to a subset of tyrosine-phosphorylated proteins in the cell. In addition to the 125 kDa substrate, a prominent protein that migrated as a broad 68 kDa band (p68) (Figure 5, lane 1, lower left arrow) was dephosphorylated in cells infected with IP17/pYOPH (lane 3). However, this protein was not hyperphosphorylated reproducibly in cells infected with IP17/pYOPHC403S (compare lanes 1 and 4). In addition, as shown in the next experiment, this 68 kDa protein did not co-precipitate

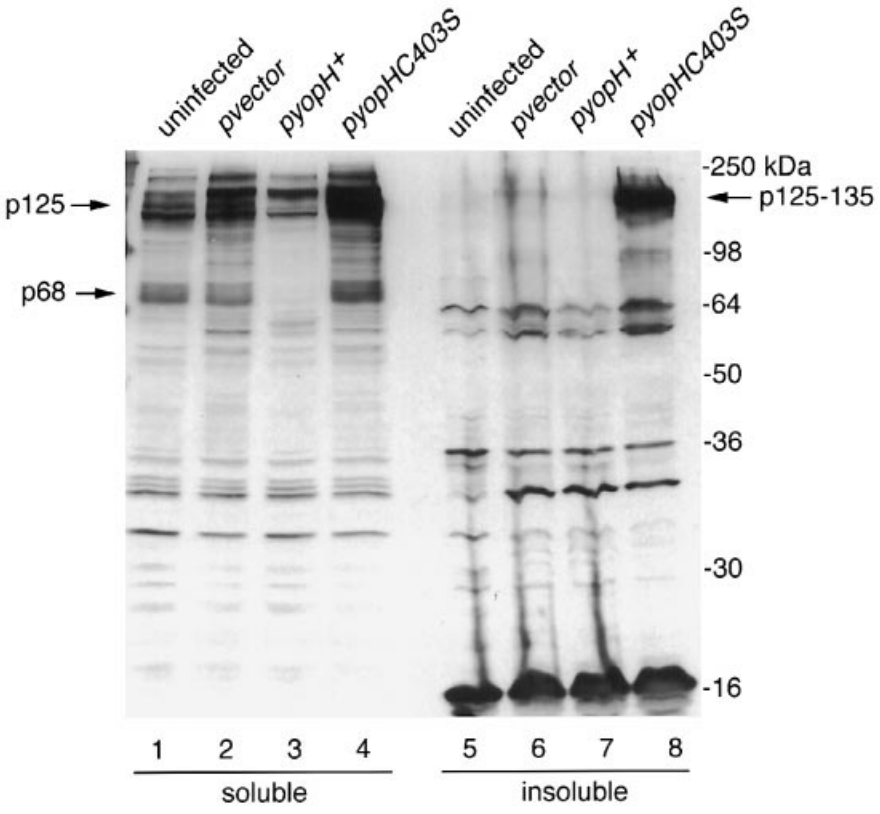

Fig. 5. A 125–135 kDa protein is hyperphosphorylated in response to translocation of YopHC403S into HeLa cells. Uninfected HeLa cells (lanes 1 and 5), or HeLa cells infected with IP17/pVector (lanes 2 and 6), IP17/pYOPH (lanes 3 and 7) or IP17/pYOPHC403S (lanes 4 and 8) were lysed in RIPA and separated into soluble (lanes 1–4) and insoluble (lanes 5–8) protein fractions (see Materials and methods). Soluble and insoluble protein fractions (equivalent to 5×10^4 and 1×10^6 HeLa cells, respectively) were separated on a 10% SDS–polyacrylamide gel and analyzed by immunoblotting with 4G10 antibody. The positions of pre-stained molecular weight standards and p125-135 (arrow) are shown on the right. The arrows on the left indicate the positions of major phosphoproteins (p125 and p68) that are dephosphorylated by YopH.

with YopHC403S (Figure 6). Although it was likely that the 68 kDa protein was dephosphorylated by YopH, it was also possible that it was dephosphorylated in an indirect fashion, for example by stimulation of endogenous PTPase activity during infection with IP17/pYOPH. Because of this uncertainty, we focused our attention on the protein that was hyperphosphorylated in the presence of YopHC403S since, based on previous studies (Herbst *et al.*, 1996; Plas *et al.*, 1996), this molecule was more likely to be a direct substrate of the PTPase.

A hyperphosphorylated 125–135 kDa protein that co-precipitates with YopHC403S is recognized by anti-Cas antibodies

To determine if p125-135 was physically associated with YopHC403S, lysates of infected HeLa cells prepared in Triton X-100 were subjected to immunoprecipitation with RAY51. The immunoprecipitates were analyzed by immunoblotting with 4G10. The results indicated that p125-135 was bound to YopHC403S, since p125-135 was largely depleted from cell lysates following immunodepletion of >94% of total cellular YopHC403S (Figure 6A, compare lanes 4 and 8), and a broad band of phosphorylated protein which migrated in the 125–135 kDa range co-precipitated with YopHC403S (lane 9, arrow). p125-135 specifically co-precipitated with YopHC403S, since it was not precipitated from lysates of uninfected cells (Figure 6A, lane 12), IP17/pVector-infected cells (lane 11) or IP17/pYOPH-infected cells (lane 10). Immunoblotting with RAY51 demonstrated that equivalent amounts of PTPase protein were recovered from IP17/pYOPHC403S- and IP17/pYOPH-infected cells (Figure 6B, lanes 9 and 10).

In an attempt to identify p125-135, immunoblots containing immunoprecipitated YopHC403S were probed with antibodies specific for the proteins focal adhesion kinase (Fak; Schaller *et al.*, 1992), vinculin (Sefton *et al.*, 1981), HEF-1 (Law *et al.*, 1996) and Cas (Sakai *et al.*, 1994), all of which are in the 125–135 kDa range and are known to be tyrosine phosphorylated. A monoclonal anti-Cas antibody specifically recognized a broad 125–135 kDa protein band that co-precipitated with YopHC403S (Figure 6C lane 9), while the other antibodies gave negative results (data not shown). A rabbit polyclonal antibody specific for Cas (Sakai *et al.*, 1994) also specifically recognized p125-135. These results suggested that the p125-135 protein associated with YopHC403S was Cas. Significantly, Cas recently has been shown to be phosphorylated on tyrosine in response to integrin engagement and to localize to focal adhesions (Nojima *et al.*, 1995; Petch *et al.*, 1995; Vuori and Ruoslahti, 1995; Harte *et al.*, 1996). Cas is detected as multiple species on immunoblots, with two distinct bands migrating at 115 and 125 kDa (CasA and B, respectively) (Figure 6C, lane 1) and a broad, diffuse band spreading between 125 and 135 kDa (CasC) (Sakai *et al.*, 1994). CasC is converted into CasA

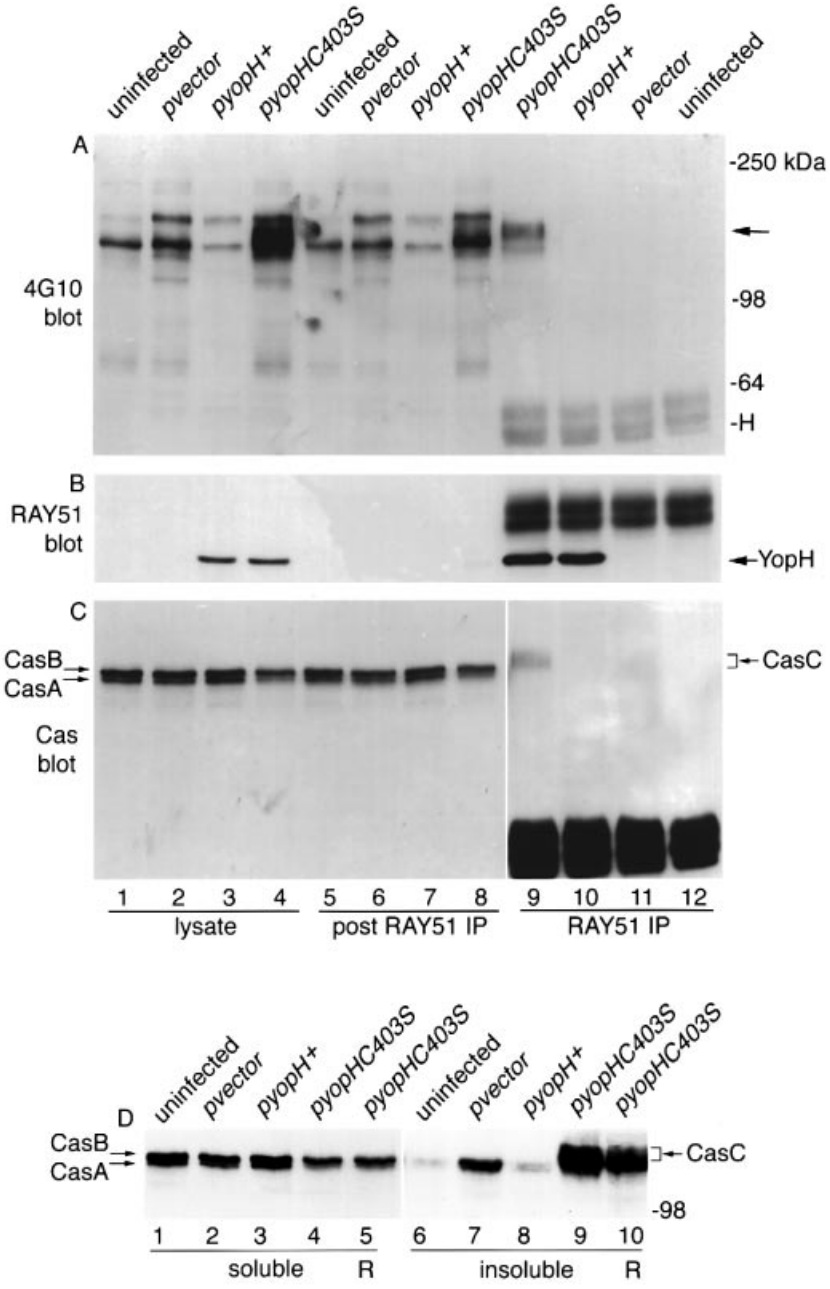

Fig. 6. A hyperphosphorylated protein that co-precipitates with YopHC403S and partitions to the insoluble fraction is recognized by a monoclonal anti-Cas antibody. (**A**–**C**) Uninfected HeLa cells (lanes 1, 5 and 12), or HeLa cells infected with IP17/pVector (lanes 2, 6 and 11), IP17/pYOPH (lanes 3, 7 and 10) or IP17/pYOPHC403S (lanes 4, 8 and 9) were lysed in Triton X-100, and the lysates were subjected to immunoprecipitation with RAY51 (see Materials and methods). Samples of lysates pre- and post-immunoprecipitation (lanes 1–4 and 5–8, respectively) and the immunoprecipitates (lanes 9–12) were separated on a 7.5% SDS–polyacrylamide gel and analyzed by immunoblotting with 4G10 (A), RAY51 (B) or monoclonal anti-Cas (C) antibodies. In (A), H corresponds to heavy chain of immunoprecipitation antibody (also visible in B and C). (**D**) Uninfected HeLa cells (lanes 1 and 6), or HeLa cells infected with IP17/pVector (lanes 2 and 7), IP17/pYOPH (lanes 3 and 8) or IP17/pYOPHC403S (lanes 4, 5, 9 and 10) were lysed in Triton X-100 (lanes 1–4 and 6–9) or RIPA (lanes 5 and 10) and separated into soluble and insoluble protein fractions (see Materials and methods). Soluble and insoluble protein fractions (equivalent to 2×10^4 and 1×10^6 HeLa cells, respectively) were separated on a 7.5% SDS–polyacrylamide gel and analyzed by immunoblotting with monoclonal anti-Cas antibody.

and B by phosphatase treatment and appears to be heavily phosphorylated on tyrosine, which may account for its diffuse gel mobility and poor reactivity with antibodies (Sakai *et al.*, 1994). The phosphorylated protein that co-precipitated with YopHC403S appeared to correspond to the slower migrating CasC (Figure 6C, lane 9), suggesting that YopHC403S was binding specifically to the tyrosine-phosphorylated form of Cas.

Translocation of YopHC403S into HeLa cells is associated with increased detergent insolubility of Cas

Infection of HeLa cells with IP17/pYOPHC403S was associated with the appearance of a 125–135 kDa tyrosine-phosphorylated protein in the RIPA-insoluble cell fraction (Figure 5, lane 8). We wanted to determine if this protein was Cas and whether its appearance in the RIPA-insoluble

fraction was due to increased tyrosine phosphorylation of insoluble protein or a net increase in the amount of insoluble protein. For this purpose, infected HeLa cells were lysed in Triton X-100, separated into soluble and insoluble fractions, and samples of the resulting fractions (equivalent to 2×10^4 and 1×10^6 HeLa cells, respectively) were analyzed by immunoblotting with the monoclonal anti-Cas antibody. RIPA-soluble and RIPA-insoluble fractions of IP17/pYOPHC403S-infected cells were also analyzed to allow for a comparison to the data shown in Figure 5. As shown in Figure 6D, there was significantly increased detection of Cas in the insoluble fractions of IP17/pYOPHC403S-infected cells relative to uninfected cells (Figure 6D, compare lanes 6, 9 and 10). Quantitation by densitometry indicated that the insoluble Cas in IP17/pYOPHC403S-infected cells represented only a small fraction (~1%) of total cellular Cas, although much of the insoluble Cas appeared to correspond to CasC. There was also a small increase in insoluble Cas in IP17/pVector-infected cells that was not seen in IP17/pYOPH-infected cells (Figure 6D, compare lanes 7 and 8), indicating that YopH activity was causing solubilization of Cas. Taken together, these results were consistent with previous data showing increased partitioning of Cas into a particulate cell fraction in response to tyrosine phosphorylation (Sakai *et al.*, 1994).

Cas is hyperphosphorylated in the presence of YopHC403S and dephosphorylated in the presence of YopH

To confirm the identity of the YopHC403S-associated p125-135 protein as Cas, a rabbit polyclonal antibody was used to immunoprecipitate Cas from lysates of infected HeLa cells, and the resulting immunoprecipitates were analyzed by immunoblotting with 4G10. As shown in Figure 7A, p125-135 was largely depleted from cell lysates after immune depletion of Cas (compare lanes 4 and 8), showing that Cas was a major component of p125-135. The tyrosine-phosphorylated molecule migrating just below p125-135 that was not depleted (Figure 7A, lane 8) corresponds to a distinct protein. Analysis of the anti-Cas immunoprecipitates indicated that Cas was phosphorylated at a low level in cells infected with IP17/pVector and in uninfected cells (Figure 7A, lanes 11 and 12). Phosphorylation of Cas increased significantly in cells infected with IP17/pYOPHC403S (Figure 7A lane 9) and diminished to nearly undetectable levels in cells infected with IP17/pYOPH (lane 10). Immunoblotting with anti-Cas antibody served as the control for loading (Figure 7B, lanes 9–12). Thus, translocation of YopHC403S into HeLa cells was associated with hyperphosphorylation of Cas, while translocation of YopH was associated with dephosphorylation of Cas.

We had already shown that Cas co-precipitated with YopHC403S (Figure 6). To strengthen the argument that YopHC403S and Cas were physically associated, we wanted to show that these proteins co-precipitated in the reciprocal fashion. This was demonstrated by probing the immunoblot containing anti-Cas immunoprecipitates with RAY51. As shown in Figure 7C, YopHC403S co-precipitated with Cas (lane 9), confirming that these two proteins were interacting physically. YopH did not co-precipitate with Cas (Figure 7C, lane 10), again suggesting that the interaction of these two proteins requires tyrosine phosphorylation of Cas.

Cas is dephosphorylated rapidly and selectively by YopH

Cas and Fak are tyrosine phosphorylated with similar kinetics in response to cell adhesion on fibronectin, with maximal phosphorylation occurring ~80 min after plating (Nojima *et al.*, 1995). To determine if YopH was capable of selectively reversing the phosphorylation of Cas after cell adhesion on fibronectin, HeLa cells were plated on fibronectin for 60 min and then infected with IP17/pYOPH for 15, 30 or 60 min. Cas was immunoprecipitated from RIPA lysates with a rabbit polyclonal antibody and analyzed by immunoblotting with 4G10. As a control for the selectivity of YopH, Fak was immunoprecipitated with rabbit polyclonal antibody and analyzed in the same fashion. Duplicate samples were also analyzed by immunoblotting with anti-Cas and anti-Fak antibodies to confirm that approximately equal amounts of each protein were recovered in the immunoprecipitations (Figure 7D, lower panel) As shown in the upper panel of Figure 7D, Cas and Fak were phosphorylated in cells adherent to fibronectin for 60 min (lanes 2 and 7), but not in cells held in suspension for 60 min (lanes 1 and 6). Dephosphorylation of Cas was detected as early as 30 min after infection with IP17/pYOPH, and by 60 min Cas was completely dephosphorylated (lanes 3–5). In contrast, the phosphorylation state of Fak was essentially unchanged (lanes 8–10), except that a small decrease in phosphorylation was apparent at the 60 min time point when the bands were scanned by laser densitometry and normalized for the amount of immunoprecipitated Fak. These results showed that translocation of YopH into HeLa cells was associated with rapid dephosphorylation of Cas molecules that were phosphorylated in response to cell adhesion on fibronectin and that Cas was dephosphorylated preferentially as compared with Fak. When HeLa cells were infected for different lengths of time with IP17/pYOPH and processed for immunofluorescence microscopy using 4G10, we observed that focal adhesions were dephosphorylated within 30 min of infection (data not shown). Thus, the time course of focal adhesion dephosphorylation closely paralleled the kinetics of Cas dephosphorylation.

To determine the level of Fak dephosphorylation after a 2 h infection such as that used in Figure 7A, we immunoprecipitated Fak from HeLa cell lysates and analyzed the resulting complexes by immunoblotting with 4G10 and anti-Fak antibodies. Levels of Fak tyrosine phosphorylation were quantitated by laser densitometry and normalized for the amount of immunoprecipitated Fak. After a 2 h infection with IP17/pYOPH, the level of Fak tyrosine phosphorylation was reduced to one-third the level seen in uninfected cells. As shown in Figure 7D, Cas was dephosphorylated completely after only 1 h of infection (lane 5). These results confirmed that Fak was dephosphorylated much less efficiently as compared with Cas. The selectivity of YopH for Cas over Fak was particularly striking since Fak binds directly to Cas through an SH3 domain-mediated interaction (Polte and Hanks, 1995; Burnham *et al.*, 1996; Harte *et al.*, 1996). When anti-Fak immunoprecipitates from IP17/pYOPHC403S-infected cells were analyzed by immunoblotting with anti-

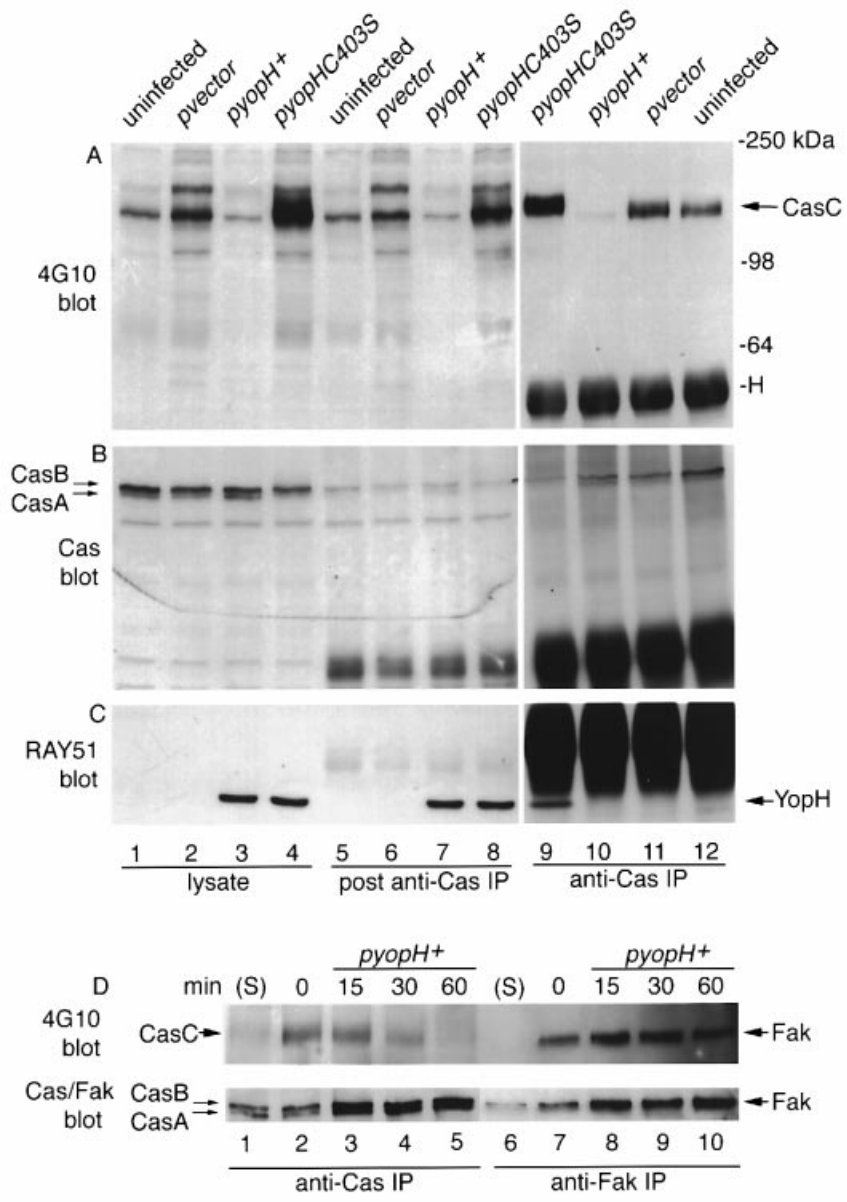

Fig. 7. Cas is hyperphosphorylated by YopHC403S and dephosphorylated by YopH. (**A**–**C**) Uninfected HeLa cells (lanes 1, 5 and 12), or HeLa cells infected with IP17/pVector (lanes 2, 6 and 11), IP17/pYOPH (lanes 3, 7 and 10) or IP17/pYOPHC403S (lanes 4, 8 and 9) were lysed in Triton X-100, and the lysates were subjected to immunoprecipitation with rabbit polyclonal anti-Cas antibody (see Materials and methods). Samples of lysates pre- and post-immunoprecipitation (lanes 1–4 and 5–8, respectively) and the immunoprecipitates (lanes 9–12) were separated on a 7.5% SDS–polyacrylamide gel and analyzed by immunoblotting with 4G10 (A), rabbit polyclonal anti-Cas (B) or RAY51 (C) antibodies. All samples were run on the same gel, except for those shown in (C), lanes 9–12, which were separated on a gel containing 8% acrylamide and 0.21% bis-acrylamide to increase the separation between YopHC403S and heavy chain. (**D**) HeLa cells were held in suspension or plated on fibronectin-coated dishes for 60 min. At this time, the suspended cells (lanes 1 and 6) and one dish of plated cells (lanes 2 and 7) were lysed in RIPA. Bacteria (IP17/pYOPH) were added to the remaining dishes at an m.o.i. of 50:1. The infected cells were lysed in RIPA at the indicated times (lanes 3–5 and 8–10). Lysates were divided and subjected to immunoprecipitation with rabbit polyclonal anti-Cas (lanes 1–5) or anti-Fak (lanes 6–10). Immunoprecipitates were analyzed by immunoblotting with 4G10, anti-Cas or anti-Fak antibodies.

Cas or RAY51 antibodies, Cas and YopHC403S were detected (data not shown). This result suggested that Fak was capable of binding to Cas even when it was complexed with YopHC403S. Cas also co-precipitated with Fak in lysates of IP17/pYOPH-infected cells (data not shown), which is consistent with the idea that interaction between Cas and Fak does not require tyrosine phosphorylation (Polte and Hanks, 1995; Burnham *et al.*, 1996; Harte *et al.*, 1996). Our inability to detect Fak co-precipitating with YopHC403S in earlier experiments appeared to be due to a technical limitation in that the anti-Fak antibody we used was not suitable for detecting very small amounts of Fak by immunoblotting.

Translocation of YopHC403S into HeLa cells is associated with recruitment of Cas to focal adhesions

Cas has been shown to concentrate primarily in focal adhesions (Harte *et al.*, 1996; Law *et al.*, 1996), although significant labeling of stress fibers and nuclear or cytoplasmic compartments with anti-Cas antibodies has been reported (Petch *et al.*, 1995; Law *et al.*, 1996). These

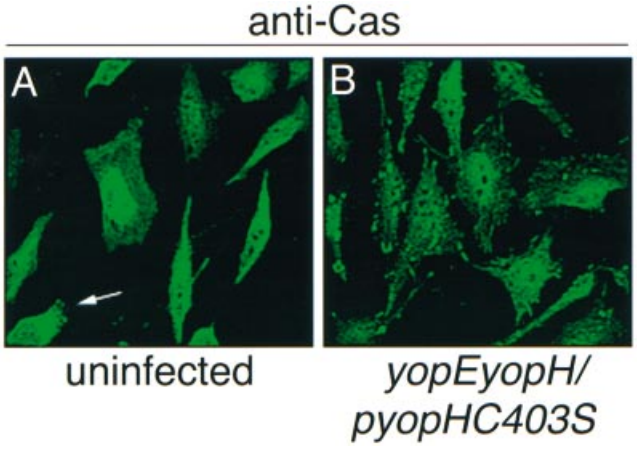

Fig. 8. Expression of YopHC403S is associated with increased localization of Cas to focal adhesions. Uninfected HeLa cells (**A**) or HeLa cells infected with IP17/pYOPHC403S (**B**) were processed for confocal immunofluorescence microscopy using rabbit polyclonal anti-Cas antibody. Fluorescein images are shown.

observations suggest that the association of Cas with focal adhesions is regulated, possibly by tyrosine phosphorylation. To determine if translocation of YopHC403S into HeLa cells was associated with recruitment of Cas into focal adhesions, uninfected HeLa cells and HeLa cells infected with IP17/pYOPHC403S were prepared for confocal immunofluorescence microscopy using a polyclonal rabbit anti-Cas antibody (the monoclonal anti-Cas antibody was found to be unsuitable for detecting Cas in this assay). As shown in Figure 8A, Cas localized primarily to the cytoplasmic compartment of uninfected HeLa cells, although there was also detectable labeling of Cas in focal adhesions (arrow). In comparison, the labeling of Cas in focal adhesions was significantly increased in cells infected with IP17/pYOPHC403S (Figure 8B). Cas was recruited to characteristic focal adhesions by YopHC403S, since it co-localized with paxillin and vinculin (data not shown). These results demonstrated that additional amounts of Cas were recruited into focal adhesions by YopHC403S, further strengthening the argument that these two proteins directly interact *in vivo*. Cas was also found to co-localize with phosphotyrosine, vinculin and paxillin in the focal adhesion-like structures we detected in IP15/pYOPHC403S-infected cells (Figure 4 and data not shown).

YopHC403S binds directly to Cas in a phosphotyrosine-dependent manner

To provide conclusive evidence that Cas is a direct substrate of YopH, we utilized an overlay assay to show that YopHC403S was capable of binding directly to Cas in a phosphotyrosine-dependent manner. Lysates prepared from HeLa cells infected with IP17/pYOPHC403S were subjected to immunoprecipitation with rabbit polyclonal anti-Cas antibody. In parallel, Fak was immunoprecipitated with rabbit polyclonal antibody as a control. The immunoprecipitated proteins were subjected to SDS–PAGE, transferred to membranes and probed with one of two purified GST fusion proteins: either GST fused to YopHC403S (GST–YopHC403S) or GST fused to the SH2 domain of Crk (GST–CrkSH2). The latter protein has been shown to bind to Cas in an overlay assay (Vuori *et al.*, 1996). After washing, binding of the fusion proteins to the membrane was detected by immunoblotting with anti-GST antibody. As shown in Figure 9A, GST–YopHC403S and GST–CrkSH2 bound to a region of the membrane corresponding to CasC (lanes 3 and 5), while GST–YopHC403S did not bind to the control lane containing immunoprecipitated Fak (lane 4). The positions of Cas and Fak on the membrane were verified by reprobing with anti-Cas (lanes 1 and 2), 4G10 (lanes 6 and 7) or anti-Fak antibodies (lanes 8 and 9). Since YopH contains a proline-rich region that potentially could bind to the SH3 domain of Cas *in vitro*, we wanted to determine if the binding of GST–YopHC403S to Cas was dependent on tyrosine phosphorylation. For this purpose, the experiment was repeated, except in this case immunoprecipitated Cas was divided, both halves were boiled to denature the complexes and one half was incubated with purified YopH to dephosphorylate Cas *in vitro*. The samples were then analyzed in the overlay assay. As shown in Figure 9B, GST–YopHC403S did not bind to Cas that was dephosphorylated by YopH *in vitro* (lane 4). Dephosphorylation of Cas by YopH was verified by reprobing the membrane with anti-Cas (lanes 1 and 2) or 4G10 (lanes 5 and 6) antibodies. These results demonstrated that YopHC403S bound directly to Cas in a phosphotyrosine-dependent manner and confirmed that Cas was recognized as a substrate by YopH *in vitro*.

Discussion

Recent studies have demonstrated that several bacterial pathogens are capable of translocating virulence proteins directly into the cytoplasmic compartment of mammalian cells (Rosqvist *et al.*, 1995). However, little or nothing is known about the eukaryotic targets of these translocated virulence proteins. Here we have identified Cas as a direct target of YopH, a bacterial PTPase that is translocated into mammalian cells by pathogenic *Yersinia* species.

Our strategy to identify a direct substrate of YopH was based on the observation that catalytically inactive PTPases form stable complexes with substrates and, in some cases, promote substrate hyperphosphorylation (Bliska *et al.*, 1992; Milarski *et al.*, 1993; Sun *et al.*, 1993; Herbst *et al.*, 1996; Plas *et al.*, 1996). In an extension of this observation, we used YopHC403S as a substrate-specific probe to determine the location of target molecules in host cells infected with *Y.pseudotuberculosis*. Confocal immunofluorescence microscopy demonstrated that YopHC403S co-localized with tyrosine-phosphorylated proteins in focal adhesions, sites where integrin receptors serve as a transmembrane bridge between extracellular matrix proteins and intracellular signaling proteins. This result suggested that YopHC403S was recognizing a substrate in focal adhesions. Confocal immunofluorescence microscopy also demonstrated that focal adhesions labeled 2- to 4-fold more brightly with anti-phosphotyrosine antibodies in cells containing YopHC403S as compared with control cells. These results suggested that YopHC403S was stabilizing focal adhesions by directly binding to a substrate and protecting it from the action of an endogenous PTPase that is present in focal adhesions, such as LAR (Serra-Pages *et al.*, 1995) or PTP-1D (Miyamoto *et al.*, 1995).

Anti-phosphotyrosine immunoblotting demonstrated

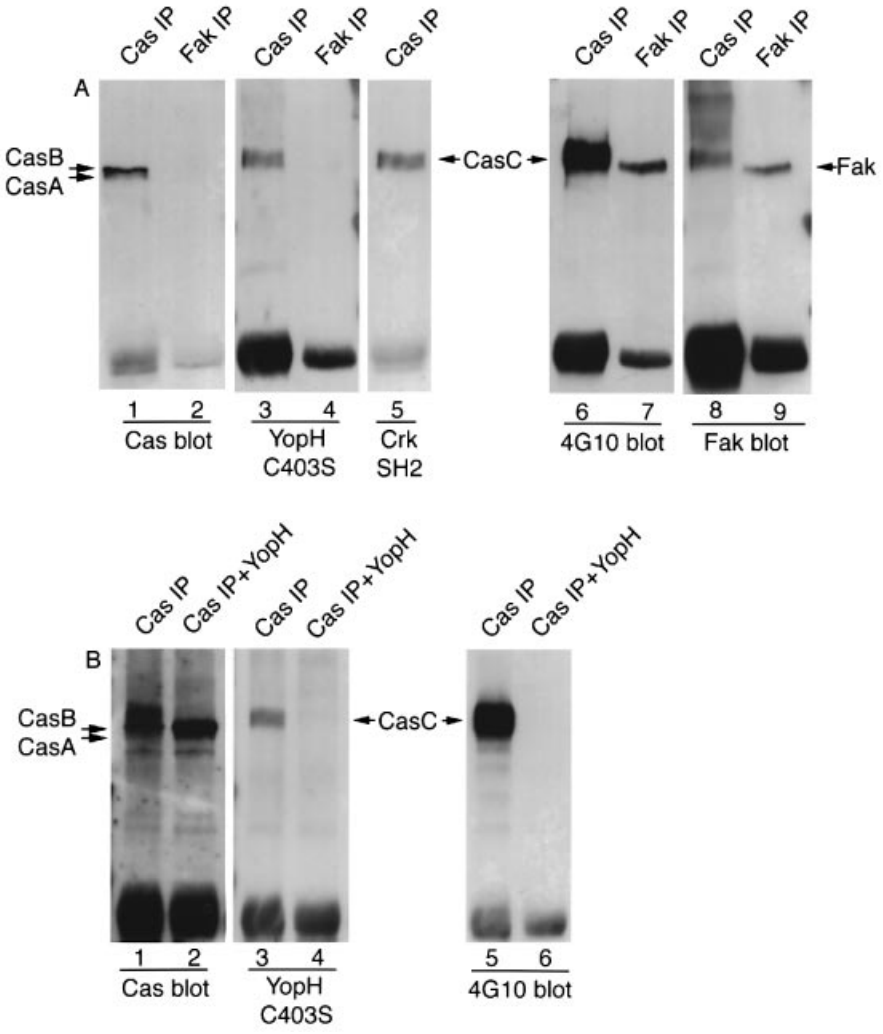

Fig. 9. YopHC403S binds directly to Cas in a phosphotyrosine-dependent manner. (**A**) Rabbit polyclonal antibodies were used to immunoprecipitate Cas (Cas IP) or Fak (Fak IP) from lysates of IP17/pYOPHC403S-infected cells. The immunoprecipitated proteins were subjected to SDS–PAGE and transferred to Immobilon membranes. Membranes were probed with purified GST–YopHC403S (lanes 3 and 4) or GST–CrkSH2 (lane 5). Binding of the fusion protein was detected by immunoblotting with monoclonal anti-GST antibody. Subsequently, the membrane was stripped and subjected to immunoblotting with anti-Cas (lanes 1 and 2), 4G10 (lanes 6 and 7) or anti-Fak (lanes 8 and 9) antibodies. (**B**) Immunoprecipitated Cas was divided, boiled and one half was treated with purified YopH (Cas IP+YopH) to dephosphorylate Cas *in vitro*. After SDS–PAGE and transfer to Immobilon, membranes were analyzed as above by probing with GST–YopHC403S (lanes 3 and 4), anti-Cas (lanes 1 and 2) or 4G10 antibodies (lanes 5 and 6).

that translocation of YopHC403S into HeLa cells was associated with the hyperphosphorylation of a diffusely migrating protein in the 125 to 135 kDa range (p125-135). Numerous lines of evidence indicate that p125-135 is identical to Cas. First, p125-135 was recognized specifically by two different anti-Cas antibodies, one monoclonal and one polyclonal. p125-135 is not a Cas-related protein, since an antibody that recognizes HEF-1 (Law *et al.*, 1996) failed to react, and Efs/Sin which is ~79 kDa (Ishino *et al.*, 1995; Alexandropoulos and Baltimore, 1996) could be ruled out. Second, p125-135 was depleted from cell lysates following immunoprecipitation of Cas. Third, immunoblotting demonstrated that Cas was hyperphosphorylated in the presence of YopHC403S and that Cas and YopHC403S co-precipitated with each other in a reciprocal fashion. Fourth, translocation of YopHC403S into HeLa cells was associated with increased detergent insolubility of Cas and increased localization of Cas to focal adhesions. Fifth, Cas was dephosphorylated rapidly and selectively when YopH was translocated into HeLa cells plated on fibronectin. Sixth, YopHC403S bound directly to Cas in a phosphotyrosine-dependent manner in an overlay assay, and Cas was recognized as a substrate by YopH *in vitro*. All of these data are entirely consistent with the idea that Cas is a direct and preferential target of YopH *in vivo*. However, there may be additional proteins that are preferential targets of YopH *in vivo*. For example, we detected a 68 kDa protein that was dephosphorylated in a YopH-specific fashion. In addition, Fak was partially dephosphorylated by YopH but with much slower kinetics as compared with Cas and only after significant levels of YopH had accumulated in the cell. Taken together, our results suggest that Cas is a preferential target of YopH, but once Cas is completely dephosphorylated and YopH accumulates to a high level in the cell, other proteins in the vicinity of Cas, such as Fak, may be subject to dephosphorylation. Our results also do not eliminate the possibility that other proteins are important for the interaction of YopHC403S or YopH with Cas *in vivo*, or

that these proteins interact outside of focal adhesions. However, we favor a model in which YopHC403S or YopH is recruited to focal adhesions by the presence of a small amount of tyrosine-phosphorylated Cas. Cas may become hyperphosphorylated in the presence of YopHC403S because sites of tyrosine phosphorylation are bound to the inactive PTPase domain. Sites of tyrosine phosphorylation in Cas that are not bound directly to YopHC403S could also be protected if the binding of YopHC403S locks Cas in a conformation that is no longer recognized by an endogenous PTPase. This is one possible explanation for why there was increased staining of focal adhesions with the 4G10 antibody after fixation and permeabilization of the cells containing YopHC403S (Figure 2D). An alternative possibility is that the interaction of YopHC403S and Cas promoted the accumulation of other tyrosine-phosphorylated proteins (other than vinculin or paxillin) to focal adhesions. The accumulation of Cas in focal adhesions in the presence of YopHC403S (Figure 8) could be explained if tyrosine phosphorylation of Cas stabilizes its interactions with other focal adhesion proteins.

Cas contains multiple Src homology 2-binding motifs that conform to an optimal substrate recognition sequence for YopH

Cas contains a single N-terminal SH3 domain followed by a 'substrate region' which contains 15 tyrosine phosphorylation motifs (YXXP) that are predicted to interact with the SH2 domains of several proteins, including Crk and tensin (Sakai *et al.*, 1994; Polte and Hanks, 1995). The Cas SH3 domain recently has been shown to bind to a proline-rich region of Fak (Polte and Hanks, 1995; Burnham *et al.*, 1996; Harte *et al.*, 1996). The C-terminus of Cas also contains binding sites for the SH2 and SH3 domains of Src (Burnham *et al.*, 1996; Nakamoto *et al.*, 1996). The presence of an SH3 domain and multiple SH2- and SH3-binding motifs in Cas suggests that it functions as a docking site for a large number of signaling proteins (Sakai *et al.*, 1994). Interestingly, most of the SH2-binding motifs in the substrate region of Cas conform to an optimal substrate recognition sequence for YopH which was derived from the analysis of phosphopeptide substrates (Zhang *et al.*, 1993). The presence of a proline at position +3 and acidic residues at positions –2 or –1 relative to the phosphotyrosyl residue was shown to be critical for high affinity binding and catalysis of phosphopeptides by YopH (Zhang *et al.*, 1993). All 15 SH2-binding motifs in the substrate region of Cas contain a proline at position +3 (Sakai *et al.*, 1994) (Table I). Ten of these also contain an acidic residue in the –2 position, and one contains acidic residues in the –2 and –1 positions. Thus, the vast majority of the Crk SH2-binding motifs in Cas correspond to sequences that would be recognized with high affinity by YopH and YopHC403S. This idea is consistent with the observation that Cas was converted into a diffusely migrating protein band in the presence of YopHC403S, suggesting that multiple tyrosine residues in the substrate region of Cas were being protected from dephosphorylation. The extremely high catalytic activity of YopH (Zhang *et al.*, 1992) may reflect the fact that its natural substrate is potentially phosphorylated at as many as 15 residues.

Table I. Multiple sites of tyrosine phosphorylation in the substrate region of Cas conform to the optimal phosphopeptide recognition motifs for YopH and the SH2 domain of Crk

Protein	(residue[a])	Sequence	Reference
YopH[b]		**DE**pYXX**P**	Zhang *et al.* (1993)
CrkSH2[c]		XXpYDX**P**	Birge *et al.* (1993)
Cas[d]	213	NVpYLV**P**	Sakai *et al.* (1994)
	226	GLpYQA**P**	
	263	**D**LpYQV**P**	
	277	**D**IpYQV**P**	
	290	**D**IpYQV**P**	
	332	**DE**pYDT**P**	
	347	**D**IpYDV**P**	
	365	**E**VpYDT**P**	
	385	**D**VpYDV**P**	
	404	SVpYDV**P**	
	425	**E**TpYDV**P**	
	460	**D**VpYDV**P**	
	470	**D**LpYDV**P**	
	485	TLpYDV**P**	
	508	GVpYAV**P**	

[a]The position of the indicated tyrosine residue within the sequence of Cas.
[b]The optimal substrate recognition for YopH was determined by alanine-scanning mutagenesis of a synthetic phosphopeptide based on the sequence of the epidermal growth factor receptor.
[c]The optimal binding motif for the SH2 domain of Crk was based on affinity purification of a partially degenerate phosphopeptide library.
[d]Potential sites of tyrosine phosphorylation based on the sequence of the substrate region of Cas.

A model for inhibition of Cas function by YopH

Cas is rapidly tyrosine phosphorylated in response to β1 integrin-mediated cell adhesion and is localized to focal adhesions, suggesting that it functions during integrin-mediated signal transduction (Nojima *et al.*, 1995; Petch *et al.*, 1995; Vuori and Ruoslahti, 1995; Harte *et al.*, 1996; Vuori *et al.*, 1996). Both Src and Fak, which localize to focal adhesions and associate with Cas, appear to be important for phosphorylation of Cas in response to integrin ligation (Kanner *et al.*, 1991; Sakai *et al.*, 1994; Clark and Brugge, 1995; Polte and Hanks, 1995; Harte *et al.*, 1996). Studies using fibroblasts deficient in either Src or Fak suggest that while Src is required for phosphorylation of Cas, Fak is not required, but may function to recruit Src kinases into the vicinity of Cas (Vuori *et al.*, 1996). It is likely that additional proteins with SH2 domains, such as Crk and tensin, are recruited into a signaling complex in response to tyrosine phosphorylation of Cas (Polte and Hanks, 1995; Vuori *et al.*, 1996). After docking on Cas, these molecules may activate a variety of signaling pathways important for regulating growth, differentiation or cytoskeletal anchoring. Dephosphorylation of Cas by YopH is likely to prevent the recruitment of these signaling proteins into focal adhesion complexes, thereby blocking the subsequent steps required for signal transduction or focal adhesion assembly. In this context, the possibility that actin-binding proteins such as tensin interact with Cas is particularly intriguing, since interference with tensin binding to Cas may disrupt a critical contact between actin microfilaments and focal adhesions. This may explain the ability of YopH to promote cell detachment from the extracellular matrix (Bliska *et al.*, 1993a), since interactions between focal adhesions and actin stress fibers are thought to play an important role in

modulating cell adhesion and regulating cell shape changes (Clark and Brugge, 1995). Dephosphorylation of Cas by YopH (or an endogenous PTPase) may be required for focal adhesion disassembly. The fact that the interaction between YopHC403S and Cas had a dominant-negative effect on YopE in this context is consistent with the idea that dephosphorylation of Cas is necessary for focal adhesion disassembly. This finding also underscores the idea that YopH and YopE probably function in a synergistic fashion to disengage the cytoskeleton of the host cell from the extracellular matrix. YopH probably functions first to immediately sever the contact between focal adhesions and actin filaments, while YopE functions subsequently to destabilize actin filaments and disassemble focal adhesions.

Although the pathological significance of this process remains to be determined, YopH-mediated dephosphorylation of Cas could be of particular importance during the interaction of *Yersiniae* with leukocytes. For example, during the inflammatory response, integrin-mediated adhesion of phagocytes to endothelia or extracellular matrix proteins plays a critical role in a number of processes, including endothelial transmigration, the release of oxidants and proteases and the synthesis of pro-inflammatory cytokines (Juliano and Haskill, 1993; Lin *et al.*, 1994). While uptake mediated by β_1 integrins is not associated with killing of bacteria by phagocytes, ingestion of complement-coated microorganisms triggers an important antibacterial pathway in macrophages and neutrophils. YopH has been shown to inhibit internalization of *Y.enterocolitica* into neutrophils via the complement receptor $\alpha_m\beta_2$ (Ruckdeschel *et al.*, 1996), suggesting that Cas may be important for signaling through β_2 integrin receptors. Consistent with this idea is the recent demonstration that adhesion of lymphocytes mediated by binding of $\alpha_L\beta_2$ (LFA-1) to ICAM-1 induces tyrosine phosphorylation of Cas (Petruzzelli *et al.*, 1996). In the future, it will be important to determine the role of Cas in focal adhesion assembly/disassembly and signaling and to identify Cas-dependent signaling pathways that are inhibited by YopH in different cell types.

Materials and methods

Cell culture

HeLa cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL) and 1 mM sodium pyruvate in a 5% CO_2 humidified incubator at 37°C.

Y.pseudotuberculosis strains

The *Y.pseudotuberculosis* strains used are derived from IP2666(pIB1) which harbors a wild-type virulence plasmid (Bliska *et al.*, 1991). IP15 (*yopH*) and IP17 (*yopHyopE*) harbor virulence plasmids with deletions in the indicated genes. The expression plasmids pYOPH and pYOPHC403S were derived from pMMB67EH and produce native levels of protein under the conditions used (Bliska and Black, 1995). pMMB67EH (referred to in the text and figure legends as pVector), pYOPH and pYOPHC403S were introduced into IP15 and IP17 by conjugation (Bliska and Black, 1995). For infection assays, bacteria were grown overnight at 26°C with shaking in Luria broth containing 100 μg/ml of ampicillin (LB-Amp). Bacteria were subcultured into fresh LB-Amp supplemented with 2.5 mM $CaCl_2$ to an OD_{600} = 0.1. Cultures were shaken at 37°C for 2 h. Bacteria were pelleted by centrifugation and resuspended in warm Hank's balanced salt solution (HBSS) to an OD_{600} = 1.0 (~1×10^9 c.f.u./ml).

Antibodies

The rabbit anti-Yop51 antibody (RAY51) (Bliska *et al.*, 1992) was provided by J.Clemens and J.Dixon. Monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology, Inc. Monoclonal anti-vinculin antibody (V-9131) was purchased from Sigma. Monoclonal anti-paxillin (P13520) and anti-$p130^{Cas}$ (P27820) antibodies were purchased from Transduction Laboratories. Rabbit anti-Fak (C-20) and monoclonal anti-GST (12) antibodies were purchased from Santa Cruz Biotechnology, Inc. The rabbit anti-$p130^{Cas}$ antibody αCas2 (Sakai *et al.*, 1994) was provided by H.Hirai. The rabbit anti-$p130^{Cas}$ antibody mixture CASB/CASF (Harte *et al.*, 1996) was provided by A.Bouton and T.Parsons. Rabbit anti-HEF1 (Law *et al.*, 1996) was provided by S.Law and E.Golemis. Anti-mouse and anti-rabbit IgG conjugated to horseradish peroxidase (HRP) were purchased from Sigma. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, FITC-conjugated $F(ab')_2$ goat anti-mouse IgG and lissamine rhodamine (LRSC)-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc.

Immunofluorescence assays

HeLa cells (1×10^{5}) were seeded in 1 ml of media onto sterile glass coverslips placed in a 24-well tissue culture plate ~20 h prior to the assay. The coverslips were overlayed with fresh media 15–30 min before the assay. Cells were left uninfected or infected with bacteria (m.o.i. of 50:1). After a brief centrifugation step (5 min at 100 *g*), the dishes were incubated for 1 or 2 h at 37°C in a 5% CO_2 incubator. The following steps were performed at room temperature. Samples were washed twice with phosphate-buffered saline (PBS) containing 1 mM sodium vanadate, fixed with 4% paraformaldehyde for 10 min, and then permeabilized with 0.2% Triton X-100 for 10 min. Samples were washed twice with PBS containing 1% bovine serum albumin (BSA) and then incubated for 1 h with primary antibody, RAY51 (1:1000), 4G10 (5 μg/ml), anti-paxillin (5 μg/ml), anti-vinculin (1:200) or CASB/F (1:1000). The monoclonal anti-Cas antibody was found to be unsuitable for detecting Cas in this assay. Antibodies were diluted in PBS containing 3% BSA. Cells were washed and then incubated for 1 h with FITC-conjugated goat anti-rabbit IgG, FITC-conjugated $F(ab')_2$ goat anti-mouse IgG or LRSC-conjugated goat anti-rabbit IgG diluted 1:200. Coverslips were washed well with PBS before mounting in 10% Airvol (Air Products, Inc.) in 100 mM Tris (pH 8.5), 25% glycerol, with 2.5% DABCO (Sigma) to help prevent fading. When DAPI (Sigma) was used, it was included in the final PBS wash at 0.1 μg/ml. Samples were analyzed by epifluorescence microscopy using a Nikon Diaphot microscope equipped with a 40× (NA 1.4) PlanApo objective oil immersion lens, or by confocal laser scanning microscopy using the same microscope equipped with an Odyssey laser. Epifluorescent images were captured with an Optronics color camera. Images were processed using Image I software and Adobe Photoshop 3.0.

Immunoprecipitations and immunoblotting

For immunoprecipitation studies, 2×10^6 cells in 10 ml of medium were plated in 100 mm tissue culture dishes. The following day, the medium was changed, and the cells were infected with bacteria (m.o.i. of 50:1) for 2 h at 37°C in a 5% CO_2 incubator or left uninfected. Dishes were placed on ice and washed twice with 10 ml of ice-cold PBS containing 10 mM NaF and 1 mM Na_3VO_4. Cells were lysed with 0.5 ml of ice-cold modified RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 1 mM Na_3VO_4, 10 mM NaF, 200 μM AEBSF, 20 μM leupeptin and 1 μM pepstatin) or Triton X-100 lysis buffer (10 mM Tris pH 7.6, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, 200 μM AEBSF, 20 μM leupeptin and 1 μM pepstatin) for 15 min on ice with occasional rocking. Cells were scraped into Eppendorf tubes and centrifuged in a Sorvall microfuge for 10 min at 12 000 r.p.m. at 4°C. The cleared cell lysates (RIPA- or Triton X-100-soluble fractions) were transferred to new tubes, and protein concentrations were determined using the Bio-Rad Protein Assay. The pellets (RIPA- or Triton X-100-insoluble fractions) were washed carefully in lysis buffer and resuspended in 60 μl of 2× Laemmli buffer for SDS–PAGE analysis. Cell lysates were adjusted for equivalent protein (500–1000 μg) and volume and then pre-cleared with 50 μl of 50% protein A–Sepharose beads (Pharmacia) for 30 min at 4°C with rotation followed by brief centrifugation. Samples of the supernatants were saved for pre-cleared lysate reference. αCas2 (2 μl), anti-Fak (C-20) (10 μl) or RAY51 (5 μg) was added, and immunoprecipitations were performed for 1 h (or overnight for RAY51) at 4°C with rotation. Immune complexes were recovered by incubation with 50 μl of 50% protein A–Sepharose beads

for 1–3 h at 4°C with rotation followed by a brief centrifugation step. Samples of the supernatants were saved for post-immunoprecipitation reference. The beads were washed three times with 1 ml of lysis buffer, resuspended in 60 µl of Laemmli sample buffer, and boiled for 5 min to dissociate the immune complex.

Samples containing equal amounts of protein (or sample volume for insoluble fractions) were separated by SDS–PAGE under reducing conditions and electrophoretically transferred onto nitrocellulose (Schleicher & Schuell). The nitrocellulose filters were blocked in TBST (50 mM Tris pH 8.0, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween-20) containing 1% BSA (Fraction V; Boehringer Mannheim) for 1 h. Filters were then incubated with the specified primary antibody, RAY51 (1:1000), αCas2 (1:2000), 4G10 (1:1000), monoclonal anti-Cas P27820 (1:1000) or anti-Fak (C-20) (1:100) for 1 h. Filters were rinsed three times with TBST and then incubated with the appropriate secondary antibody, HRP-conjugated anti-mouse or anti-rabbit IgG, for 1 h. The filters were washed four times with TBST and developed using the Renaissance (DuPont NEN) chemiluminescence system. In some cases, the blots were stripped of bound antibodies by incubating in 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol at 50–55°C for 30 min. Exposed films were scanned on an LKB laser densitometer.

Adhesion of HeLa cells to fibronectin

Tissue culture dishes (100 mm) were coated in PBS containing 20 µg/ml fibronectin (Gibco BRL) at 4°C overnight. Dishes were rinsed twice with PBS before use. HeLa cells were detached with 0.05% trypsin, 0.5 mM EDTA (Gibco BRL), followed by two washes in medium containing serum and two washes in serum-free medium. Then 2×10^6 cells in serum-free medium were seeded onto fibronectin-coated dishes or held in suspension for 60 min. At this time, the suspended cells and plated cells in one dish were lysed in RIPA as described above. The remaining dishes of plated cells were infected with IP17/pYOPH at an m.o.i. of 50:1. At 15, 30 or 60 min post-infection, the cells were lysed in RIPA. The lysates were divided into equal portions and subjected to immunoprecipitation with anti-Cas or anti-Fak antibodies as described above.

Gel overlay assay and in vitro dephosphorylation

Cas and Fak were immunoprecipitated from Triton X-100 lysates of IP17/pYOPHC403S-infected cells as described above. The immunoprecipitates were washed in 50 mM imidazole (pH 7.3). The Cas immunoprecipitate was divided in half and then boiled for 2 min. Once the samples had cooled to room temperature, purified Yop51 (400 U, New England BioLabs) was added to one of the halves, and both halves were incubated for 10 min at room temperature. Samples were then boiled for 5 min in 2× Laemmli sample buffer. The immunoprecipitates were resolved by SDS–PAGE and transferred to Immobilon-P transfer membrane (Millipore). Blots were blocked with PBS–1% BSA and then probed with 5 µg/ml of purified GST–YopHC403S or GST–Crk (provided by H.Hanafusa) for 1 h at room temperature. The construction of GST–YopHC403S will be described elsewhere. GST fusion proteins were purified as described (Guan and Dixon, 1991). The blots were washed with PBS–1% BSA–0.1% Tween-20 and then probed with monoclonal anti-GST antibody (1 µg/ml) for 1 h. Following washing, blots were incubated with HRP-conjugated anti-mouse IgG for 1 h. Filters were washed and developed using enhanced chemiluminescence.

Acknowledgements

We thank J.Clemens, J.Dixon, H.Hirai, H.Hanafusa, A.Bouton and T.Parsons for generously providing antibodies used in this study, and J.Galan, J.Konopka, C.Roy and members of the laboratories for helpful discussions and comments on the manuscript. Microscopy was performed by D.Colflesh of the University Microscopy and Imaging Facility. This research was supported by Grants from the National Institutes of Health (R29-AI35175-02) and the Sinsheimer Foundation. D.S.B was supported by a National Institutes of Health training grant 2T32CA09176-16. J.B.B. is a PEW Scholar in the Biomedical Sciences.

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Received on December 31, 1996; revised on February 3, 1997