

# Neural Wiskott–Aldrich syndrome protein is implicated in the actin-based motility of *Shigella flexneri*

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***Shigella*, the causative agent of bacillary dysentery, is capable of directing its own movement in the cytoplasm of infected epithelial cells. The bacterial surface protein VirG recruits host components mediating actin polymerization, which is thought to serve as the propulsive force. Here, we show that neural Wiskott–Aldrich syndrome protein (N-WASP), which is a critical target for filopodium formation downstream of Cdc42, is required for assembly of the actin tail generated by intracellular *S.flexneri*. N-WASP accumulates at the front of the actin tail and is capable of interacting with VirG *in vitro* and *in vivo*, a phenomenon that is not observed in intracellular *Listeria monocytogenes*. The verprolin-homology region in N-WASP was required for binding to the glycine-rich repeats domain of VirG, an essential domain for recruitment of F-actin on intracellular *S.flexneri*. Overexpression of a dominant-negative N-WASP mutant greatly inhibited formation of the actin tail by intracellular *S.flexneri*. Furthermore, depletion of N-WASP from *Xenopus* egg extracts shut off *Shigella* actin tail assembly, and this was restored upon addition of N-WASP protein, suggesting that N-WASP is a critical host factor for the assembly of the actin tail by intracellular *Shigella*.**  
**Keywords:** actin polymerization/N-WASP/*Shigella*/VirG

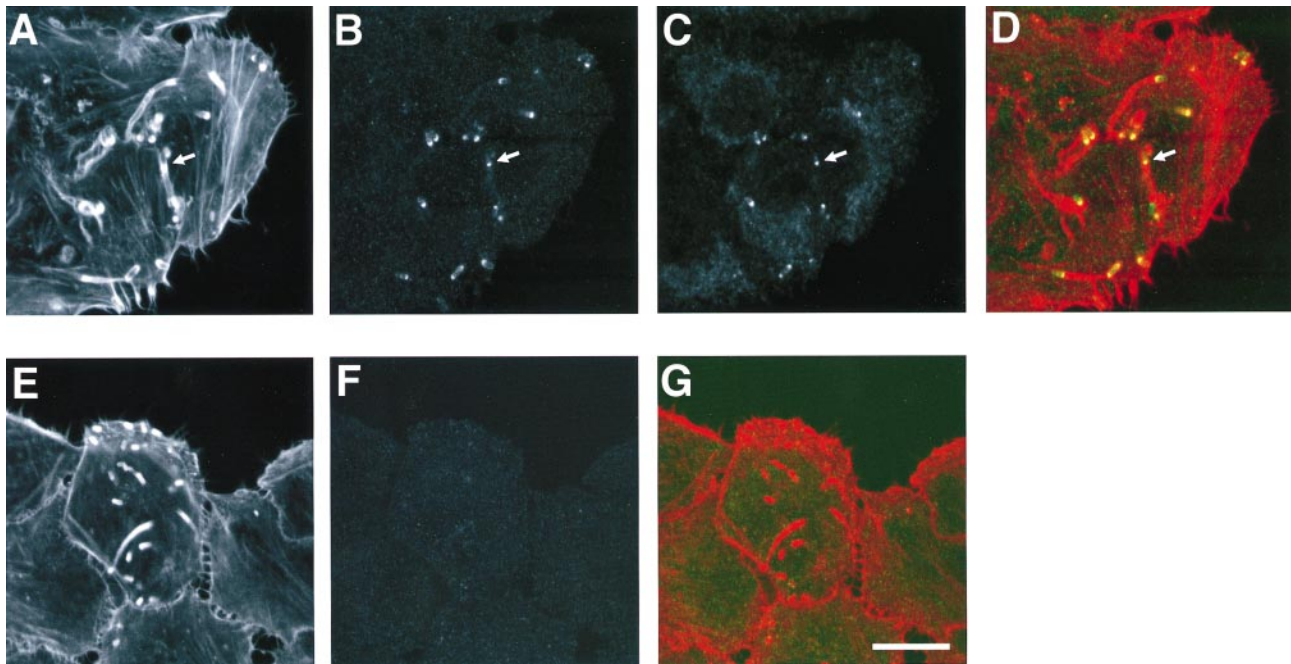
## Introduction

The abilities of *Shigella* to enter colonic epithelial cells and subsequently to spread within and between the cells are a prerequisite for causing dysentery. The capacity of the bacteria to spread in the cytoplasm and then to move into adjacent epithelial cells is mediated by the bacterial functions encoded by the *virG* (*icsA*) gene (Makino *et al.*, 1986; Bernardini *et al.*, 1989; Lett *et al.*, 1989). After invasion of epithelial cells, *Shigella* become surrounded by filamentous actin (F-actin). This F-actin ‘clot’ then rearranges into a tail (also known as a ‘comet’) which remains stationary in the cytoplasm and is left behind by bacteria moving ahead (Bernardini *et al.*, 1989; Prévost *et al.*, 1992; Goldberg *et al.*, 1993). When the spreading bacterium contacts the inner surface of the host cell plasma membrane, a long membrane protrusion (filopodium) develops with the F-actin tail behind it that is endo-

cytosed by the adjacent cells, resulting in the bacterium being surrounded by a double membranous vacuole (Kadurugamuwa *et al.*, 1991; Allaoui *et al.*, 1992; Goldberg *et al.*, 1993; Suzuki *et al.*, 1994). After disruption of the vacuole, bacteria are released into the new host cell cytoplasm and multiply again. Thus, this actin-based bacterial motility is critical for allowing invading *Shigella* to spread horizontally among colonic epithelial cells.

VirG is a surface-exposed outer membrane protein, and the sequence of the *virG* gene revealed that VirG is a 1102 amino acid protein (Lett *et al.*, 1989). VirG protein consists of three distinctive domains, the N-terminal signal sequence (amino acids 1–52), the 706 amino acid  $\alpha$ -domain (amino acids 53–758) and the 343 amino acid C-terminal  $\beta$ -core (amino acids 759–1102) (Goldberg *et al.*, 1993; Suzuki *et al.*, 1995). The  $\alpha$ -domain is presented on the surface of *Shigella* and contains repetitive sequences rich in glycine residues, while the  $\beta$ -core is embedded in the outer membrane (Suzuki *et al.*, 1995). The assembly of F-actin near the surface of intracellular *Shigella* is absolutely dependent on the surface presentation of VirG  $\alpha$ -domain, and the formation of an actin tail depends on the asymmetric distribution of VirG (Goldberg *et al.*, 1993; Rajakumar *et al.*, 1994; Sandlin *et al.*, 1995; Suzuki *et al.*, 1996). Recently, to demonstrate that VirG is the sole bacterial factor needed for movement in infected mammalian cells, experiments on actin-based movement were carried out using *Xenopus laevis* egg extracts (Goldberg and Theriot, 1995; Kocks *et al.*, 1995). In these studies, heterologous VirG expression in *Escherichia coli* K-12 was achieved by exploiting an OmpT<sup>-</sup> (OmpT is a trypsin-like outer membrane protease) VirG-expressing strain, in which the cleavage of VirG and, thus, the release of the  $\alpha$ -domain into the bacterial environment, was prevented (Nakata *et al.*, 1993). This strain displayed an asymmetric distribution of VirG on the bacterial body similar to *Shigella*, and was capable of intra- and intercellular movement (Kocks *et al.*, 1995).

The movement of *S.flexneri* (and *Listeria monocytogenes*) within the host cells resembles the formation of filopodia of locomoting cells, since those are mediated by actin polymerization and cause membrane protrusions (Bernardini *et al.*, 1989; Prévost *et al.*, 1992; Nobes and Hall, 1995). However, no known protein has yet been reported to be involved specifically in both phenomena. Several host proteins such as vinculin (Kadurugamuwa *et al.*, 1991; Suzuki *et al.*, 1996; Laine *et al.*, 1997), plastin (fimbrin) (Prévost *et al.*, 1992), filamin (Prévost *et al.*, 1992),  $\alpha$ -actinin (Zeile *et al.*, 1996) and vasodilator-stimulated phosphoprotein (VASP) (Chakraborty *et al.*, 1995) have been identified as being associated with the F-actin tail generated by intracellular *S.flexneri*. Amongst these proteins, only vinculin can interact directly with VirG (Suzuki *et al.*, 1996; Laine *et al.*, 1997), in which



**Fig. 1.** N-WASP accumulation at one pole of intracellular *Shigella*. HeLa cells were infected with *S.flexneri* YSH6000 (A–D) or *L.monocytogenes* 1/2a EGD (E–G). Triple immunofluorescence stainings (A–D) were performed with rhodamine–phalloidin (A), Cy2-labeled anti-N-WASP antibody (B) and Cy5-labeled rat VRG-N2 antibodies (C). Double immunofluorescence stainings (E–G) were performed with rhodamine–phalloidin (E) and Cy2-labeled anti-N-WASP antibody (F). The yellow color in the combined image shown in (D) and (G) indicates co-localization between F-actin (red) and N-WASP (green). Arrows indicate an intracellular bacterium recruiting N-WASP and F-actin. Bar, 10  $\mu$ m.

the 95 kDa vinculin head domain interacting with the VirG  $\alpha$ -domain is involved in promoting the formation of actin tail from intracellular *Shigella*. However, vinculin is not only associated with *Shigella* actin tail but is also distributed widely in focal adhesions, filopodia and lamellipodia. Recently, neural Wiskott–Aldrich syndrome protein (N-WASP) was identified as the Ash/Grb2-binding protein in brain, but its expression was also noted in other tissues including the colon (Miki *et al.*, 1996). The sequence of N-WASP is homologous to WASP (Derry *et al.*, 1994), and they both possess various functional motifs, such as a pleckstrin-homology (PH) domain, an IQ motif, a GBD/CRIB motif, a proline-rich region (P-region), a verprolin-homology region (V-region) and an ADF/cofilin-homology region (C-region) (Miki *et al.*, 1996). Importantly, when N-WASP or WASP is over-expressed in mammalian cells, only N-WASP induces long filopodia (Miki *et al.*, 1998). These data led us to investigate whether N-WASP is involved directly in actin comet formation by intracellular *Shigella*.

## Results

### **Intracellular *S.flexneri* can recruit N-WASP at one pole of the bacterium**

HeLa, Cos-7, PtK2 or Caco-2 cells and *Xenopus* egg extracts, which all have been used for studies of bacterial motility, were investigated for their expression of N-WASP. Although expression of N-WASP in each cell lysate, as assayed by an immunoblot with an anti-N-WASP antibody, varied slightly, they expressed significant amounts of the protein (data not shown). HeLa cells infected with wild-type *S.flexneri* 2a strain YSH6000 were examined further for the cellular distribution of N-WASP

by triple immunostainings with rhodamine–phalloidin, Cy2-labeled rabbit anti-N-WASP antibody and Cy5-labeled rat anti-VirG antibody. After 60 min of infection, N-WASP was highly concentrated at one pole of the bacterium, from which F-actin was also assembled as a comet tail (Figure 1A, B and D). Under the same conditions, N-WASP did not accumulate at all on the surface of M94, a *virG*-deficient derivative of YSH6000 unable to form an actin comet tail (data not shown). Indeed, the N-WASP accumulated at one pole of intracellular YSH6000 was always confined to the area expressing VirG on the bacteria (Figure 1C). Similar results were obtained with human Caco-2 cells (data not shown). To assess further the role of N-WASP in the *Shigella*-induced actin tail, HeLa cells were infected with YSH6000 for 20, 40 and 60 min, and then examined for changes in the distributions of N-WASP, VirG and F-actin on the bacteria by confocal microscopy. After 20 min of infection, all intracellular YSH6000 accumulated VirG at one pole of bacterium and approximately half of the bacteria had detectable F-actin and N-WASP accumulation. At 60 min after infection, ~90% of bacteria possessed an assembled F-actin. At this time point, the N-WASP associated with *Shigella* was still confined to the front of the tail, suggesting that N-WASP plays a role in assembly of F-actin in infected cells.

### **N-WASP cannot be recruited by intracellular *Listeria***

Like *Shigella*, *L.monocytogenes*, a Gram-positive bacterium, moves in mammalian cells by generating an actin comet (for reviews, see Lasa and Cossart, 1996; Machesky, 1997), an ability which requires the expression of ActA protein on the bacterial surface (Domann *et al.*, 1992;

Kocks *et al.*, 1992). Hence, we infected HeLa cells with *L. monocytogenes* 1/2a EGD and examined its ability to recruit N-WASP on the bacterial surface by double immunostaining with Cy2-labeled anti-N-WASP antibody and rhodamine-phalloidin. As shown in Figure 1E–G, at 120 min after infection, none of the bacteria in the HeLa cells had recruited localized N-WASP in the region of the actin comet tail, suggesting that accumulation of N-WASP at one pole of the intracellular bacterium is a *Shigella*-specific event.

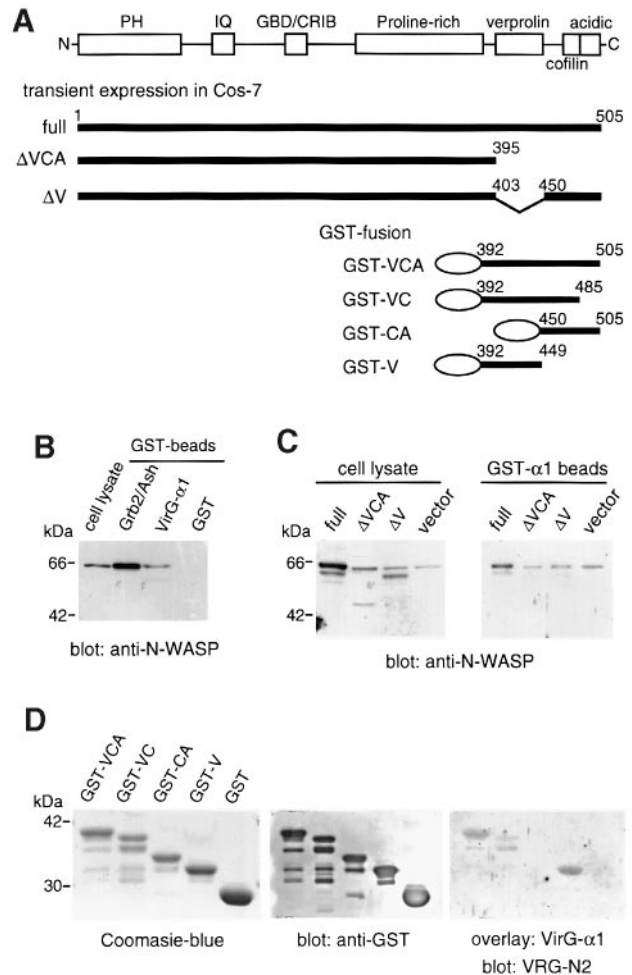
### *Shigella* VirG binds to the verprolin-homology region of N-WASP

Since N-WASP accumulated at the site of VirG expression on intracellular *Shigella*, the ability of N-WASP to interact with VirG was first investigated by co-precipitation experiments. HeLa cell lysates were incubated with GST- $\alpha$ 1 and GST-Ash/Grb2 fusion proteins or GST alone immobilized to glutathione-Sephadex. GST- $\alpha$ 1 contained residues Thr53–Arg779 of the VirG peptide, an essential portion for eliciting actin comet tail formation (Suzuki *et al.*, 1996), while GST-Ash/Grb2 (Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992) was exploited originally for the isolation of N-WASP (Miki *et al.*, 1996). Proteins co-precipitating with the GST beads were analyzed by immunoblots with anti-N-WASP antibody. As shown in Figure 2B, N-WASP co-precipitated with GST- $\alpha$ 1 as well as with GST-Ash/Grb2, but not with GST alone, suggesting that N-WASP was capable of interacting with VirG *in vitro*. To assess which domains in N-WASP are responsible for interacting with VirG, Cos-7 transfectants (Figure 2A), that overexpressed either the full-length N-WASP (Met1–Asp505),  $\Delta$ VCA protein (lacking the verprolin- and cofilin-homology regions) or  $\Delta$ V protein (lacking the verprolin-homology region), were lysed and incubated with GST- $\alpha$ 1. The proteins precipitated by GST- $\alpha$ 1 beads were then analyzed by immunoblotting with anti-N-WASP antibody. The full-length N-WASP, but not the other products,  $\Delta$ VCA and  $\Delta$ V, were precipitated with GST- $\alpha$ 1 (Figure 2C), indicating that the verprolin-homology region of N-WASP could be involved in the interaction with VirG.

To investigate further whether this verprolin-homology region can interact directly with VirG, we constructed four GST fusion proteins containing various portions of the VCA region of N-WASP (Figure 2A) and examined their ability to interact with the VirG  $\alpha$ -domain. The resulting GST fusion proteins were blotted onto a membrane filter, overlaid with VirG- $\alpha$ 1 (see Materials and methods), and its binding to the VCA segments was examined by immunostaining with rabbit VRG-N2 antibody (a VirG-specific antibody). The results showed that GST-VCA, GST-VC and GST-V, but not GST-CA or GST alone, were bound by VirG- $\alpha$ 1 (Figure 2D). We thus concluded that the 58 amino acids of the verprolin-homology region of N-WASP take part in the interaction with VirG.

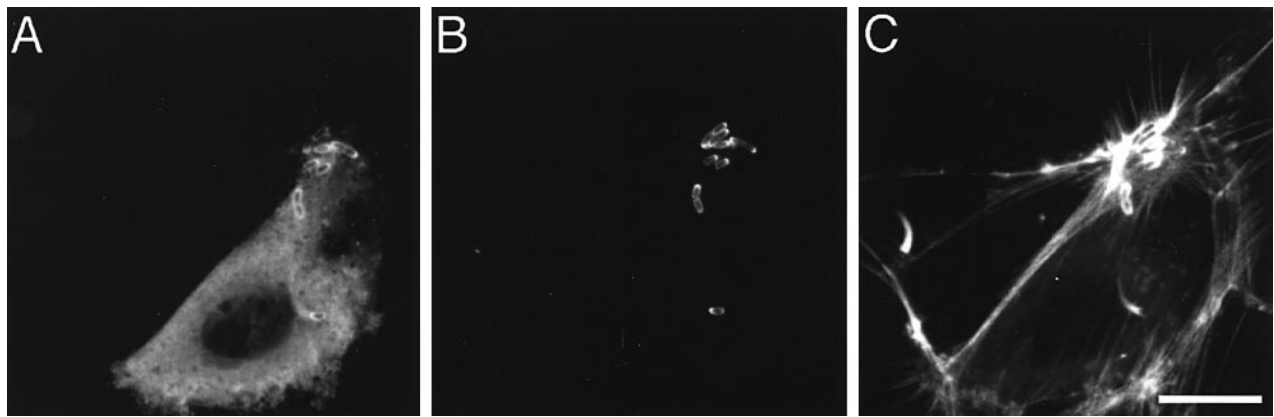
### The verprolin-homology region of N-WASP interacts with VirG *in vivo*

To investigate whether the verprolin-homology region of N-WASP is able to interact with VirG *in vivo*, Ptk2 cells infected with *S. flexneri* M94 (a *virG* mutant of YSH6000)



**Fig. 2.** Association of N-WASP with VirG is mediated by its verprolin-homology region. (A) Schematic representation of the structure of N-WASP and its variants. Bars indicate deleted constructs expressed in Cos-7 or recombinant GST fusion proteins. (B) Cell lysates from HeLa cells were incubated with Sepharose-immobilized GST-fusion proteins (20  $\mu$ g). Proteins bound to the beads were analyzed by immunoblotting with anti-N-WASP antibody. (C) Cell lysates from Cos-7 cells expressing N-WASP variants were used for a binding assay with GST- $\alpha$ 1. Immunoblots of whole cell lysates are shown in the left panel. The 65 kDa protein bands appearing in all the lanes indicate endogenous N-WASP expressed in Cos-7 cells. The thick 65, ~47 and ~59 kDa bands correspond to full-length,  $\Delta$ VCA and  $\Delta$ V constructs, respectively. Immunoblots of N-WASP bound to GST- $\alpha$ 1 beads are shown in the right panel. (D) Recombinant GST fusion proteins of N-WASP (5  $\mu$ g/lane) were analyzed by a gel blot overlay assay with VirG- $\alpha$ 1. Coomassie blue staining and immunoblots with anti-GST antibody are shown in the left and middle panel, respectively. Proteins transferred onto nitrocellulose membrane were overlaid with recombinant VirG- $\alpha$ 1 (10  $\mu$ g/ml), and bound protein was detected with rabbit VRG-N2 antibody.

carrying pD10-1, a plasmid expressing VirG at a high level (Suzuki *et al.*, 1996), were microinjected with purified GST-V. Fifteen minutes after microinjection, the Ptk2 cells were immunostained with Cy2-labeled goat anti-GST antibody, Cy5-labeled rabbit VRG-N2 antibody and rhodamine-phalloidin. As shown in Figure 3, the injected GST-V was accumulated around the VirG-expressing area on *Shigella* (Figure 3A and B), where the actin tail was generated (Figure 3C). When GST-V was injected into Ptk2 cells infected with *L. monocytogenes*, it did not accumulate at all on the intracellular bacteria

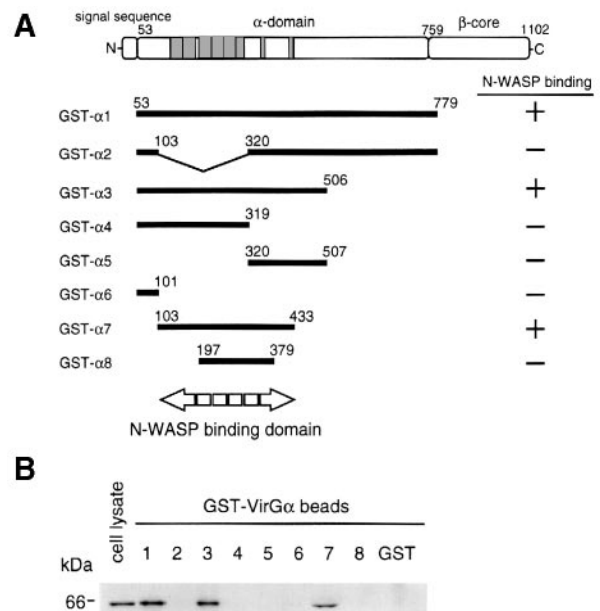


**Fig. 3.** Targeting of GST-verprolin-homology region of N-WASP to surface-exposed VirG on intracellular *Shigella*. PtK2 cells infected with *S. flexneri* (M94 carrying pD10-1) were microinjected with GST-V fusion protein and immunostained using Cy2-labeled anti-GST (A), Cy5-labeled rabbit VRG-N2 antibodies (B) and rhodamine-phalloidin (C). Bar, 10  $\mu$ m.

(data not shown). These data further confirmed that the verprolin-homology region of N-WASP is involved in specific interaction with VirG expressed on intracellular *Shigella*.

#### Identification of the VirG domain interacting with N-WASP

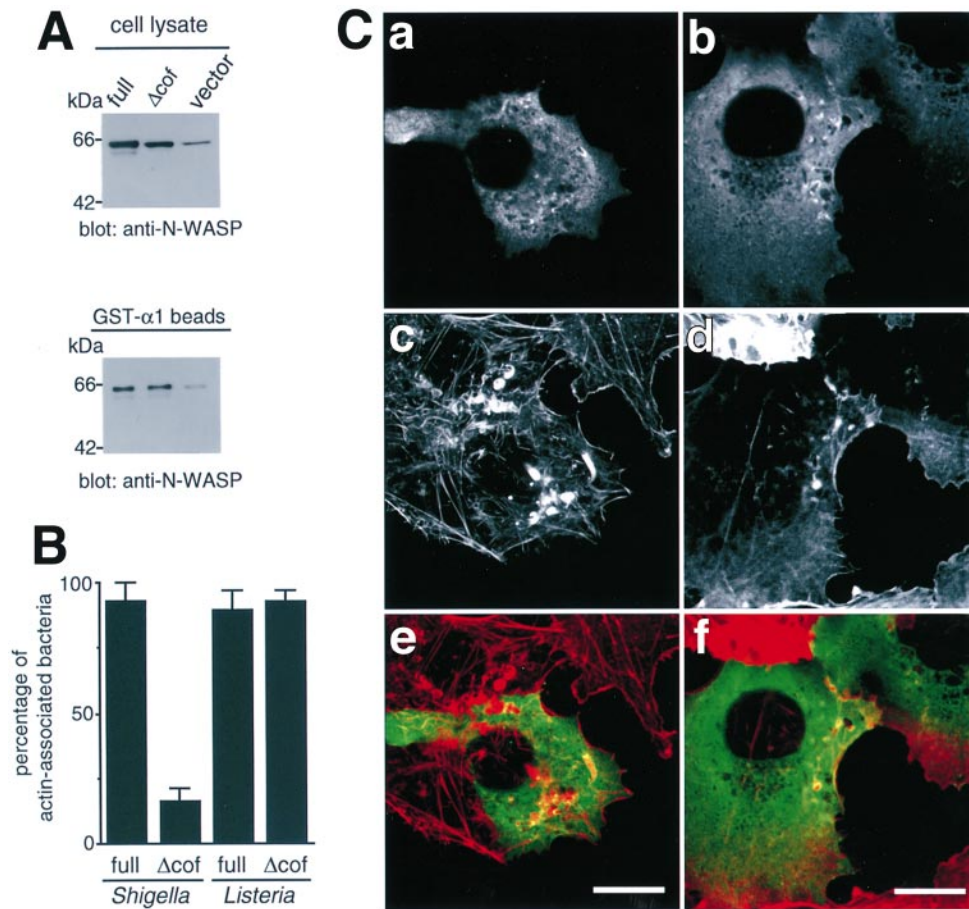
Our recent studies have indicated that the VirG  $\alpha$ -domain (the surface-exposed VirG sequence) consists of two distinct regions, an N-terminal portion corresponding to amino acids 53–508, and the remaining C-terminal portion corresponding to amino acids 509–758 (Suzuki *et al.*, 1995, 1996). The N-terminal region contains eight glycine-rich repeats, and is essential for eliciting an actin comet tail in intracellular *Shigella*, while the following C-terminal region is required for the unipolar deposition of VirG on the bacterium (Suzuki *et al.*, 1996). In order to identify which VirG portion takes part in the interaction with N-WASP, we constructed various GST- $\alpha$ -domain derivatives, named GST- $\alpha$ 1 to GST- $\alpha$ 8 (see Figure 4A), and tested each of them for their ability to interact with N-WASP by incubating them in HeLa cell lysates followed by precipitation with the beads to which the GST derivatives were attached. Analysis of the precipitated proteins by immunoblotting with anti-N-WASP antibody indicated that GST- $\alpha$ 1, GST- $\alpha$ 3 and GST- $\alpha$ 7 constructs containing all of the eight glycine-rich repeats could be bound by N-WASP (Figure 4B). To confirm that these three VirG  $\alpha$ -domain portions were capable of binding N-WASP in infected cells, *Shigella* strain M94 carrying pD10-1 (the whole VirG  $\alpha$ -domain), pD10-*virG3* (VirG3, lacking amino acids 509–729 of the VirG  $\alpha$ -domain) or pD10-*virG4* (VirG4, lacking amino acids 104–506 of the VirG  $\alpha$ -domain) (Suzuki *et al.*, 1996) was examined for its ability to recruit an N-WASP clot as well as recruit an F-actin assembly on the intracellular bacterium. The results showed that bacteria expressing wild-type VirG and VirG3 but not VirG4 were capable of eliciting accumulation of N-WASP and F-actin assembly in the infected cells as examined by immunostaining (data not shown). These results indicated that the minimum VirG domain required for the interaction with N-WASP lies within the Arg103–Ala433 region in the VirG polypeptide.



**Fig. 4.** VirG interacts with N-WASP via its glycine-rich repeat region. (A) Schematic representation of the construction of GST- $\alpha$  domain fusion proteins and summary of N-WASP binding assays. (B) Cell lysates from HeLa cells were incubated with GST-VirG $\alpha$  fusions immobilized on Sepharose beads. Proteins bound to the beads were analyzed by immunoblotting with anti-N-WASP antibody.

#### A dominant-negative N-WASP affects actin assembly on intracellular *Shigella*

It has been indicated that activated N-WASP promotes the depolymerization activity of actin filaments through its ADF/cofilin-homology region, and that overexpression of  $\Delta$ cof, a mutant lacking four amino acids highly conserved in cofilin-family members, can inhibit filopodium formation, thus indicating that the  $\Delta$ cof mutant functions in a dominant-negative manner (Miki *et al.*, 1998). Thus, we exploited the  $\Delta$ cof mutant to check the observed interaction of N-WASP with VirG involved in the assembly of F-actin on *Shigella* in infected mammalian cells. As shown in Figure 5A, the  $\Delta$ cof mutant was capable of interacting with VirG *in vitro*. The effect of overexpression

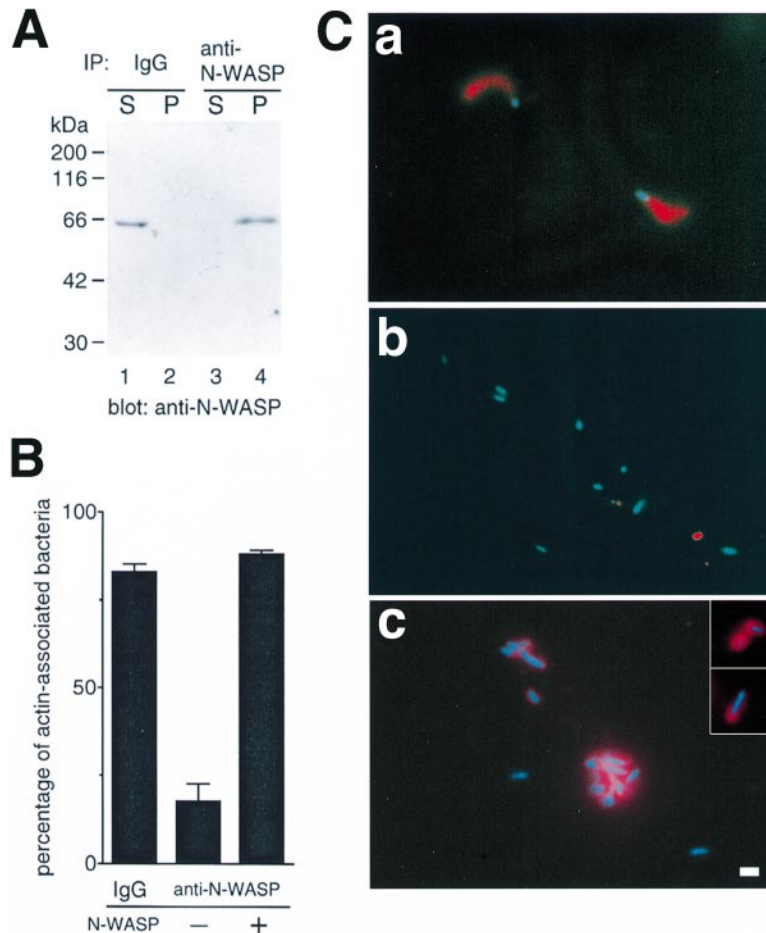


**Fig. 5.** Actin assembly by *Shigella* is inhibited by transient expression of dominant-negative N-WASP in Cos-7 cells. (A) Cell lysates from Cos-7 cells expressing N-WASP variants were used for a binding assay with GST- $\alpha$ 1. Immunoblots of whole cell lysates are shown in the upper panel. Immunoblots of N-WASP bound to GST- $\alpha$ 1 beads are shown in the lower panel. (B) Quantitation of the actin-associated intracellular bacteria in transfected cells. The transfected cells were infected with *S.flexneri* (M94 carrying pD10-1) or *L.monocytogenes* 1/2a EGD. The data shown are the means of triplicate experiments. The top bars show the standard deviation of the mean. (C) Cos-7 cells overexpressing full-length N-WASP (a, c and e) or  $\Delta$ cof mutant (b, d and f) were infected with *Shigella* and immunostained using anti-N-WASP antibody (a and b) and rhodamine-phalloidin (c and d). The yellow color in the combined image shown in (e) and (f) indicates co-localization between N-WASP (green) and F-actin (red). Bars, 10  $\mu$ m.

of the  $\Delta$ cof mutant on the formation of actin comet tail by *Shigella* infecting Cos-7 cells was examined by counting the number of actin assemblies associated with the intracellular bacteria in Cos-7 cells overexpressing  $\Delta$ cof or the full-length N-WASP. As shown in Figure 5B and C, after 60 min of infection, the intracellular bacterium had an actin tail in Cos-7 cells overexpressing the full-length N-WASP, whereas it was almost abolished in Cos-7 cells overexpressing the  $\Delta$ cof mutant. Approximately 90% ( $92.8 \pm 7.2\%$ ,  $n = 85$ ) of intracellular bacteria possessed an actin clot in Cos-7 cells overexpressing the full-length N-WASP, whereas only ~16% ( $16.4 \pm 4.9\%$ ,  $n = 138$ ) had an actin assembly in Cos-7 cells overexpressing the  $\Delta$ cof mutant, even though  $\Delta$ cof accumulation at one pole of the bacteria was observed (Figure 5C, panel b). The overexpression of  $\Delta$ cof or the full-length N-WASP had no effect on actin comet formation from intracellular *L.monocytogenes* (Figure 5B). These results show that the overexpression of a dominant-negative N-WASP greatly inhibits formation of actin tail by intracellular *S.flexneri*.

#### Depletion of N-WASP from *Xenopus* egg extracts affects the ability of VirG-expressed bacteria to recruit actin assembly

To investigate further the functional requirement for N-WASP for the assembly of an actin tail by intracellular *Shigella*, we depleted N-WASP from *Xenopus* egg extracts by immunoprecipitation with anti-N-WASP antibody immobilized on protein A beads. The immunodepleted extracts were then tested for their ability to support the possession of an actin clot or tail from *E.coli* MC1061 *ompT::Km* carrying pD10-1, according to the methods described by Goldberg and Theriot (1995). Upon depletion of N-WASP, generation of an actin tail was shut off, and only  $17.6 \pm 5.0\%$  ( $n = 229$ ) of the bacteria associated with a weak actin clot around the bacterial body (Figure 6B and C, panel b), whereas ~80% of bacteria ( $82.9 \pm 2.3\%$ ,  $n = 198$ ) possessed an actin clot or tail in a mock-depleted extract (Figure 6B and C, panel a), this level being similar to that in the untreated extract ( $83.7 \pm 4.8\%$ ,  $n = 135$ ). When a similar amount of N-WASP, prepared by a baculovirus system, to that in the



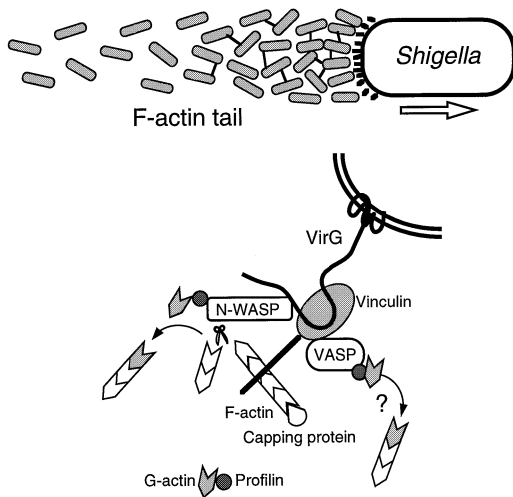
**Fig. 6.** N-WASP is essential for VirG-induced actin assembly in *Xenopus* egg extracts. **(A)** Pre-immune IgG and anti-N-WASP antibody were used for immunodepletion of N-WASP from *Xenopus* egg extracts. Depleted extracts and precipitated proteins bound to the beads were analyzed by immunoblotting with anti-N-WASP antibody. **(B)** Quantitation of the actin-associated bacteria in N-WASP-depleted extracts. The data shown are the means of triplicate experiments. The top bars show the standard deviation of the mean. **(C)** Tail formation in N-WASP-depleted *Xenopus* egg extracts. Bacteria and actin tails were visualized by adding DAPI-labeled *E.coli* expressing VirG (blue) and rhodamine-labeled G-actin (red), respectively: (a) mock-depleted extracts, (b) N-WASP-depleted extracts, (c) N-WASP-depleted extracts plus purified N-WASP. Insets in (c) show that the bacteria possessed a short actin tail. Bar, 1  $\mu$ m.

original extracts (~45 nM) was added to the N-WASP-depleted extracts, although the formation of a long actin tail from the bacteria was less efficient than in the original extract (Figure 6C, panels a and c), the bacterial population associated with an actin clot was greatly increased ( $88.0 \pm 1.1\%$ ,  $n = 233$ ) (Figure 6B and C, panel c). These data demonstrate that N-WASP is functionally involved in the promotion of actin assembly by *Shigella*.

## Discussion

Recent studies have shown that some bacteria such as *Shigella*, *Listeria* and *Rickettsia* or viruses such as vaccinia virus have gained an ability to control dynamic remodeling of the actin cytoskeleton in order to achieve actin-based motility in mammalian cells in their efforts to spread within and between the cells and evade the host defense system (Lasa and Cossart, 1996; Higley and Way, 1997; Machesky, 1997). Although the precise mechanisms underlying actin-based motility still remain to be elucidated, the systems exploited by such pathogens have provided a simplified model that mimics the actin filament dynamics

involved in lamellipodial or filopodial protrusions. To gain further insights into *Shigella* actin-based motility, we investigated the possibility of N-WASP involvement in actin tail formation by intracellular *Shigella*, since N-WASP has been shown to bind actin directly, depolymerize actin filaments and regulate cortical cytoskeletal rearrangement, including formation of filopodia (Miki *et al.*, 1996, 1998). Furthermore, although N-WASP is expressed predominantly in brain, it is also distributed in other tissues including the colon (Miki *et al.*, 1996). In this study, we found that N-WASP can interact with VirG under *in vitro* and *in vivo* conditions and plays a critical role in eliciting *Shigella* actin tail formation in infected cells. This conclusion was deduced from the following results: (i) N-WASP accumulation at one pole of intracellular *Shigella* is always confined to the VirG deposition area on the bacterial surface; (ii) N-WASP is able to interact with a VirG domain ( $\alpha$ -domain), which we previously showed to be required for assembly of F-actin on intracellular *Shigella* (Suzuki *et al.*, 1996); (iii) overexpression of a dominant-negative N-WASP mutant in Cos-7 cells infected with *Shigella* resulted in a decrease in actin tail formation; and (iv) actin tail formation by



**Fig. 7.** Hypothetical model for VirG-induced actin polymerization on *Shigella* in infected mammalian cells. The VirG  $\alpha$ -domain exposed on the bacterial surface binds vinculin and N-WASP. The vinculin could then interact with actin filaments and/or other signaling molecules such as VASP. The actin filaments recruited may be partially depolymerized by N-WASP and the uncapped barbed ends become exposed. At the uncapped barbed ends, actin polymerization would occur, possibly promoted by profilin. In this model, we are not sure whether or not the vinculin and N-WASP are recruited by VirG at the same time and by the same polypeptide.

*Shigella* supported by *Xenopus* egg extracts was shut off upon depletion of N-WASP.

N-WASP contains at least six distinct domains: a PH domain; an IQ motif; a GBD/CRIB motif; a proline-rich region; a verprolin-homology region; and an ADF/cofilin-homology region (Miki *et al.*, 1996). The proline-rich region (P-region), verprolin-homology region (V-region) and ADF/cofilin-homology region (C-region) are likely to participate directly in generation of the actin tail from the surface of intracellular *Shigella*. The V-region was shown to be able to bind directly to VirG under *in vitro* and *in vivo* conditions. The amino acid sequence of the P-region, which contains four Gly(Pro)<sub>5</sub> sequences, was equivalent to the putative profilin-binding motifs distributed among the VASP (Haffner *et al.*, 1995), WASP (Derry *et al.*, 1994) and Mena (Gertler *et al.*, 1996) proteins, although the number of motifs varied. Since it has been indicated that those proline-rich repeats interact with profilin (Reinhard *et al.*, 1995; Gertler *et al.*, 1996; Higley and Way, 1997), and the P-region of N-WASP can interact with profilin *in vitro* (Miki *et al.*, 1998), we presume that N-WASP-bound profilin could mediate local actin polymerization on the surface of intracellular *Shigella* (Figure 7). Recent studies have indicated that activation of N-WASP by binding of Cdc42 to the GBD/CRIB motif results in exposure of the VCA actin-depolymerizing region, which is thought to be masked in unstimulated full-length N-WASP through the intramolecular interaction between the acidic residues at the C-terminal end and the basic region near the GBD/CRIB motif region (Miki *et al.*, 1996, 1998). In this context, the VCA region of N-WASP may be important for the formation of actin tail from intracellular *Shigella*, in that the stable capped actin filaments may be partially depolymerized by N-WASP and the uncapped barbed ends become exposed to serve as sites for actin polymerization (Zigmond, 1996; Welch

*et al.*, 1997). At the uncapped barbed ends, actin polymerization would occur and it could be promoted by profilin (Pantaloni and Carlier, 1993).

It has been postulated that host cytoskeletal proteins responsible for promoting actin filament nucleation and elongation from intracellular *Shigella* and *Listeria* should be localized only at the front of the tail, and perhaps associated with the surface of the bacterium (Sanger *et al.*, 1992; Theriot *et al.*, 1992; Goldberg and Theriot, 1995). Hence, we investigated the distribution of N-WASP together with that of VASP in *Shigella* actin tail in infected cells and compared it with that of intracellular *Listeria*. Although the N-WASP clot appearing on intracellular *Shigella* was always immediately adjacent to the back half of the bacterium, it was never detected on intracellular *Listeria*. However, VASP has been reported to be accumulated around the area expressing ActA on intracellular *L.monocytogenes*, and the VASP protein was shown to bind directly to the proline-rich repeats domain in ActA (Niebuhr *et al.*, 1997). In contrast to *Listeria*, VASP was shown to be unable to bind to VirG *in vitro*, perhaps due to the absence of proline-rich repeats in the VirG polypeptide (Chakraborty *et al.*, 1995). Examination of the distribution of VASP in HeLa cells infected by *S.flexneri* or *L.monocytogenes* using immunostaining confirmed that the distribution of VASP in the actin tail generated by *Shigella* and *Listeria* was different (T.Suzuki, unpublished data). In the experiment, we noted that the distribution of VASP, but not of N-WASP, was similar to the distribution of vinculin in the *Shigella* actin tail (Suzuki *et al.*, 1996; Laine *et al.*, 1997). Since it has been shown that VASP is bound by the proline-rich domain in vinculin (Brindle *et al.*, 1996; Reinhard *et al.*, 1996), it is thus possible that vinculin and VASP could be co-localized in the actin tail in *Shigella*-infected cells. Based on our results together with other studies (Chakraborty *et al.*, 1995; Zeile *et al.*, 1996), we assume that the role of VASP in the assembly of actin in *Listeria* is different from that in *Shigella*.

In this study, we have identified the VirG region involved in interacting with N-WASP, which corresponds to Arg103–Ala433 in the VirG polypeptide. This region contains eight glycine-rich repeats (G-region) and is essential for recruiting F-actin assembly on intracellular *Shigella* (Suzuki *et al.*, 1996). In our previous study, we found that the G-region of VirG had a capacity to interact with the vinculin head *in vitro*. Indeed, we observed that the ability of the vinculin head to bind to to GST- $\alpha$ 1, which contains the G-region of VirG, was reduced upon addition of the V-region of N-WASP in a dose-dependent manner *in vitro*, suggesting that the domains in VirG binding to N-WASP and vinculin could overlap at least in part (T.Suzuki, unpublished data). The vinculin head has been shown to bind talin, while the vinculin tail portion can bind and cross-link F-actin (Johnson and Craig, 1994, 1995). Micro-injection studies have indicated that injection into PtK2 cells infected with *S.flexneri* of one of the proline-rich sequences of ActA (FEFPPPPTDE) or that of VASP (GPPPPPGPPPPPGPPPPP) results in decreased *Shigella* movement that is restored upon co-injection of profilin (Zeile *et al.*, 1996). Therefore, in this way, vinculin may serve as an adaptor protein for VirG on intracellular bacteria, in which the vinculin may recruit VASP-bound

profilin or another host protein that facilitates elongation from the barbed end of nascent actin filaments at the bacterial surface (Figure 7). Alternatively, the VirG-bound vinculin might serve as an actin filament recruiter (Johnson and Craig, 1994; Jockusch and Rüdiger, 1996) so that actin filament elongation on the *Shigella* surface with the aid of other VirG-bound host proteins such as N-WASP would be feasible (Figure 7). Since the affinity of GST- $\alpha 1$  for the vinculin head portion, as measured by its  $K_d$  value ( $\sim 0.3 \mu\text{M}$ ) (T.Suzuki, unpublished data), is similar to the affinity of the vinculin tail for F-actin ( $0.6\text{--}0.8 \mu\text{M}$ ) (Johnson and Craig, 1995), it is possible that the vinculin, once having interacted with VirG, could in turn be diffused into the elongated actin tail, leaving the N-WASP bound by VirG on the *Shigella* surface. Although we have discussed an important role for vinculin in *Shigella* actin-based motility, there have been two controversial reports on the requirement for vinculin. Goldberg (1997) examined the functional role of vinculin in *Shigella* actin-based motility in a vinculin-deficient F9 cell line variant 5.51, and observed that *Shigella* are able to form actin tails and are motile as in the F9 cells, suggesting that vinculin is not the essential factor. In contrast, Laine *et al.* (1997) found that vinculin proteolysis occurred in *Shigella*-infected cells and that the cleaved head portion of vinculin is a rate-limiting factor in *Shigella* motility. In the latter study, they proposed that the cleaved vinculin head bound by VirG can interact with VASP, through which profilin could be recruited, thus promoting assembly of actin on the *Shigella* surface. In this regard, it should be noted that cleavage of vinculin in HeLa cells infected by *S.flexneri* 2a YSH6000 or M94 (a *virG* mutant of YSH6000) strains was not detected after 60 min of infection in our study (T.Suzuki, unpublished data). At present, therefore, we are not sure how these discrepant observations were made in each study, whether they merely reflect the different cell types or the absence of appropriate control. In any case, we must await further studies to elucidate the exact role of VirG-bound vinculin and N-WASP in generating a *Shigella* actin tail.

In this study, we performed the functional assay for the requirement for N-WASP for the assembly of an actin clot and tail by intracellular *Shigella* by using *Xenopus* egg extracts, and revealed that depletion of N-WASP from the *Xenopus* egg extracts affected the ability of VirG-expressed bacteria to recruit actin assembly. Although the purified N-WASP was sufficient to support the assembly of dense actin clouds and sometimes a short actin tail (insets in Figure 6C, panel c), it was still less efficient than in the original extract for the formation of a long actin tail by the bacteria. This may result from the elimination of other host factor(s) required for facilitating the elongation of actin tail which could have been co-precipitated together with N-WASP, or it may result from the use of heterogenous N-WASP which was added to the depleted extract (see Materials and methods).

In conclusion, our results show that N-WASP is required to trigger both actin assembly and movement of *Shigella* in mammalian cells, although this was not the case in intracellular *Listeria*. Although the precise role of N-WASP in assembly of actin filaments on the *Shigella* surface still awaits further study, the results presented in this study will provide some insight into understanding the systems

underlying actin-based motility of other pathogens as well as the formation of filopodia from locomoting mammalian cells.

## Materials and methods

### Bacterial strains, cell culture and media

The *S.flexneri* 2a YSH6000 and *L.monocytogenes* serotype 1/2a EGD strains have been described previously (Sasakawa *et al.*, 1986; Domann *et al.*, 1992). M94 (*S.flexneri virG::Tn5*) carrying pD10-1, pD10-1*virG3* or pD10-1*virG4* were prepared as described previously (Suzuki *et al.*, 1996). All strains were grown routinely in brain-heart infusion (BHI) broth (Difco) at 37°C. HeLa cells were maintained in minimal essential medium (MEM) (Nissui, Tokyo, Japan) with 10% fetal calf serum (FCS) (Nichirei, Tokyo, Japan). Caco-2 cells were grown in MEM supplemented with 10% FCS and 0.1 mM non-essential amino acids (Gibco), and Cos-7 cells and PtK2 cells (ATCC CCL 56) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) containing 10% FCS.

### Antibodies

The anti-N-WASP-specific rabbit antibody has been described previously (Miki *et al.*, 1996). The rabbit VRG-N2 and anti-GST antibodies were prepared as described by Suzuki *et al.* (1996). The rat VRG-N2 was obtained by immunization of rats with the same synthesized peptides as for rabbit antibody.

### Infection of cultured cells and immunofluorescence microscopy

All cells were grown on glass coverslips to  $\sim 60\%$  confluency in the absence of antibiotics. Cells were infected with bacteria at a multiplicity of infection of 100 per cell. In the case of *S.flexneri*, the plates were centrifuged at 700 *g* for 10 min after adding bacteria. After 20–60 min, the plates were washed extensively with phosphate-buffered saline (PBS) and fresh medium supplemented with gentamycin (100  $\mu\text{g}/\text{ml}$ ) and kanamycin (60  $\mu\text{g}/\text{ml}$ ) added. The infected cells were incubated for an additional 1 h (*S.flexneri*) or 2 h (*L.monocytogenes*) at 37°C before fixation in 4% paraformaldehyde in PBS for 20 min. The coverslips were incubated in 50 mM  $\text{NH}_4\text{Cl}$  in PBS for 10 min and permeabilization carried out in 0.2% Triton X-100 in PBS for 20 min. After blocking for 30 min in 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS), the coverslips were incubated with rat VRG-N2 or anti-N-WASP antibody in TBS. CyDye-conjugated secondary antibodies (Amersham) were used to visualize VirG and N-WASP, and rhodamine-phalloidin (Molecular probes) was used to visualize F-actin. The coverslips were mounted in Vectashield (Vector) and observed with a confocal laser-scanning microscope (MRC-1024, Bio-Rad). Accumulation of N-WASP or F-actin around the intracellular bacteria was defined as areas where the intensity was higher than an arbitrary threshold of 80 estimated in non-infected cells by using image processing software (LaserSharp version 2.0, Bio-Rad).

### GST fusion proteins

GST-Ash/Grb2 and GST-VirG  $\alpha$ -domain fusion protein series GST- $\alpha 1$  to  $\alpha 6$  were constructed as described previously (Miki *et al.*, 1994; Suzuki *et al.*, 1996). GST fusion proteins of N-WASP were constructed as previously described (Miki *et al.*, 1996). For preparation of GST- $\alpha 7$  and  $\alpha 8$ , the DNA fragments encoding the amino acids shown in Figure 4A were generated by PCR and cloned into pGEX-2T (Pharmacia). These GST fusion proteins were purified using protocols supplied by the manufacturer. For use in the gel blot overlay assay, the GST fusion proteins were cleaved with 0.4 mg/ml human thrombin (Sigma) in thrombin buffer [100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2.5 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol (DTT)] at 37°C for 1 h. The thrombin was removed by adding *p*-aminobenzamidine-agarose beads (Sigma) for 30 min at 4°C. Purified proteins were dialyzed against TBS and concentrated by ultrafiltration using Centricon-10 (Amicon).

### In vitro binding assay using GST fusion proteins

HeLa and Cos-7 cells grown in 100 mm dishes were lysed in ice-cold RIPA buffer [25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu\text{g}/\text{ml}$  leupeptin]. Recombinant GST fusion proteins immobilized on glutathione-Sepharose were incubated with 1 ml of lysate overnight at 4°C. Samples were washed three times in RIPA buffer without deoxycholate and SDS and subjected to immunoblotting.

For gel blot overlay assays, proteins to be analyzed were electrophoresed on an SDS-polyacrylamide gel followed by blotting onto a nitrocellulose membrane. After incubating in blocking solution (2% BSA, 0.05% sodium azide in TBS), purified recombinant VirG- $\alpha$ 1 (10  $\mu$ g/ml) in blocking solution was incubated with the filter. After washing with TBS-0.05% Tween-20, bound protein was detected with rabbit VRG-N2 antibody.

#### Transient expression of N-WASP and its mutants in Cos-7 cells

Full-length and  $\Delta$ VCA constructs were prepared as described previously (Miki *et al.*, 1996). For preparation of  $\Delta$ V and  $\Delta$ cof constructs, the DNA fragments encoding the amino acids shown in Figure 2A were generated by PCR and cloned into pCDL-SR $\alpha$ II mammalian expression vector. A total of  $0.5 \times 10^7$  cells were mixed with each purified plasmid (20  $\mu$ g) and transfected by electroporation. The cells were re-plated and cultured for 48 h before bacterial infection. Transfected cells were defined as cells where the total cellular intensity of N-WASP was >10-fold higher than that of non-transfected cells.

#### Microinjection

Purified GST-V fusion protein was microinjected into the cytoplasm of PtK2 cells infected with bacteria. The needle concentration of proteins was 100  $\mu$ M, and ~10% of the total cell volume was injected. Cells were then returned to the incubator for 15 min before fixation.

#### Actin tail assay in *Xenopus* egg extracts

Meiotically arrested cytoplasmic extracts of *X.laevis* were prepared as described previously (Theriot *et al.*, 1994; Rosenblatt *et al.*, 1997). Pre-immune rabbit IgG or anti-N-WASP antibody was bound to 30  $\mu$ l of protein A-conjugated Sepharose 4B (Sigma) in TBS. The pellets were washed twice with 1 ml of XB (100 mM KCl, 50 mM sucrose, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.7) and incubated with 30  $\mu$ l of extracts for 1 h at 4°C. The supernatant was removed by centrifugation and treated as the immunodepleted extract. Aliquots of the pellets and supernatant were analyzed by immunoblotting. Purified G-actin from rabbit skeletal muscle was covalently labeled with tetramethylrhodamine iodoacetamide (Molecular Probes) as an actin tail tracer. *Escherichia coli* MC1060 *ompT::Km* carrying pD10-1 (Suzuki *et al.*, 1996) was labeled with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and fixed with 4% paraformaldehyde in PBS. Cells were washed with XB and resuspended in buffer containing 20% glycerol. Bacterial motility was assayed by mixing 6  $\mu$ l of depleted extracts with 0.5  $\mu$ l each of DAPI-labeled bacteria and 0.5 mg/ml of rhodamine-labeled G-actin. Recombinant baculovirus expressing rat N-WASP was prepared by using the BAC-TO-BAC expression system (Gibco) (Miki *et al.*, 1998). Cell lysates of infected Sf9 cells were applied to a HiTrap Heparin column (Pharmacia). After washing, the column was eluted with a gradient of 0–1000 mM NaCl. Purified protein was dialyzed against XB and concentrated by ultrafiltration.

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