Microtubule-dependent regulation of Rho GTPases during internalisation of *Yersinia pseudotuberculosis*

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Abstract Internalisation of the human pathogen *Yersinia pseudotuberculosis* via interaction of bacterial invasin with host β1 integrins depends on the actin cytoskeleton and involves Src family kinases, focal adhesion kinase, p130Crk-associated substrate, proline-rich tyrosine kinase 2, Rac, Arp 2/3 complex and WASP family members. We show here that Rho GTPases are regulated by the microtubule system during bacterial uptake. Interfering with microtubule organisation using nocodazole or paclitaxel suppressed uptake by HeLa cells. The nocodazole effect on microtubule depolymerisation was partially inhibited through overexpression of Rac, Cdc42, RhoG or RhoA and completely prevented by expression of Vav2. This suggests that microtubules influence Rho GTPases during invasin-mediated phagocytosis and in the absence of functional microtubules Vav2 can mimic their effect on one, or more, of the Rho family GTPases. Lastly, overexpression of p50 dynamitin partially inhibited bacterial uptake and this effect was also blocked by co-expression of Vav2, thus further implicating this guanine nucleotide exchange factor in activating Rho GTPases for internalisation during loss of microtubule function.

Key words: Invasin; Internalization; Microtubule; Rho GTPase; Vav2; *Yersinia*

1. Introduction

Entry of the bacterial pathogen *Yersinia pseudotuberculosis* into non-professional phagocytic cells is initiated subsequent to the binding of the bacterial surface protein invasin to host cell β1 integrins [1]. While much is known regarding the transduction pathways that govern membrane remodelling during phagocytosis through Fcγ receptor (FcγR) and complement receptor 3, the molecular machinery required for invasin-promoted internalisation via β1 integrins is only beginning to be understood. We, and others, have shown a requirement for actin, a subset of Rho GTPases and WASP family proteins during invasin-stimulated internalisation of *Yersinia* by non-phagocytic cells [2–4] as well as professional phagocytes [5]. These proteins have been shown to regulate various cellular processes [6–10]. In this current study, a possible additional role for microtubules during *Yersinia* uptake was investigated given the recent observations that co-ordination of signals between the actin and microtubule cytoskeletons is mediated through Rho family GTPases during cell locomotion [11–13]. Invasion of cultured cells by certain pathogenic bacteria is known to be significantly affected by drug-induced disruption of the microtubule system [14–17]. The role of microtubules in bacterial invasion has not been clarified, some bacteria however can interact with microtubules and possibly move intracellularly via a dynein-dependent mechanism, e.g. *Campylobacter jejuni* and *Orientia tsutsugamushi* [18,19]. More recently it was demonstrated that the *Shigella flexneri* VirA effector protein, which is essential for cell entry, interacts with tubulin dimers leading to destabilisation of microtubules [20]. Here we show that invasin-stimulated uptake of bacteria is abrogated when microtubule organisation is perturbed through use of microtubule-disrupting drugs. However, overexpression of Rho GTPase family members and their regulators can, to a large extent, prevent this drug-induced inhibition. Hence invasin-promoted internalisation involves interplay between the microtubule and actin cytoskeletons, which is mediated by Rho GTPases.

2. Materials and methods

2.1. Transfection, bacterial infection and immunofluorescence analyses

The pCB6-EGFP-Cdc42 constructs have been described [4], cloning of Rac1 and RhoA constructs into the pCB6-EGFP expression vector will be reported elsewhere. Transfection of HeLa cells and subsequent infection followed by immunofluorescence analysis of bacterial internalisation was performed as previously described [4] except that infection was carried out for 10 min. Numbers of extracellular and total cell-associated bacteria were counted for 40 random transfected and untransfected cells per coverslip.

For the normalised uptake graphs, internalisation of bacteria by drug-treated, transfected cells is normalised to that of untreated, transfected cells. Likewise, uptake by drug-treated, untransfected cells is normalised to that of untreated, untransfected cells. Subcellular localisation of tubulin was detected with an anti-α-tubulin monoclonal antibody, B-5-1-2 (Sigma-Aldrich, Deisenhofen, Germany), followed by rhodamine anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA), total *Yersiniae* were stained with rabbit anti-*Yersinia* antiserum followed by FITC-conjugated swine anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) and the cell nuclei were stained with DAPI (Sigma-Aldrich). For the nocodazole and paclitaxel titration assays the infected untransfected cells were stained as described before [4] with the modification that AMCA-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) was used to stain total bacteria and Alexa 488 (Molecular Probes, Eugene, OR, USA) was used to label F-actin. Coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA, USA). Images were collected with a Zeiss Axiophot microscope (Carl Zeiss AB, Oberkochen, Germany) using a C4742-95 SC Matrix digital camera and Twain 32 imaging software

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Abbreviations: FcγR, Fcγ receptor; GEF, guanine nucleotide exchange factor

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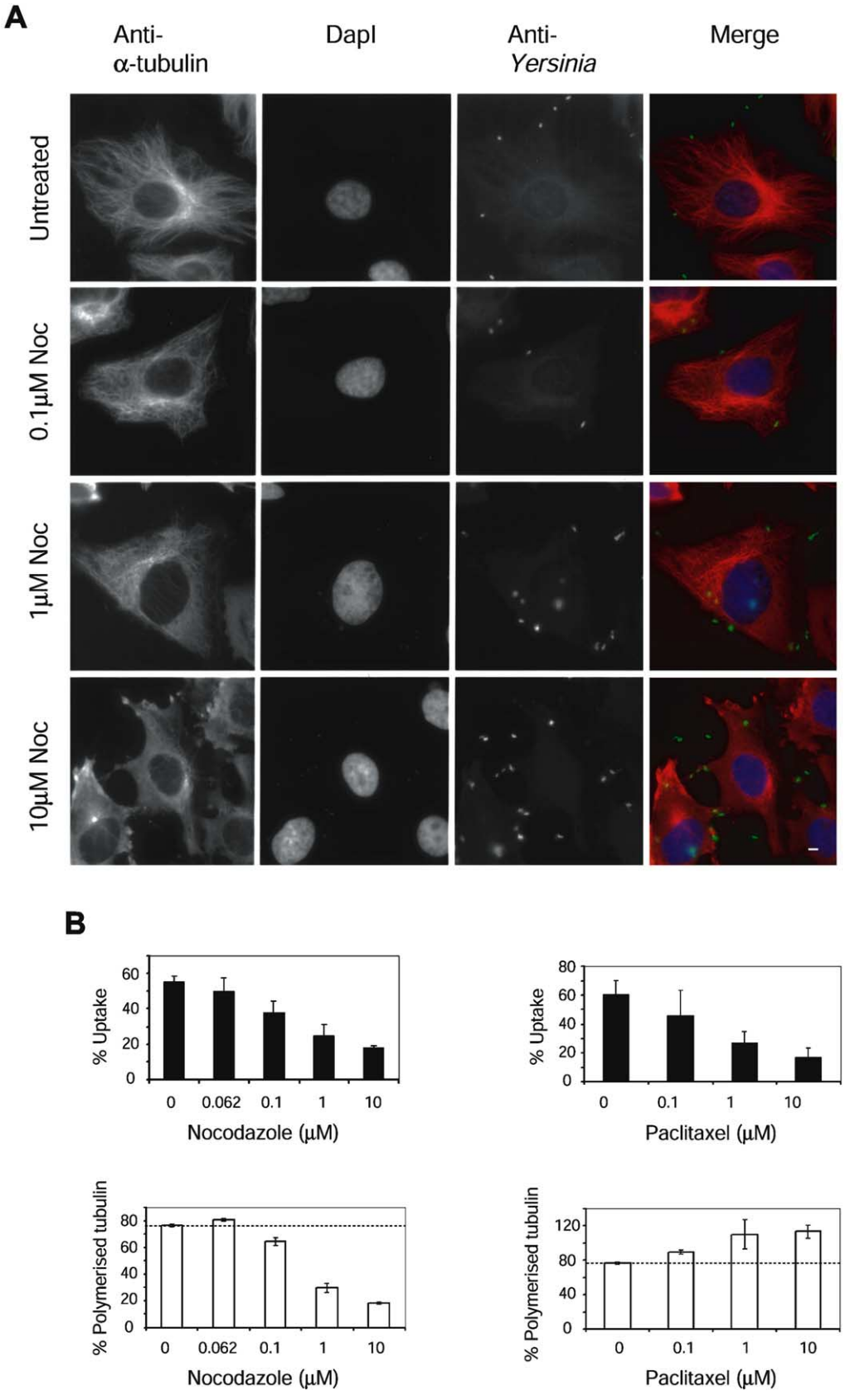
A
Anti-
α-tubulin
DapI
Anti-
Yersinia
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0.1μM Noc
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% Uptake
Paclitaxel (μM)
% Polymerised tubulin
Nocodazole (μM)
% Polymerised tubulin
Paclitaxel (μM)

Fig. 1. Drugs that interfere with microtubule dynamics block invasin-promoted internalisation of *Yersinia*. A: Immunofluorescence analysis of HeLa cells treated with nocodazole 30 min prior to infection with YPIII. Cells are stained for α-tubulin (left panel) and the nucleus is stained with DAPI (right panel). *Yersiniae* are stained with an anti-*Yersinia* antiserum (centre panel). Scale bar, 5 μm, indicated in lower right corner. B: Determination of YPIII uptake and microtubule polymer content in nocodazole- and paclitaxel-treated HeLa cells. Cells were pretreated with increasing concentrations of the drugs for 30 min prior to infection. Each drug was present throughout infection. Results are the mean ± S.D. of five separate experiments. Subsequent to nocodazole/paclitaxel treatment HeLa cells were extracted, fixed and stained with an α-tubulin antibody and propidium iodide. Total polymerised microtubule content was then assessed by flow cytometry. The data obtained for untreated cells have been normalised to the value of the steady-state level of tubulin, that is 76.5% in HeLa cells [48]. Results are representative of the mean ± S.E.M. of duplicate samples from two experiments.
←

(Hamamatsu Photonics, Germany) and processed using Adobe software (Adobe, San Jose, CA, USA).

2.2. Quantification of cellular microtubule content by flow cytometry

This was carried out by a method modified from Holmfeldt et al. [21]: HeLa cells were seeded at a density of 5×10^4 cells per well in a 24-well tissue culture plate. After 15 h the cells were treated with nocodazole or paclitaxel (Sigma-Aldrich) for 1 h. Then the medium was aspirated and soluble tubulin was extracted with a microtubule-stabilising buffer, containing 4 μM paclitaxel and 0.05% saponin. Subsequently the cells were detached from the substratum by gentle scraping and then fixed in 2% paraformaldehyde. Cells were washed, and tubulin was labelled with anti-α-tubulin monoclonal antibody, B-5-1-2 (Sigma-Aldrich), followed by FITC rabbit anti-mouse immunoglobulin (Dako). When staining green fluorescent protein (GFP)-transfected cells Cy5 donkey anti-mouse IgG (Jackson Immunoresearch) was used instead of the FITC secondary antibody. Propidium iodide solution was used to stain the nuclear content. Finally the cells were subjected to flow cytometry on a FACs-calibur in combination with Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

3. Results and discussion

3.1. Perturbing microtubule organisation inhibits entry of Yersinia into HeLa cells

To determine whether the microtubule cytoskeleton is required during invasin-promoted uptake we examined the consequence of depolymerising microtubules with nocodazole on the uptake of the avirulent strain of *Y. pseudotuberculosis* (YPIII) by HeLa cells. Immunofluorescent analysis of bacterial internalisation revealed that *Yersinia* uptake was inhibited by nocodazole in a dose-responsive fashion (Fig. 1B). A reduction in uptake was also observed when cells were treated with the microtubule-disrupting agent vinblastine (data not shown). Examination of treated HeLa cells by flow cytometry confirmed that increasing concentrations of nocodazole resulted in a corresponding decrease in microtubule polymer mass (Fig. 1B). In addition, epifluorescence examination of cells incubated with an α-tubulin antibody showed the effect of nocodazole on the arrangement of microtubules, where the higher drug concentrations caused a visible reduction in the number of polymerised microtubules (Fig. 1A). To ascertain whether it was microtubule dynamics, or the presence of polymer, that was involved in *Yersinia* uptake we also examined the effect of the microtubule stabiliser paclitaxel. We found that this treatment, which caused an increase in the total cellular content of microtubules, also resulted in a dose-dependent reduction in *Yersinia* uptake (Fig. 1B). At low concentrations ($\leq$100 nM) of either drug, we expect microtubule dynamics to be influenced, whereas at the higher concentration the gross organisation of microtubules is also affected [22–24]. The relatively small effects on bacterial uptake at low concentrations compared to the more prominent blocking at higher concentrations indicate that while dynamic behaviour cannot be completely ruled out it is mostly microtubule function and organisation that is required for efficient internalisation of *Yersinia*.

3.2. Rho family GTPases, and their upstream regulators, can prevent nocodazole-induced blocking of uptake

How is the microtubule cytoskeleton linked to *Yersinia* uptake? Recent reports have shown a coupling between Rho GTPases and the microtubule system [25,26]. Our previous observations have shown that *Yersinia* uptake involves Rac1, a known regulator of protrusive events [27], which can also bind tubulin [28]. Rac as well as Cdc42 can activate PAK, which in turn inactivates the microtubule destabiliser Op18, promoting polymer growth [25]. In addition, Rac and Cdc42 have been shown to interact with microtubules through a common effector, IQ-GAP, which binds CLIP-170, a microtubule-associated protein that is recruited to the plus ends of polymerising microtubules [26]. Given these observations, we examined whether expression of constitutively activated variants of Rac or Cdc42 (GFP-RacL61 and GFP-Cdc42L61 respectively), would affect *Yersinia* uptake in nocodazole-treated HeLa cells. We found that the activated variants of either Rac or Cdc42 partially rescued the inhibiting effect of nocodazole (Fig. 2B). It was also clear that the previously reported recruitment of GFP-RacL61 and GFP-Cdc42L61 to internalised *Yersinia* [4] was unaffected by nocodazole (Fig. 2A and data not shown). Expression of dominant negative GFP-RacN17 and GFP-Cdc42N17 neither further inhibited nor promoted uptake of *Yersinia* in the nocodazole-treated cells although they were recruited to the few bacteria that were internalised by these cells (Supplementary data[1] and data not shown). Interestingly, although activated Rac or Cdc42 rescued bacterial uptake there were no appreciable differences in the amount of polymerised microtubules in transfected compared to untransfected nocodazole-treated cells (Fig. 2B). This suggests that the rescue of uptake by expression of activated Rac or Cdc42 is not mediated through an effect on the microtubule cytoskeleton. Rather it is the microtubule organisation that affects Rho GTPases, thus promoting bacterial uptake by affecting the actin cytoskeleton. Similarly, Waterman-Storer and coworkers have shown that microtubule growth leads to activation of Rac, promoting lamellipodial formation [29]. Their work, however, indicated involvement of microtubule dynamics, whereas our work with bacterial uptake is more consistent with an effect of gross organisation of microtubules rather than dynamics.

Given that Rac and Cdc42 partially rescued the inhibitory effect of nocodazole on *Yersinia* uptake we wondered whether an upstream guanine nucleotide exchange factor (GEF) might

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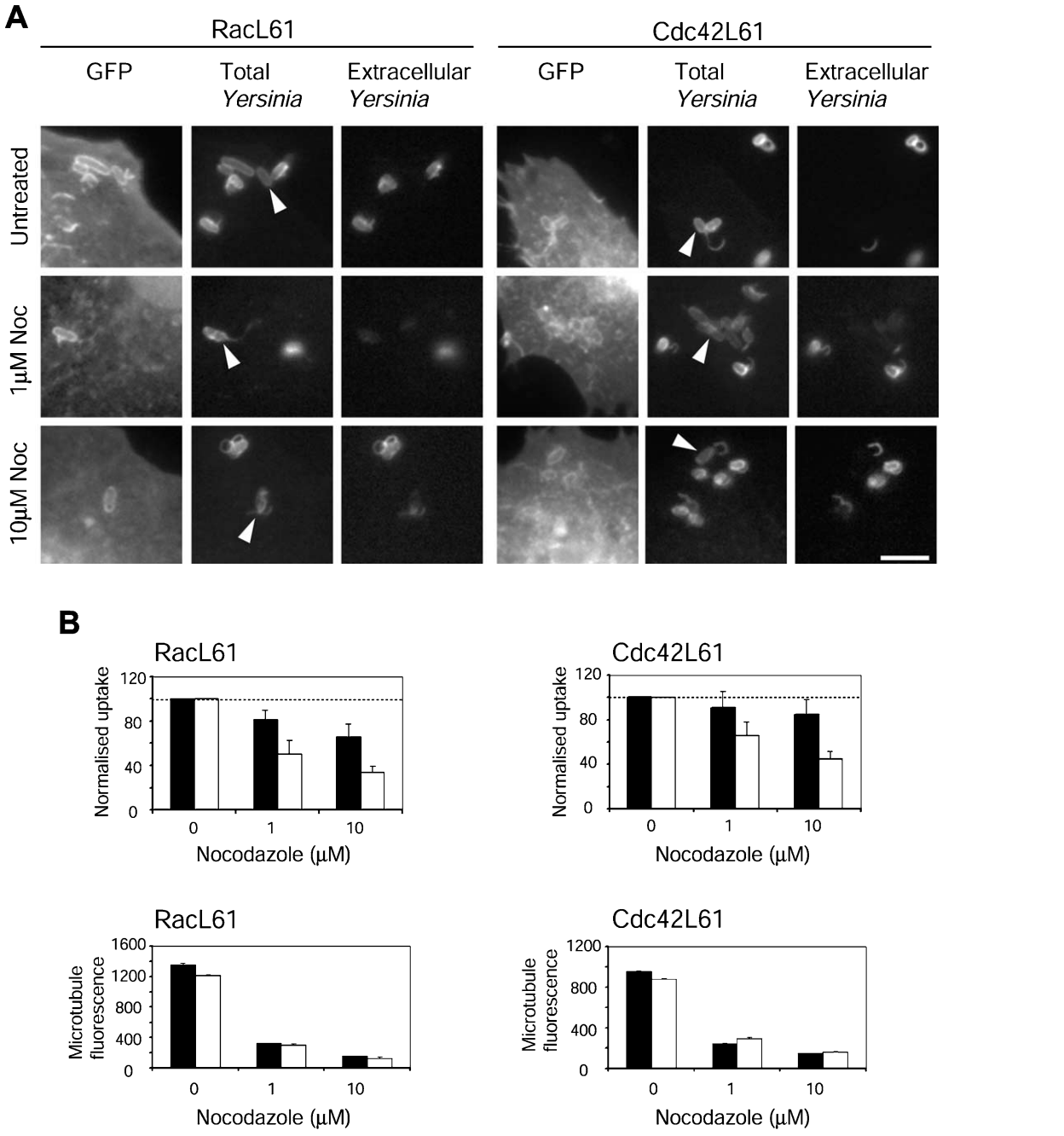

Fig. 2. Activated Rac or Cdc42 partially rescues nocodazole-mediated inhibition of uptake. A: Immunofluorescence of *Yersinia* uptake in HeLa cells transfected with GFP-RacL61 or GFP-Cdc42L61 and treated with increasing nocodazole prior to infection. Shown are localisation of the GFP proteins, total cell-associated bacteria and extracellular bacteria. Arrowheads indicate internalised bacteria. Scale bar, 5 μm. B: Determination of YPIII uptake and microtubule polymerisation in nocodazole-treated HeLa cells transfected with GFP-RacL61 or GFP-Cdc42L61 (filled bars) or untransfected (open bars). Results are the mean ± S.D. of five separate experiments for the uptake analysis and mean ± S.D. of duplicate samples from one experiment for the flow cytometry analysis.

have a role in this process. Vav family proteins are GEFs for Rho family GTPases [30–34], involved in cell spreading and protrusive events [35,36]. Hitherto reported data are conflicting as to which subsets of Rho family GTPases are activated by Vav [33,34,37], however, for FcγR-mediated phagocytosis in macrophages Vav has been shown to regulate activation of Rac but not Cdc42 [38]. Nevertheless, overexpression of an activated variant of the ubiquitous family member Vav2, Δ184N-Vav2-GFP, or an inactive variant, C-terminal Vav2-GFP [34], had little effect on bacterial uptake levels (91.3 ± 10.7% and 95.8 ± 7.5% respectively, $n = 7$ for both). Both proteins were weakly recruited to internalised bacteria, although for the activated variant this localisation became more apparent in the presence of nocodazole (Fig. 3A and data not shown). In addition, Δ184N-Vav2-GFP localised more intensely to the plasma membrane in drug-treated cells and co-stained with tubulin in these discrete areas (Supplementary data[1]), which may reflect association of Vav2 with tubulin. Interestingly, expression of Δ184N-Vav2-GFP completely rescued the nocodazole effect on uptake of *Yersinia*,

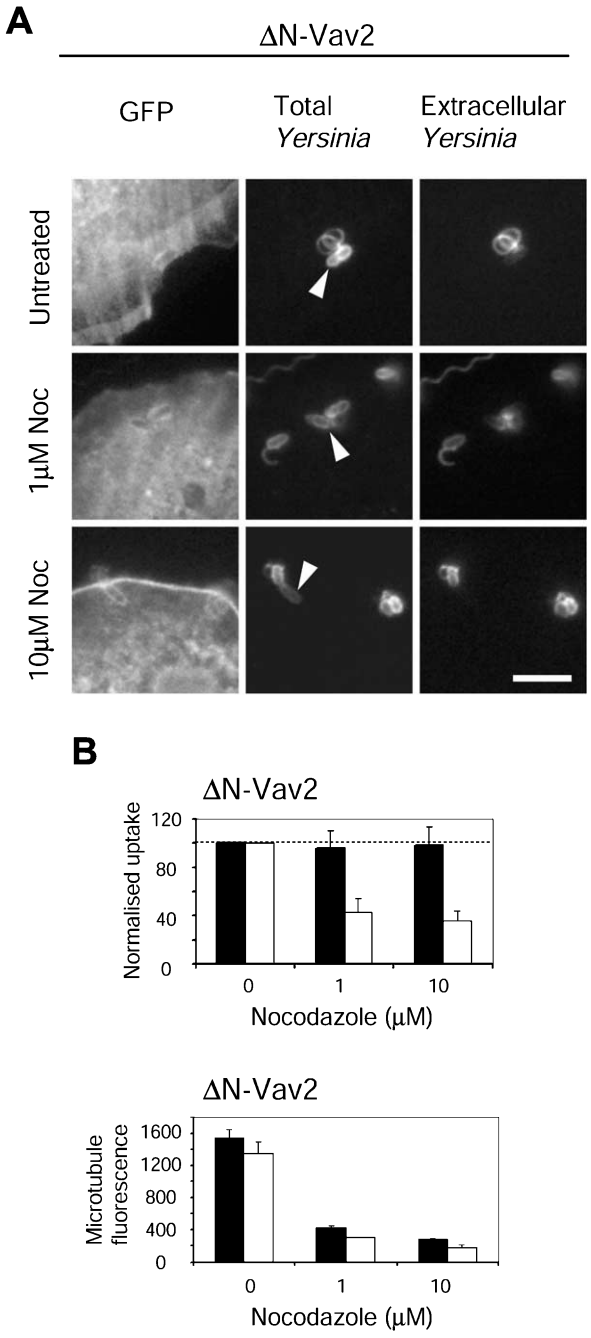

Fig. 3. Activated Vav2 prevents nocodazole-mediated suppression of uptake. A: Immunofluorescence analysis of *Yersinia* uptake in HeLa cells transfected with Δ184N-Vav2-GFP and pretreated with nocodazole prior to infection. Shown are localisation of Δ184N-Vav2-GFP, total cell-associated bacteria and extracellular bacteria. Arrowheads indicate internalised bacteria. Scale bar, 5 μm. B: Determination of YPIII uptake and microtubule polymerisation in nocodazole-treated HeLa cells transfected with Δ184N-Vav2-GFP (filled bars) or untransfected (open bars). Results are the mean ± S.D. of four separate experiments for the uptake analysis and mean ± S.D. of duplicate samples from one experiment for the flow cytometry analysis.

which contrasts with the partial effects of activated Rac or Cdc42 (compare Fig. 2B with Fig. 3B). Expression of C-terminal Vav2-GFP had no significant rescue effect (Supplementary data[1]). The differences in rescue efficiency of *Yersinia* uptake in nocodazole-treated cells by expressing Cdc42, Rac or Vav2 would suggest that Vav2 most likely activates both Rac and Cdc42 and possibly other additional GTPases. This is consistent with our previous observation that toxin B completely inhibits *Yersinia* uptake whereas dominant negative Rac causes partial inhibition [4]. Flow cytometry analyses of cells revealed that expression of Δ184N-Vav2-GFP only modestly affected microtubule polymerisation, thus further sub-

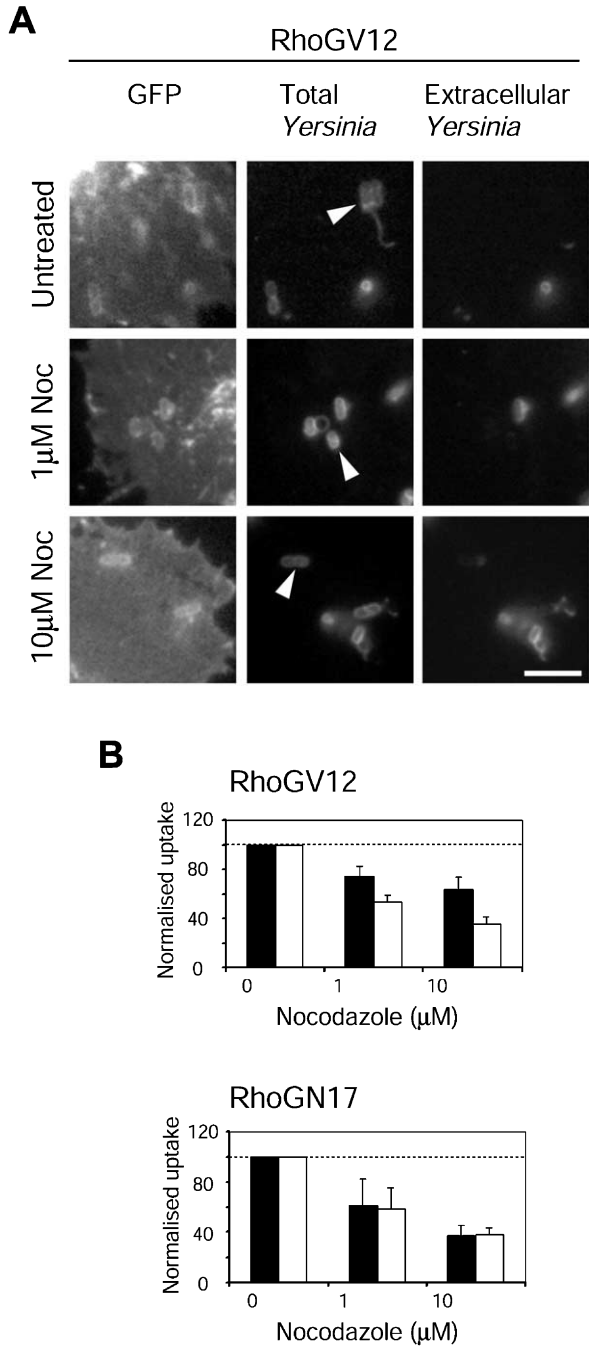

Fig. 4. Activated RhoG partially rescues nocodazole-mediated inhibition of uptake. A: Immunofluorescence analyses of *Yersinia* uptake in HeLa cells transfected with GFP-RhoGV12 and treated with increasing concentrations of nocodazole prior to infection. Shown are localisation of GFP-RhoGV12, total cell-associated bacteria and extracellular bacteria. Arrowheads indicate internalised bacteria. Scale bar, 5 μm. B: Determination of YPIII uptake by nocodazole-treated HeLa cells transfected with GFP-RhoGV12 or GFP-RhoGN17 (filled bars) or untransfected (open bars). Results are the mean ± S.D. of four separate experiments.

stantiating the proposal that this GEF does not regulate microtubules themselves but rather governs GTPase activity (Fig. 3B).

RhoG, which is a widely expressed GTPase, can activate Rac- and Cdc42-dependent ruffles, lamellipodia and filopodia formation [39]. Furthermore, depolymerisation of microtubules by nocodazole results in the loss of RhoG-induced Rac and Cdc42 morphological effects [39]. Given these observations we examined the effect of expressing activated RhoG (GFP-RhoGV12) and dominant negative RhoG (GFP-RhoGN17) on invasin-mediated phagocytosis of *Yersinia*. Expression of GFP-RhoGV12 was found to slightly stimulate uptake levels (111.8 ± 11.6%, $n = 6$). We have previously seen a similar level of stimulation of bacterial uptake when activated Rac (GFP-RacL61) is expressed [4]. However, in contrast to that of dominant negative RacN17 [4], we found that GFP-RhoGN17 had no inhibitory effect on *Yersinia* uptake (96.4 ± 16.2%, $n = 6$). The ability of RhoGV12 to rescue bacterial uptake in nocodazole-treated cells was however comparable to that seen with activated Rac or Cdc42, but not the sum of the two (compare Fig. 4B with Fig. 2B). Furthermore, co-expression of RhoGV12, RacL61 and Cdc42L61 failed to recover the level of nocodazole-affected uptake to the same degree of rescue as that seen with Vav2 (Supplementary data[1]). As seen with the other GTPases, both variants of RhoG localised to internalised bacteria (Fig. 4A and data not shown). Taken together, our observations suggest a signalling pathway where RhoG acts downstream of, or parallel to, Vav2 and upstream of either Rac or Cdc42 but not both.

Nevertheless, the limited effects of co-expressing activated variants of Rac, Cdc42 and RhoG indicated that additional factors, downstream of Vav2, were required for a complete rescue.

One possible additional factor is RhoA, which can be activated by Vav2 and also through microtubule destabilisation [32,34,40,41]. In accordance with reports by others, we observed an increase in stress fibre formation in nocodazole-treated cells, which would suggest upregulation of RhoA activity (data not shown). Thus, we expressed GTPase-active (GFP-RhoAV14) or GTPase-inactive (GFP-RhoAN19) variants of RhoA and examined uptake in the presence of nocodazole. However, overexpression of either protein resulted in partial rescue of bacterial uptake, where the inactive variant was the more efficient of the two in rescuing at the higher drug concentration (Supplementary data[1]). As RhoA is known to act antagonistically against Rac [42] the rescue with the dominant negative protein could be explained by Rac activation. This is consistent with a previous study demonstrating inactivation of Rac but activation of RhoA as a consequence of microtubule depolymerisation whereas the opposite result was observed when microtubules were allowed to regrow [43]. We have previously seen that both the constitutively active and dominant negative RhoA proteins generate a repressive effect on uptake as opposed to the stimulatory effect of activated Rac [4]. It is thus likely that phases of activation as well as inactivation of RhoA are influential in modulating uptake of *Yersinia*.

3.3. *Disruption of the dynein–dynactin complex partially suppresses invasin-mediated uptake but this effect is abrogated by co-expression of activated Vav2*

Our data so far indicate a role for Vav2 in imitating the effect of microtubule involvement in directing actin dynamics through Rho GTPases. Other factors which influence microtubule function are microtubule motors. Purified phagosomes have been shown to interact with microtubules in vitro and to move on microtubules in a mainly minus-end directional and dynein-dependent manner [44,45]. The dynein–microtubule interaction is mediated by the dynactin complex, and disruption of the dynein–dynactin interaction by expressing the p50 dynamitin subunit of dynactin inhibits dynein-dependent activity [46].

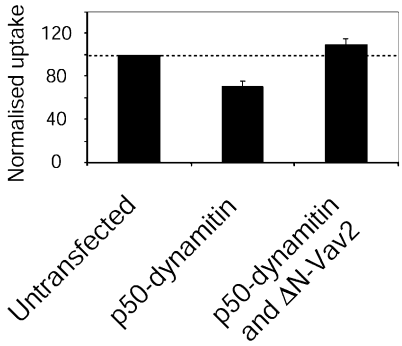

Fig. 5. Activated Vav2 rescues the effect of disruption of the dynein–dynactin complex on invasin-mediated uptake. Uptake of YPIII by nocodazole-treated HeLa cells co-expressing either p50 dynamitin and a GFP vector or p50 dynamitin and Δ184N-Vav2-GFP. Internalisation of bacteria by transfected cells is normalised to that of untransfected cells. Results are the mean ± S.D. of four separate experiments.

Hence, to determine whether this motor is involved in invasin-promoted uptake we co-expressed p50 dynamitin and a GFP vector (as a marker for transfection) in HeLa cells and analysed the effect on uptake of *Yersinia*. In transfected cells internalisation of bacteria was decreased by approximately 30% (uptake value; 70.5 ± 5.4%, $n = 4$) compared to untransfected cells. We have not yet pursued this aspect further, therefore we can only speculate why dynein may be involved in bacterial uptake. One possibility is that there may be microtubule-dependent trafficking, which may involve endocytosis or exocytosis of a factor(s) participating in early events such as integrin activation or clustering, or phagocytic cup formation. Indeed, microtubules and their motors have roles in endocytic regulation [47]. Given the apparent involvement of dynein we wondered whether upregulation of the actin cytoskeleton might overcome the effect of disrupting dynein activity. We found that co-expressing Δ184N-Vav2-GFP with p50 dynamitin elevated uptake compared to cells expressing p50 dynamitin alone (Fig. 5). This further supports our hypothesis that Vav2 can step in and assume control of GTPases when the microtubule network is no longer functional.

This present work demonstrates that internalisation of *Y. pseudotuberculosis* requires the integrity of the microtubule cytoskeleton. Others have reported that Rho GTPases can influence, or are influenced by, microtubule dynamics [25,26,29,43]. Our data indicate that an intact microtubule network, which implies a functional microtubule rail system, also controls actin dynamics via Rho GTPases. We show that GTPases and their regulators, such as the GEF Vav2, can compensate for a disrupted microtubule function allowing bacterial internalisation to occur. Many studies are currently focused on unravelling the precise mechanism of cross-talk between the actin and microtubule systems. We propose

that invasin-stimulated uptake can be used as a model system to dissect signalling between Rho GTPases and the microtubule and actin cytoskeleton networks.

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