A role for N-WASP in invasin-promoted internalisation

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Abstract Phagocytosis of *Yersinia pseudotuberculosis* occurs through interaction of the bacterial protein invasin with β1-integrins. Here we report that N-WASP plays a role in internalisation of an invasin-expressing, avirulent strain of *Y. pseudotuberculosis*. Ectopic expression of N-WASP mutants, which affect recruitment of the Arp2/3 complex to the phagosome, reduces uptake of *Yersinia*. In addition, expression of the Cdc42/Rac-binding (CRIB) region of N-WASP has an inhibitory effect on uptake. Using GFP-tagged Rho GTPase mutants, we provide evidence that Rac1, but not Cdc42, is important for internalisation. Furthermore, activated Rac1 rescues Toxin B, CRIB and Src family kinase inhibitor PP2-mediated impairment of uptake. Our observations indicate that invasin-mediated phagocytosis occurs via a Src and WASP family-dependent mechanism(s), involving the Arp2/3 complex and Rac, but does not require Cdc42. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Invasin; Internalization; N-WASP; Rac; Src; *Yersinia*

1. Introduction

Pathogenic *Yersinia* species target host lymphoid tissues and possess ability to out manoeuvre the host immune defence by an antiphagocytic mechanism [1]. This talent is conferred by expression of effector molecules, Yops (*Yersinia* outer proteins), which are translocated from the bacterium into target cells via a type III secretion apparatus. The intracellular roles of the various Yops are still being elucidated, but they have been demonstrated to induce apoptosis, inhibit host inflammatory responses, disrupt the host actin cytoskeleton, focal complexes and phosphotyrosine signalling [2]. The latter effects, which are critical for the process of phagocytic evasion, are attributable to the *Yersinia* effectors YopE and YopH [3].

In the absence of the virulence plasmid-encoded Yops, *Yersinia* strains are not prevented from being ingested by either professional or non-professional phagocytes. Ingestion is mediated by β1 integrins that bind invasin on the bacterial surface and during uptake the membrane of the host cell rearranges around the bacterium in a sequential 'zipper-like' mechanism [4]. Morphologically, this resembles Fcγ receptor (FcγR)-mediated phagocytosis of bacteria, which has been shown to require the Rho GTPases Rac and Cdc42 [5,6]. The initial signalling that occurs upon receptor ligation differs however between β1 integrins and FcγRs. Moreover, uptake via β1 integrins and FcγRs is clearly distinct from complement receptor 3 (CR3)-mediated uptake, which is a RhoA GTPase-dependent process where no extending pseudopods are formed and the bacterium sinks passively into the cell [5–7].

More recently both FcγR- and CR3-promoted phagocytic processes have been shown to be dependent on the Arp2/3 complex [8]. In recent years it has emerged that the Arp2/3 complex plays an important role in dynamic actin filament assembly during numerous cell processes [9]. As with many Arp2/3-dependent processes both FcγR- and CR3-promoted phagocytosis are dependent on WASP family members [8]. WASP family proteins have emerged as key integrators of signalling cascades that lead to Arp2/3-dependent actin polymerisation by virtue of their ability to bind the GTPase Cdc42, the phosphoinositide PIP_2, the adapters Nck and Grb2, and other proteins such as WASP-interacting protein (WIP) and WISH [9].

Given that invasin-stimulated uptake differs from FcγR- and CR3-promoted phagocytosis with respect to signalling and morphology we examined the possible role of WASP family proteins in *Yersinia* internalisation. By using an approach of expressing GFP-tagged variants of N-WASP and potential cooperating proteins we show a role for this protein, its interactor WIP, and also Rac GTPase in uptake of *Yersinia*.

2. Materials and methods

2.1. Plasmids and reagents

The cDNA encoding N-WASP, WIP, N-WASP, Cdc42 and their variants were subcloned from their pE/L-GFP counterparts [10,11] into the *Not*I–*Eco*RI sites of the pCB6 expression vector into which EGFP had been inserted [12]. Cdc42/Rac-binding (CRIB) H208D was amplified by PCR from pE/L-GFP-WH1-CRIB H208D using primers previously designed for PCR amplification of the CRIB domain [11]. Cloning of Rac1 and RhoA into pCB6-EGFP will be reported elsewhere. WAVE2, WAVE2 WA and WAVE2 proline-rich region were amplified by PCR using pEF-BOS WAVE2 as a template (kindly provided by Tadaomi Takenawa). The following primers were used: (for full-length WAVE2 encoding amino acids 1–498) KpnI-WAVE2-For 5′-GGGGGGTACCACCATGCCGTTAGTAACGAGGAACA-TC-3′ and WAVE2-NotI-Rev 5′-GGGGGCGGCCGCGGCGGCA-TCGGACCAGTCGTCCTCATCAAA-3′; (for WAVE2 WA encoding amino acids 437–498) KpnI-WAVE2 WA-For 5′-GGGGGGT-ACCACCATGCGTAGCGACCTGCTTTCAGCCATC-3′ and same reverse primer for full-length WAVE2; (for WAVE2 polyproline encoding amino acids 265–402) KpnI-WAVE2 Pro-For 5′-GGGG-GGTACCACCATGGACAACTTGCCTCCTCCACCAGCA-3′ and WAVE2 Pro-NotI-Rev 5′-GGGGGCGGCCGCGGCGGCGGGC-CCCGGAGGAGGAGGAGGAGG-3′. The resulting PCR products were cloned into the *Kpn*I–*Not*I sites of pCB6-GFP. The HA-CRIB

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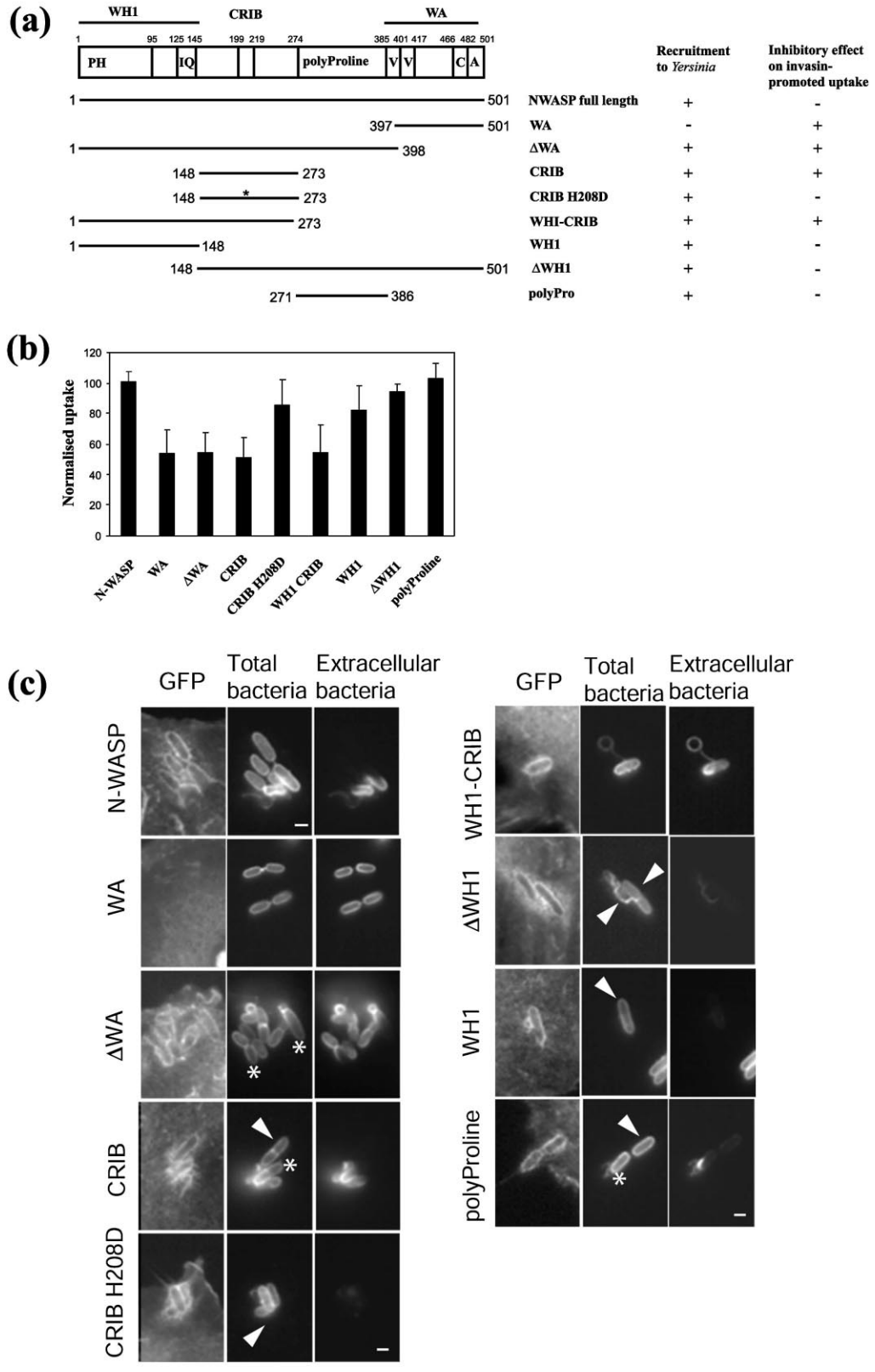

Fig. 1. N-WASP is involved in invasin-stimulated engulfment. a: Schematic diagram of N-WASP and the GFP-tagged constructs used in this study. Recruitment to *Yersinia* and inhibitory effect on invasin-mediated uptake are summarised to the right. b: Uptake of YPIII by HeLa cells expressing N-WASP mutants. Results are the mean ± S.E.M. of six separate experiments. c: Immunofluorescence analysis of infected HeLa cells expressing GFP-N-WASP variants, showing recruitment of proteins to either fully (arrowheads) or partially (asterisks) internalised bacteria. Scale bar, 1 μm.

construct was subcloned, via the *Not*I–*Eco*RI sites, from pCB6-GFP-CRIB into the pCB6-HA expression vector [13]. *Clostridium difficile* Toxin B was kindly provided by Klaus Aktories, Freiburg, Germany. PP2 was obtained from Alexis Corporation, San Diego, CA, USA.

2.2. Transfection, bacterial uptake and immunofluorescence

All CB6 expression constructs were transfected into HeLa cells using a standard calcium phosphate method. 15 h later the cells were washed and allowed to recover for 1–2 h before infection. An overnight culture of the virulence plasmid-cured YPIII strain of *Yersinia pseudotuberculosis* [14], grown in Luria broth at 26°C, was diluted 1:200 in cell culture medium and incubated for 1 h at 26°C. This culture was used to infect HeLa cells at a multiplicity of infection of 10:1 in 24 well plates. Infection was carried out for 15 min at 37°C in an atmosphere of 5% CO_2.

Following infection cells were fixed as described previously [11]. Double immunofluorescence labelling was used to differentiate extracellular and total cell-associated bacteria [15]. Rabbit anti-*Yersinia* antiserum was used as the primary antibody [16] followed by Texas Red-conjugated donkey anti-rabbit IgG and AMCA-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA), to stain extracellularly located bacteria and total bacteria, respectively. All antibody dilutions were in Tris-buffered saline with 1% bovine serum albumin and 2% fetal calf serum. For the Toxin B titration assay the untransfected cells were stained in the same manner as above except that FITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) was used to stain total bacteria with the addition of coumarin phallicidin (Molecular Probes, Eugene, OR, USA) to stain F-actin. To detect cells expressing HA-CRIB, a monoclonal antibody, HA.11, was used (Covance, Richmond, CA, USA). Coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA, USA). Images were collected with a DMRXA microscope (Leica, Mannheim, Germany) using a Cohu ccd 4910 camera (Cohu, San Diego, CA, USA) and NIH Imaging software (version 1.62) and processed using Adobe Software (Adobe, San Jose, CA, USA). Numbers of extracellular and total cell-associated bacteria were counted for 40 random transfected and untransfected cells per coverslip.

3. Results

3.1. Efficient entry of Yersinia into HeLa cells involves N-WASP

To examine the potential role of N-WASP during invasin-promoted internalisation we expressed GFP-tagged N-WASP proteins in HeLa cells and investigated their effect on *Yersinia* uptake (Fig. 1a,b). Immunofluorescence analysis revealed that full-length N-WASP was recruited to the engulfed bacteria and had no effect on uptake (Fig. 1c). In contrast expression of an N-WASP variant deleted of the WA domain (ΔWA) reduced uptake to 54 ± 15.1% (Fig. 1b), although localisation to the bacterial phagosome still occurred (Fig. 1c). Similarly, expression of the WA domain alone inhibited bacterial uptake, but this protein was not recruited to the phagosome. Expression of the CRIB domain alone or WH1-CRIB was found to block uptake and both molecules were recruited to the phagosome. In contrast, the CRIB H208D variant, which is deficient in interaction with Cdc42 and Rac [17,18], had only a minor effect on uptake (85 ± 16.5%), although it was still recruited to the phagosome (Fig. 1b,c). Expression of ΔWH1, WH1 or the polyproline alone had little effect on uptake, although all were targeted to the phagosome. Taken together, our observations reveal that N-WASP and Arp2/3 complex play an important role in invasin-promoted phagocytosis of *Yersinia*.

The observation that several different domains of N-WASP were targeted to phagosomes indicated existence of different routes by which N-WASP could be recruited in this process.

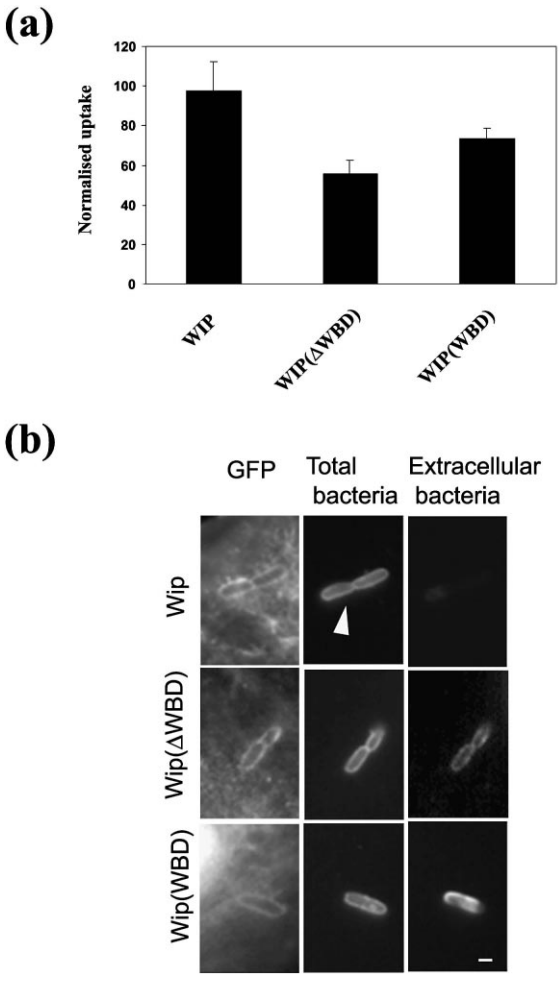

Fig. 2. WIP can interfere with invasin-activated engulfment. a: Uptake of YPIII by HeLa cells expressing full-length GFP-WIP, GFP-WIP ΔWBD and GFP-WIP-WBD. Results are the mean ± S.E.M. of six separate experiments. b: Immunofluorescence analysis of infected HeLa cells expressing the indicated GFP-WIP variants, showing recruitment of proteins to either fully (arrowheads) or partially internalised bacteria. Scale bar, 1 μm.

Therefore, we investigated the potential roles of proteins that bind these domains. One such protein is WIP, which binds the WH1 region of N-WASP [19,20]. To elucidate the possible role of this protein, full-length WIP, WIP lacking the WASP-binding domain (ΔWBD) or the WBD alone were expressed in cells and uptake was examined (Fig. 2a,b). All proteins localised to the bacterial phagosome but only ΔWBD and WBD reduced uptake to 55.6 ± 6.8% and 73.6 ± 5.1%, respectively. Hence, interfering with WIP influences invasin-stimulated uptake, suggesting that this WIP takes part in invasin-promoted phagocytosis of *Yersinia*. Proteins interacting with the N-WASP proline-rich region are Grb2 and Nck [21,22]. To test involvement of these adapter proteins, GFP-Nck1 and GFP-Grb2 as well as dominant negative variants with mutations in the SH3 or SH2 domains were expressed in cells and uptake was examined. Both wild-type proteins localised to phagosomes, but none of the mutated variants significantly affected uptake (data not shown). Hence, these adaptor proteins do not play an evident role in the uptake process.

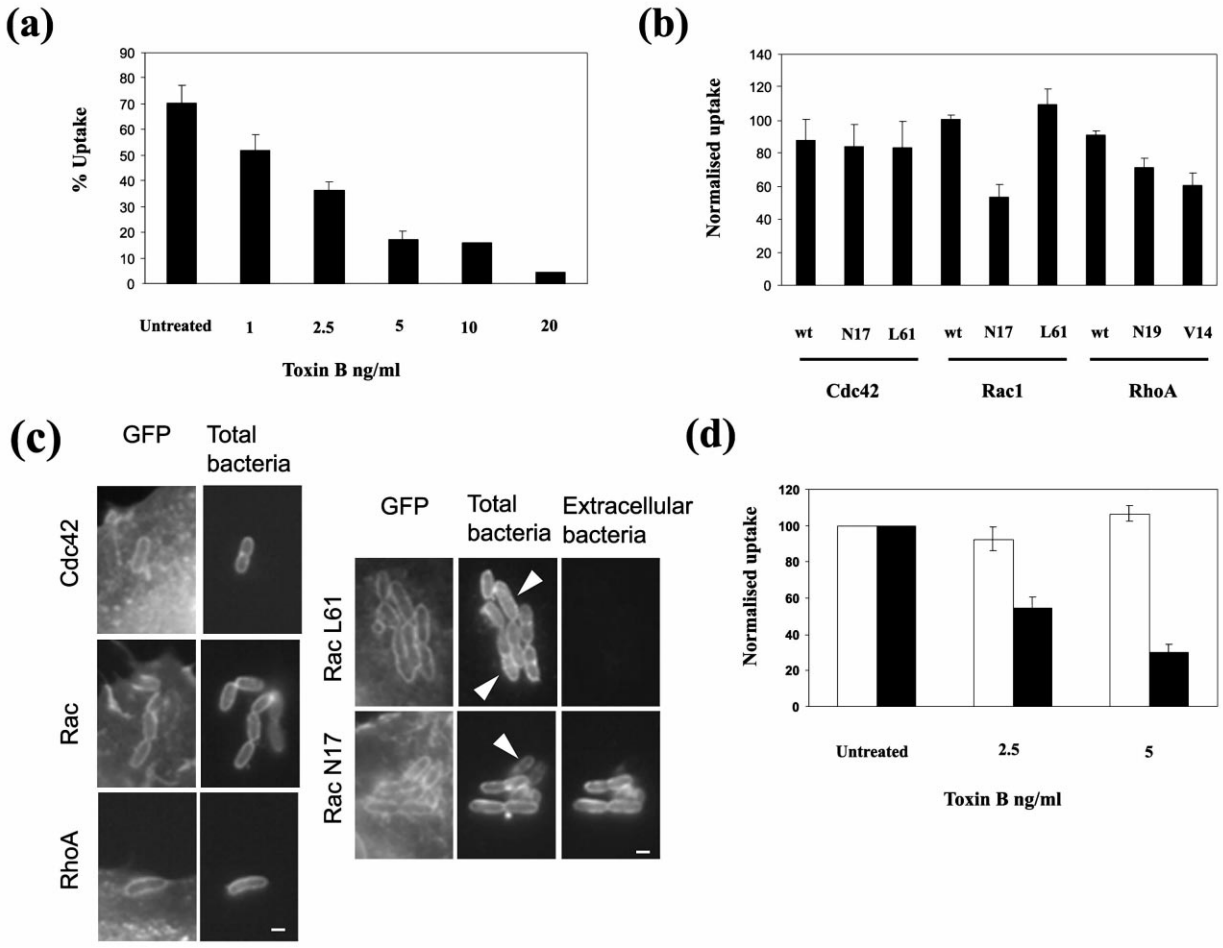

Fig. 3. Rac is involved in *Yersinia* internalisation. a: Uptake of YPIII in Toxin B-treated HeLa cells. Cells were pretreated with increasing concentrations of Toxin B 30 min before infection, and the toxin was present throughout infection. b: Uptake of YPIII in HeLa cells expressing Rho family GTPase constructs. Results are the mean ± S.E.M. of four separate experiments. c: Immunofluorescence analysis of infected HeLa cells expressing GFP-Rho family GTPase mutants, showing recruitment of the wild-type Rho GTPases (left panel) or Rac mutants (right panel) to either fully (arrowheads) or partially internalised bacteria. d: Uptake of YPIII by cells expressing GFP-RacL61 in the presence of Toxin B. Unfilled bars represent transfected cells and filled bars represent untransfected cells. Scale bar, 1 μm.

3.2. Rac1 GTPase is required for invasin-promoted uptake

Taking into account the inhibitory effect of the CRIB domain together with the general role of Rho GTPases as central regulators of actin rearrangements we explored whether these GTPases play a part in invasin-stimulated uptake. When the *C. difficile* Toxin B was used to inhibit Rho GTPase activity, a concentration-dependent abrogation of uptake was observed (Fig. 3a). To find which member(s) was involved, GFP-tagged wild-type, dominant negative and constitutively activated variants of Rho, Rac and Cdc42 were expressed in cells and uptake was examined (Fig. 3b). Unexpectedly, given the previous results with CRIB, Cdc42 mutants had little effect on phagocytosis. To exclude possible adverse effects from the GFP-tag we also analysed effects of expressing HA-tagged Cdc42 variants, but expression of these gave identical results to GFP-tagged versions (data not shown). In contrast, dominant negative Rac (RacN17) inhibited uptake to 54 ± 7.5% while the activated version (RacL61) had a slightly stimulating effect (Fig. 3b). Activated (RhoAV14) as well as dominant negative Rho (RhoAN19) also had inhibitory effects, 60.7 ± 7.2% and 71.4 ± 5.3%, respectively. All three Rho family proteins were recruited to the phagosome (Fig. 3c). In addition, cells expressing RacL61 were protected from the Toxin B-mediated phagocytic inhibition (Fig. 3d).

Cdc42, along with PIP_2, has been shown to bind and activate N-WASP [17,18,23], whereas the interaction and role of Rac are less clear [24]. To explore whether Rac is upstream of N-WASP in the signalling pathway during *Yersinia* internalisation we infected cells co-expressing activated GFP-Rac and HA-CRIB. Uptake analysis revealed that RacL61 rescued the inhibition of uptake caused by CRIB. In these double transfectants CRIB could no longer be detected around the bacteria, although RacL61 was clearly present (Fig. 4a,b). Co-expression of GFP-RacN17 and HA-CRIB did not further decrease uptake supporting the concept of Rac and N-WASP are operating within the same signalling pathway.

Another player known to participate in both invasin-promoted and Fcγ R-mediated phagocytosis is Src [25,26]. Hence, we wondered whether this kinase is involved in the same pathway as Rac/N-WASP. Control experiments revealed that treatment of cells with the Src family kinase inhibitor PP2 had a negative effect on uptake levels in a concentration-dependent manner. However inhibition of Src kinase activity by this drug failed to abrogate the uptake response in cells ex-

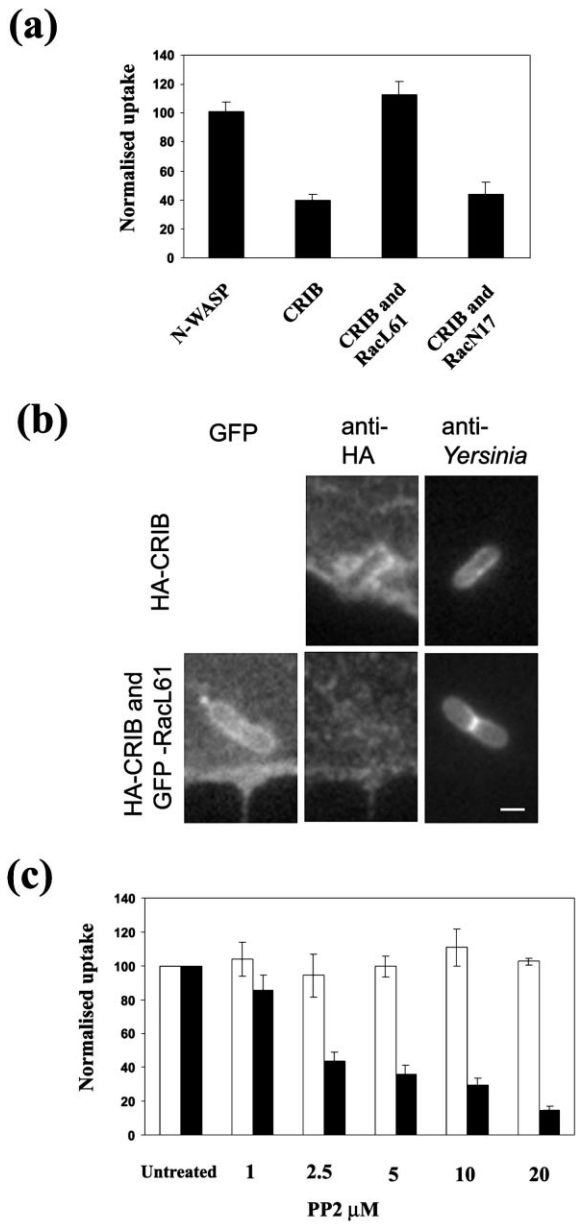

Fig. 4. Rac1 rescues CRIB-mediated and PP2-induced inhibition of *Yersinia* internalisation. a: Uptake of YPIII by HeLa cells co-transfected with GFP-RacL61 and GFP-CRIB. Results are the mean ± S.E.M. of five separate experiments. b: Immunofluorescence analysis of infected HeLa cells expressing either HA-CRIB or co-expressing HA-CRIB and GFP-RacL61. c: Uptake of YPIII by GFP-RacL61-expressing HeLa cells, which had been pretreated with increasing concentrations of PP2. Results are the mean ± S.E.M. of four separate experiments. Scale bar, 1 μm.

pressing RacL61 (Fig. 4c). This shows that Rac is a downstream effector of Src family kinases in invasin-activated bacterial internalisation.

The WASP family protein often implicated as a Rac effector is Scar/WAVE [27–29]. Having shown that Rac plays a role in *Yersinia* internalisation we examined the potential involvement of WAVE. Miki et al. [30] recently showed that the insulin receptor substrate IRSp53 forms a tri-molecular complex with these proteins, acting as a link between the GTPase and WAVE. IRSp53 binds to the proline-rich region of ubiquitously expressed WAVE2 and expression of this region alone had a dominant negative effect on WAVE2 responses [30]. To elucidate this possible pathway in invasin-stimulated uptake, the proline region of WAVE2 (WAVE2 polyPro) was expressed in HeLa cells. We found that expression of this region of the WAVE2 molecule had no appreciable inhibitory effect on uptake (96 ± 8%). Furthermore, cells expressing the WAVE2 WA region had only a slightly reduced capacity to internalise *Yersinia* (71.4 ± 12.9%) compared to cells expressing full-length WAVE2 (82 ± 18.4%). Thus, there is no obvious role for WAVE in uptake promoted by invasin.

4. Discussion

In recent years numerous studies have focused on signal transduction cascades and the role of the actin cytoskeleton and associated proteins during internalisation of bacterial pathogens into host cells [31,32]. One common theme that has emerged is the involvement of Rho family GTPases [31–41]. Our current study reveals that while Cdc42, Rac and Rho are recruited to *Yersinia* during uptake, only Rac is required for invasin-promoted engulfment of the bacterium into host cells. Our observations are consistent with the recent studies showing that Rac is important for uptake of *Yersinia* [37,38]. In this respect *Yersinia* uptake is similar to phagocytosis of *Neisseria*, which requires Rac, but is independent of Cdc42 [35]. In *Salmonella* species on the other hand, Cdc42 modulates entry into non-polarised cells [33] whereas Rac regulates invasion of polarised cells [40]. Rac is also a key regulator in phagocytosis of *Listeria* [39]. Coiling phagocytosis of *Borrelia* is controlled by Cdc42 and to a lesser extent by Rac [41], and entry of *Shigella* into cells occurs in a Rho-, Rac- and Cdc42-dependent manner [34,36]. It is clear that different internalisation mechanisms using distinct subsets of Rho GTPases exist which must ultimately reflect the stimulating bacterial ligand and corresponding receptors.

Given Rac is involved, what is the molecular basis for its role in uptake of *Yersinia*? Previous observations have shown that Src family kinases play an important role in *Yersinia* uptake [25]. Our observations showing that activated Rac is able to efficiently rescue the uptake of *Yersinia* in the presence of the Src family kinase inhibitor PP2 indicate that this GTPase acts downstream of an as yet to be identified Src family kinase member during this process. So, if Src family kinases are upstream, what is downstream of Rac? One would think that the WASP family member WAVE would be the most plausible downstream effector of Rac, that would be capable of activating the Arp2/3 complex required for actin polymerisation during uptake of *Yersinia*. As with N-WASP the WA of WAVE is the minimal essential region for Arp2/3 activation [42]. We could however find no obvious role for WAVE in the invasin-stimulated uptake of *Yersinia* as expression of WAVE2 WA has an effect on uptake similar to that of the full-length WAVE2. Surprisingly the WA of N-WASP inhibits internalisation more effectively. This however is likely due to the fact that WAVE WA is less potent than N-WASP WA in activating Arp2/3 complex [43]. In addition to our unexpected observation that N-WASP is required for uptake it also appears to be downstream of Rac. N-WASP and Rac appear to be in the same pathway, as CRIB and RacN17 together give a level of inhibition that is equivalent to either alone. While Cdc42 is generally considered as the activator of

WASP, Rac also can interact with the CRIB domain of this protein [23,24].

Expression of the CRIB H208D mutant, which contains the PIP_2-binding/activation site but is deficient in Cdc42- and Rac-binding, does not appreciably inhibit uptake. This suggests that it is the interaction of the CRIB motif and not the basic region of N-WASP with a Rho GTPase that is principally responsible for the inhibitory effect on *Yersinia* uptake. Given the lack of effects with variants of Cdc42 and the partial inhibition by dominant negative Rac we suggest that an additional 'Rac-like' Rho family member which can activate N-WASP is involved in *Yersinia* uptake. Consistent with this hypothesis, the inhibitory effect on *Yersinia* uptake by the Rho GTPase inhibitor Toxin B is far more effective than that of any of the single Rho GTPase mutants tested. Identification of other potential Rho GTPases involved in *Yersinia* uptake requires further study but obvious potential candidates are other known members of the Rho family such as Chp, RhoG, TC10 and TCL [44–46]. Lastly, although we have a clear indication that the Arp2/3 complex is involved in actin-mediated uptake of *Yersinia*, the lack of near complete inhibition, as in the case of inhibition of actin-based motility of vaccinia or EPEC pedestal formation [10,47], would suggest involvement of Arp2/3-independent actin polymerisation. Zyxin for example exhibits actin polymerisation activity without participation of Arp2/3 [48]. Another potential candidate is ezrin for which a role in actin assembly on phagosomal membranes has been shown and whose function is required for cell entry of *Shigella* [49,50].

Taken together, our present study indicates that Src, Rac, WIP, N-WASP and Arp2/3 complex participate in invasin-promoted phagocytosis of *Yersinia*. Understanding whether a single or, as is more likely the case, multiple redundant pathways are involved and their molecular interplay will require further work that will undoubtedly be aided by null cell lines for Src family kinases, Rac, Cdc42 and N-WASP which are now available [51–55].

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