Phosphorylation of Tyrosine 291 Enhances the Ability of WASp to Stimulate Actin Polymerization and Filopodium Formation*

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Wiskott-Aldrich Syndrome protein (WASp) is a key regulator of the Arp2/3 complex and the actin cytoskeleton in hematopoetic cells. WASp is capable of forming an auto-inhibited conformation, which can be disrupted by binding of Cdc42 and phosphatidylinositol 4,5-bisphosphate, leading to its activation. Stimulation of the collagen receptor on platelets and crosslinking the B-cell receptor induce tyrosine phosphorylation of WASp. Here we show that the Src family kinase Hck induces phosphorylation of WASp-Tyr291 independently of Cdc42 and that this causes a shift in the mobility of WASp upon SDS-PAGE. A phospho-mimicking mutant, WASp-Y291E, exhibited an enhanced ability to stimulate actin polymerization in a cell-free system and when microinjected into primary macrophages induced extensive filopodium formation with greater efficiency than wild-type WASp or a Y291F mutant. We propose that phosphorylation of Tyr291 directly regulates WASp function.

Wiskott-Aldrich Syndrome protein (WASp)† has long been implicated in the control of actin dynamics. The WAS phenotype is characterized by abnormal or absent actin structures in platelets, T-cells, macrophages, and dendritic cells (1–3). Murine T-cells deficient in WASp are unable to cap CD3 in response to T-cell receptor signaling (4), whereas WASp null bone marrow macrophages (BMMs) exhibit impaired phagocytosis (5, 6) and chemotaxis (7). WASp, N-WASP, and SCAR/WAVE 1–3 constitute a protein family characterized by the presence of a C-terminal tripartite VCA (verprolin, central, acidic) domain. This domain binds actin monomers (via the V region) and the Arp2/3 complex (via the C and A regions) (8–10) and displays an ability to stimulate actin polymerization when added to a mixture of G-actin, the Arp2/3 complex, and ATP (11–13). WASp and N-WASp also contain a CRIB domain, which specifically binds the active, GTP-bound form of Cdc42 (14, 15). Cdc42 is a member of the Rho family of GTPases and has been shown to induce the formation of F-actin-containing microspikes at the cell surface (filopodia) (16, 17). N-WASp cooperates with Cdc42 in fibroblasts to enhance filopodium formation (18, 19). WAS patient macrophages are deficient in WASp, display no surface microspikes, and are defective in podosome formation (7, 20).

WASp activity is believed to be regulated by the formation an auto-inhibited loop structure in which residues from the VC region bind to a region including the C-terminal part of the CRIB domain (21). This interaction may be further stabilized by the Arp2/3 complex bridging the acidic domain and a region slightly N-terminal to the CRIB domain (22). Binding of GTP-bound Cdc42 to the CRIB domain of WASp displaces the VCA domain (21), enabling it to activate the Arp2/3 complex. Actin nucleation experiments in vitro show that the addition of active Cdc42 to a mixture containing full-length WASp (or N-WASp), the Arp2/3 complex, ATP, and monomeric actin enhances actin polymerization. This is dependent on the presence of WASp and is greatly augmented by phosphatidylinositol 4,5-bisphosphate and F-actin (22–24). More recently it has been shown that SH3 domains from a variety of proteins including WISH, Grb2, and Nck are capable of stimulating WASp/N-WASp actin polymerization activity and that binding of SH3 domains to proline regions in WASp/N-WASp can replace the requirement for Cdc42 but not phosphatidylinositol 4,5-bisphosphate for maximal in vitro activity (25, 26).

WASp has been shown to be phosphorylated on tyrosine 291 (WASp-Tyr291) by Bruton’s tyrosine kinase (Btk) when both are expressed in 293T cells (27). WASp also becomes tyrosine-phosphorylated following B-cell receptor cross-linking (27) and following ligation of the collagen receptor on platelets in a pathway that may involve Btk, Src kinases, and phosphatidylinositol 3-kinase activity (28), although Tyr291 has not been conclusively identified as a tyrosine kinase target in endogenously expressed WASp. Btk and Src family tyrosine kinases have been further implicated in the regulation of WASp via in vitro interactions of WASp with their SH3 domains and an in vivo association between Fyn and WASp (29–31). Hck is a Src family kinase exclusively expressed in hematopoietic cells, most notably in granulocyte and myeloid lineages. Hck has been shown to regulate differentiation and several actin-dependent processes such as chemotaxis of monocytes in response to urokinase plasminogen activator signaling (32, 33), integrin and FcR-γ signaling/phagocytosis (34–37) and adhesion (38). Hck/Fgr knock-out macrophages show decreased mobility, adhesion, and an inability to produce filopodia (39).

In this study we report that Hck-induced phosphorylation of...
Tyr<sup>291</sup> causes a shift in the electrophoretic mobility of WASp that can be mimicked by mutation of this residue to a negatively charged glutamic acid. We used this mutant to dissect the effects of tyrosine phosphorylation upon the ability of WASp to stimulate actin polymerization in vitro and upon its effects on the macrophase actin cytoskeleton in vivo.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Mouse anti-vesicular stomatitis virus (VSV) clone PD54 and mouse M2 anti-FLAG antibodies were obtained from Sigma. For immunoprecipitation experiments anti-VSV antibody was coupled to CN-Br activated Sepharose 4B (Amersham Biosciences). Rabbit polyclonal anti-mouse antibody (H-250) was obtained from Santa Cruz. Anti-phosphotyrosine monoclonal antibody (clone 4G10) was obtained from Upstate Biotechnology. Anti-actin monoclonal antibody (clone A4700) was obtained from Sigma. Acrylamide-bisacrylamide (37:5:1) was obtained from Quantum Appligene.

Expression Constructs—The cDNA of human WASp (a kind gift from Dr. C. Kinnon, Institute of Child Health, London, UK) was cloned into the pCMV3 expression vector (obtained from Dr. L. Stephens, Babraham Institute, Cambridge, UK) driven by an extended CMV promoter. A VSV tag was incorporated at the 3'-end coding for the C-terminal peptide ASYTDIEMNRLGK following directly from the WASp coding sequence. A similar construct was made lacking the VSV tag and linker sequence; this full-length FLAG-tagged murine Hck cDNAs in EF-Bos (Amersham Biosciences) were a generous gift from Dr. G. Scholz (Ludwig Institute for Cancer Research, Melbourne, Australia). C-terminally hemagglutinin-tagged human-Btk in pCDNA3 (Invitrogen) was a kind gift from Dr. C. Kinnon, Institute of Child Health, London, UK. N-terminally FLAG-tagged Cdc42-V12 and Cdc42-N17 were expressed using pCMV5. All plasmids were purified using Qiagen endo-free maxi-preps. Mutation of WASp-Tyr<sup>291</sup> was performed on the untagged cDNA using the QuikChange protocol (Stratagene) and constructs sequences to confirm the presence of mutations. A pGEX-2T-WASp-CRIB vector coding for residues 201–321 of WASp was a generous gift from Dr. Pontus Aspenstrom (Ludwig Institute for Cancer Research, Upsala, Sweden). A mammalian GST-WASp construct in pEYF-Bos was made by cloning WASp into the pCMV3 expression vector (obtained from Dr. L. Stephens, Babraham Institute, Cambridge, UK) driven by an extended CMV promoter. A VSV tag was incorporated at the 3'-end coding for the C-terminal peptide ASYTDIEMNRLGK following directly from the WASp coding sequence. A similar construct was made lacking the VSV tag and linker sequence. A VSV tag was incorporated at the 3'-end using the pCMV3 expression vector (obtained from Dr. L. Stephens, Babraham Institute, Cambridge, UK) driven by an extended CMV promoter.

Cell Culture—All cell culture medium and antibiotics were obtained from Invitrogen. COS-7 epithelial cells were grown in Dulbecco’s modified Eagle’s medium + 10% fetal calf serum (Heelena Biosciences) plus penicillin/streptomycin at 37 °C in 10% CO<sub>2</sub>. U937 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum plus penicillin/streptomycin at 37 °C in 5% CO<sub>2</sub>. Murine BMMs were obtained as described (40) and were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and 15% L-glutamine supplement with penicillin/streptomycin at 37 °C in 5% CO<sub>2</sub>. For experiments with <sup>32</sup>P-labeled WASp, cells were washed three times with LB, twice with 0.5 M LiCl, 20 mM Tris-HCl, pH 8.0, and twice with 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0.

In Vitro Kinase Assay—EF-Bos-FLAG-Hck vector was electroporated into COS-7 cells as above. Protein was purified using anti-FLAG M2 agarose beads (Sigma) and eluted by incubation with 100 μl of FLAG elution buffer (Invitrogen) at 200 μl ml<sup>-1</sup> of beads or the appropriate antibody for 1 h followed by a 1-h incubation. Beads were washed three times with LB, twice with 0.5 M LiCl, 20 mM Tris-HCl, pH 8.0, and twice with 0.5 M NaCl, 20 mM Tris-HCl, pH 8.0.

In Vitro Actin Polymerization Assay—5 × 10<sup>5</sup> U937 cells were lysed in 0.5 ml of actin polymerization lysis buffer (apLB); 1% Nonidet P-40, 130 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM NaF, 2 mM sodium orthovanadate, 15 mM 3-TP, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM sodium orthovanadate, 20 mM sodium fluoride, 1 mM EDTA. Lysates were clarified by centrifugation at 15,000 × g for 10 min. Lysates were suspended with 2 μM MgCl<sub>2</sub> and incubated with GST-WASp (full-length) fusion protein on glutathione-Sepharose 4B beads overnight at 4 °C with rotation. Beads were then washed three times with LB before resolution on 10.6% SDS-PAGE and immunoblotting for WASp. For immunofluorescence, beads were fixed with 4% paraformaldehyde followed by staining with TRITC-phalloidin (Sigma) and mounting in moviol (Calbiochem). Images were obtained using a cooled CCD camera (Photonic Science, Robertbridge, UK) attached to a Zeiss Axioskope microscope. Exposure times were kept constant for all samples within an experiment.

Microinjection and Immunofluorescence—Growing or starved (in medium lacking CSF-1 for 18 h) BMMs on glass coverslips were microinjected using DNA at 100 ng/μl. Cells were left in the appropriate medium for 3 h before fixation with 4% paraformaldehyde for 20 min, permeabilization with 0.25% Triton X-100 for 5 min, and blocking in 20% heat-inactivated goat serum for 45 min. For immunofluorescence anti-WASp antibody was used at 10 μg/ml, TRITC-labeled phalloidin at 0.1 μg/ml, and fluorescein isothiocyanate-labeled goat anti-rabbit antibody (WASp) (Jackson Laboratories) at a dilution of 1:200.

RESULTS

Co-expression of WASp with Hck Causes Two Distinct Shifts in the Electrophoretic Mobility of WASp—The hematoepoietically expressed Src family kinases Lyn and Btk can induce tyrosine phosphorylation of WASp (27, 41), and active Cdc42 can potentiate this effect (41). We were interested to determine whether the hematopoietic kinase Hck demonstrated similar properties. Btk, Hck, or Lyn were co-expressed with a VSV-tagged WASp construct in COS-7 cells. WASp-VSV immunoprecipitated from cells not co-expressing Hck or cells co-expressing a kinase-dead mutant of Hck migrated as a single species with an apparent molecular mass of 60 kDa. WASp-VSV immunoprecipitated from cells co-expressing WT Hck migrated as three distinct species with apparent molecular masses of 60, 62, and 64 kDa (Fig. 1A), where the 62- and 64-kDa species were tyrosine-phosphorylated (Fig. 1B).

Co-expression of an active Cdc42-V12 mutant did not cause WASp tyrosine phosphorylation (data not shown) nor did Cdc42-V12 or dominant negative Cdc42-N17 affect the ability of Hck to induce tyrosine phosphorylation of WASp (Fig. 1B), showing that in this system the ability of Hck to induce WASp phosphorylation is independent of Cdc42. Btk showed no ability to phosphorylate WASp in the absence of active Cdc42 (Fig. 1B) but was able to cause low levels of phosphorylation when co-transfected with Cdc42-V12 (data not shown). Lyn was also capable of inducing WASp phosphorylation, although only low...
levels of WASp were observed in the lysates of Lyn-overexpressing cells. Hck co-immunoprecipitated (IP) using anti-VSV antibody and immunoprecipitates and whole cell lysates (WCL) immunoblotted (IB) as indicated. A, arrows indicate apparent molecular masses (kDa) of differently migrating WASp (W) species. B, phospho-WASp (W) species and co-immunoprecipitating Hck are indicated by arrows. C, region shown is ~55–60 kDa. FLAG immunoblot shows co-immunoprecipitating Hck. D, region shown is ~25–30 kDa. Co-immunoprecipitating FLAG-Cdc42 is indicated.

Fig. 1. Hck induces tyrosine phosphorylation of WASp. COS-7 cells were transfected with WASp-VSV, either WT or kinase-dead (267) FLAG-tagged Hck or FLAG-tagged Lyn-WT or hemagglutinin-tagged Btk-WT and active, V12 (V) or dominant negative, N17 (N) FLAG-tagged-Cdc42. WASp was immunoprecipitated (IP) using anti-VSV antibody and immunoprecipitates and whole cell lysates (WCL) immunoblotted (IB) as indicated. A, arrows indicate apparent molecular masses (kDa) of differently migrating WASp (W) species. B, phospho-WASp (W) species and co-immunoprecipitating Hck are indicated by arrows. C, region shown is ~55–60 kDa. FLAG immunoblot shows co-immunoprecipitating Hck. D, region shown is ~25–30 kDa. Co-immunoprecipitating FLAG-Cdc42 is indicated.

WASp bound similar amounts of Cdc42-V12 whether or not it was tyrosine phosphorylated (Fig. 1D). Interestingly, co-expression of Cdc42-V12 increased the amount of WASp-VSV detected without affecting the amount of FLAG-Hck. As both WASp-VSV and FLAG-Hck constructs are driven by CMV promoters, it is unlikely that Cdc42-V12 affects transcription of WASp. It is therefore possible that Cdc42-V12 increases WASp-VSV protein levels by inhibiting WASp degradation.

The Shifts in the Mobility of WASp Are Caused by Tyrosine Phosphorylation—To determine whether the shift in WASp electrophoretic mobility induced by Hck was caused by tyrosine phosphorylation, WASp-VSV was immunoprecipitated from COS-7 cells co-expressing Hck and treated with various phosphatases. CIP and recombinant PP1 are able to remove phosphate groups from serine, threonine, and tyrosine residues. Leukocyte antigen-related protein tyrosine phosphatase is phosphatase-specific for phosphotyrosine (42). Treatment with any of these phosphatases removed most (CIP, PP1) or all (LAR) of the phosphotyrosine (Fig. 2A, top row) and abolished the shift in mobility of WASp (Fig. 2A, bottom row). The phosphotyrosine-containing species migrating slightly faster than WASp have the same mobility as co-immunoprecipitating Hck (Fig. 2A).

To determine whether endogenously expressed WASp could undergo a tyrosine kinase-induced mobility shift, primary murine BMMs were treated with pervanadate (PV) to specifically inhibit tyrosine phosphatase activity. A slower migrating WASp species was observed in lysates from PV-treated cells (Fig. 2B). Similar results were obtained by PV treatment of the human myeloid precursor cell line, U937 (Fig. 3). These results demonstrate that an endogenous tyrosine kinase or kinases phosphorylates WASp and that this event is tightly controlled by phosphatase activity. PV was most active at low concentrations (Fig. 2B), suggesting that phosphatase activity itself is in part dependent on tyrosine kinase activity.

We next tested the effect of PV on BMM morphology. Starved cells exhibited few F-actin-rich protrusions and some punctate F-actin staining (Fig. 2C, see arrow). After 10 min of PV treatment cells had begun to produce filopodia (Fig. 2D, arrowhead) and exhibited intense and widespread podosomal structures characterized by punctate F-actin staining (Fig. 2D, arrows). After 30 min podosomes were no longer visible, and the cells exhibited dramatic filopodium formation stemming from a strong cortical actin ring (Fig. 2E, arrowhead). Some polarized cells exhibited retraction fibers in the tail (Fig. 2, C, D, and F, see asterisks). Although PV did not induce a decrease in macrophage spread area, we cannot rule out the possibility that retraction fibers may contribute to the F-actin morphology observed following PV treatment. Similar results were obtained upon PV treatment of phorbol 12-myristate 13-acetate-differentiated adherent
U937 cells (data not shown). Thus, increased tyrosine phosphorylation of proteins in these cell types promotes the sequential formation of podosomes and filopodia.

WASp Tyr291 Is a Target of Phosphorylation—Mass spectrometric analysis revealed that WASp-VSV immunoprecipitates from COS-7 cells transfected with WASp-VSV, and Hck contained WASp peptides phosphorylated at Tyr291 (data not shown). The phosphopeptides were only observed in immunoprecipitates of WASp-VSV from COS-7 cells co-expressing Hck. We mutated WASp-Tyr291 to phenylalanine (Y291F) and co-expressed the construct (without an epitope tag) with Hck in COS-7 cells. The 62-kDa WASp species was absent as shown by immunoblotting (Fig. 3A), demonstrating that phosphorylation of Tyr291 is responsible for the mobility shift of WASp from 60 to 62 kDa. So far we have been unable to identify the Hck-induced phosphorylation event that leads to the 64-kDa WASp species, although it can occur independently of Tyr291 phosphorylation (Fig. 3A, see Y291F lane).

WASp Tyr291 Is a Target of Phosphorylation—Mass spectrometric analysis revealed that WASp-VSV immunoprecipitates from COS-7 cells transfected with WASp-VSV, and Hck contained WASp peptides phosphorylated at Tyr291 (data not shown). The phosphopeptides were only observed in immunoprecipitates of WASp-VSV from COS-7 cells co-expressing Hck. We mutated WASp-Tyr291 to phenylalanine (Y291F) and co-expressed the construct (without an epitope tag) with Hck in COS-7 cells. The 62-kDa WASp species was absent as shown by immunoblotting (Fig. 3A), demonstrating that phosphorylation of Tyr291 is responsible for the mobility shift of WASp from 60 to 62 kDa. So far we have been unable to identify the Hck-induced phosphorylation event that leads to the 64-kDa WASp species, although it can occur independently of Tyr291 phosphorylation (Fig. 3A, see Y291F lane).

We next mutated WASp-Tyr291 to glutamic acid (Y291E) to mimic the negative charge introduced by tyrosine phosphorylation of this residue and expressed this construct in COS-7 cells. When cell lysates were separated by SDS-PAGE, WASp-Y291E migrated with the same mobility as the 62-kDa species of WASp-WT generated by co-expression with Hck, as did WASp from PV-treated U937 cells (Fig. 3A). This indicates that...
a negative charge at amino acid 291 of WASp is sufficient to alter its mobility in SDS-PAGE and that the Y291E mutation mimics the effect of phosphorylation of this residue.

To determine whether Hck was capable of phosphorylating WASp directly, we purified recombinant Hck from overexpressing COS-7 cells and incubated it with GST-WASp-CRIB (containing Tyr291) and full-length GST-WASp. Both fusion proteins were labeled strongly with $\gamma^32P$ATP as was recombinant Hck (Fig. 3B), whereas GST alone was poorly phosphorylated. These were the only labeled species detected by radiography, indicating that no other kinase activity was present in these assays and that Hck was not phosphorylating an intermediate kinase. Furthermore, incubation of GST-WASp proteins with kinase-dead Hck did not result in any phosphorylation, demonstrating that the kinase activity is not a contaminant of the GST-WASp preparations. Hck was also able to phosphorylate GST-WASp-Y291F (data not shown), consistent with the finding that there is more than one WASp residue that is a target of Hck activity.

**WASp-Y291E Has Enhanced Actin Polymerization-stimulating Activity in Vitro—Phosphorylation of Tyr291 is predicted to destabilize the auto-inhibited structure of WASp (21). We used the Y291E phospho-mimicking WASp mutant in a variation of a bead-based assay (43) to analyze the effects of phosphorylation of this site upon WASp activity in vitro. GST-WASp-Y291E was able to stimulate actin polymerization when added to lysates of U937 cells to a greater extent than GST-WASp-WT and Y291F, as shown by phalloidin staining of beads coated with each protein (Fig. 4A). Immunoblot analysis of WASp-coated beads also showed greatly enhanced actin polymerization by the Y291E mutant (Fig. 4B). This was sensitive to cytochalasin D treatment (Fig. 4, A and B), demonstrating that the actin observed was newly polymerized F-actin. In addition, F-actin was only observed in the presence of Mg2+ (data not shown).**

**WASp-induced Filopodium Formation Is Enhanced by the Phospho-mimicking Mutation Y291E—**We were interested to ascertain the effect of Tyr291 phosphorylation upon WASp function. The effects of wild-type and mutant WASp proteins on the actin cytoskeleton of CSF-1-deprived BMMs were therefore investigated.

The morphology of BMNAs was quite heterogeneous, and both growing and CSF-1-starved cells showed varied levels of filopodia (Fig. 5, A, D, and G, and data not shown). WASp-WT induced an increase in filopodia in BMNAs (Fig. 5A). WASp-Y291F was less efficient at inducing filopodium formation, whereas the WASp-Y291E mutant displayed the most potent activity characterized by the actin morphology in Fig. 5G. WASp-Y291E, and to a lesser extent WASp-WT, induced a similar pattern of F-actin redistribution to that induced by PV, with numerous filopodia extending from a cortical F-actin ring (Fig. 5, A and G). Actin and WASp co-localized at the base of these filopodia but were also present along the length of the filopodia (Fig. 5J). As BMNAs showed variable levels of filopodia, WASp-expressing cells from 10 separate experiments were scored as to whether they had considerably more filopodia (at least 3-fold) than surrounding, non-expressing cells. WASp-Y291E induced extensive filopodium formation in 69% of expressing cells compared with 43% for WASp-WT and 27% for WASp-Y291F (Fig. 5J). These results indicate that phosphorylation of WASp-Y291 enhances WASp-induced effects on the actin cytoskeleton, potentiating the generation of filopodia.

**DISCUSSION**

In this study we show that tyrosine phosphorylation of WASp on Tyr291 is potently induced by the hematopoietic-specific Src family kinase, Hck. This phosphorylation causes a change in the physical properties of WASp as demonstrated by a large shift in its electrophoretic mobility. To mimic this effect we used a mutated form of WASp in which Tyr291 was substituted for glutamic acid (Y291E). This mutant possessed an increased ability to stimulate actin polymerization in vitro and was more effective in inducing filopodia in macrophages than wild-type WASP or WASp-Y291F. In addition, the data presented here demonstrate that WASp overexpression induces filopodium formation in macrophages and that WASp co-localizes with F-actin at the base of filopodia. This is the first report of filopodium induction by WASp (as opposed to N-WASp), and importantly this effect is observed in hematopoietic cells that normally express WASp.

The ability of the Y291E mutation to enhance the ability of WASp to stimulate actin polymerization in a cell-free system demonstrated a direct effect of WASp phosphorylation upon its function. In addition to the binding of Cdc42 and SH3 domains, it has been proposed that phosphorylation of Tyr291 would destabilize the auto-inhibited loop structure of WASp (21). Tyr291 lies at the interface of two hydrophobic sheets that pack together within the loop. The introduction of a negatively charged phosphate group would disrupt this interface and would thereby stabilize an open conformation. The data presented here support this model and suggest that the introduction of a negative charge by phosphorylation of Tyr291 induces a conformational change in WASp, exposing the VCA domain and facilitating a functional interaction with the Arp2/3 com-
plex. We propose that this activation of WASp is responsible for the enhanced ability of WASp-Y291E to induce filopodia, although we cannot rule out the possibility that this mutation acts by facilitating WASp recruitment to sites of actin polymerization.

Overexpression of N-WASp has been shown to cause membrane protrusions in COS-7 cells in the presence of an activated mutant Cdc42 (18) and to cooperate with WIP to form filopodia in fibroblasts (19). Although it is usually assumed that Cdc42 is the major activator of WASp/N-WASp, it has recently been reported that WASp localizes to the immunological synapse between a T-cell and an antigen-presenting cell independently of Cdc42, in a mechanism that requires its polyproline region, suggesting the involvement of an SH3 domain-containing protein in recruiting WASp (44). Also, the SH3 domain-containing adapter protein Nck has been shown to be required for N-WASp localization to the EPEC Tir molecule (45). Vaccinia virus recruits N-WASp to its membrane via interaction with a Nck-WIP complex, and the resulting actin comet tail is formed independently of Cdc42 (46). The Vaccinia mechanism requires tyrosine phosphorylation of a membrane protein, A36R, and may mimic tyrosine kinase receptor signaling (47).

Our results show that the induction of filopodia by WASp is enhanced by a phospho-mimicking mutation and that WASp is subject to phosphorylation of Tyr-291 in vivo, although the stimuli that regulate phospho-WASp levels remain to be fully elucidated. We have observed filopodium formation induced by WASp-Y291E in the presence of co-microinjected dominant negative Cdc42-N17 (data not shown), suggesting that Cdc42 is not essential for its activity. Thus, there may be several regulatory inputs controlling WASp function, including Cdc42 activity, WASp tyrosine phosphorylation, and/or SH3 domain binding. The ability of the WASp-Y291F mutant to induce filopodia in 27% of expressing macrophages is likely to reflect inputs from these other signals and may be in part a consequence of high levels of exogenous WASp expression. The degree of cooperation between these signaling pathways feeding into WASp remains an important question. Phosphorylation of WASp by Hck does not appear to increase its association with Cdc42, suggesting that these two signals may be capable of acting independently of one another as has been shown for N-WASp regulation by Cdc42 and SH3 domain binding (25, 26).

Recent work has shown that the Cdc42 guanine nucleotide exchange factor, intersectin-1, binds to and is activated by N-WASp (48) and that intersectin-2 associates with WASp following T-cell receptor stimulation (49). It is therefore possible that WASp family proteins may be able to recruit GDP-bound Cdc42 and activate it through intersectin-2 or similar guanine nucleotide exchange factors suggesting a role for WASp upstream as well as downstream of Cdc42.

Phosphorylation of WASp and the concomitant stabilization of an open conformation may favor an interaction with an SH3 domain-containing protein such as Hck or may enable binding via SH2-mediated interactions. WASp bound equally well to wild-type Hck (i.e. when WASp was tyrosine-phosphorylated) and to kinase-dead Hck (i.e. when WASp was not tyrosine-phosphorylated). In addition, a panel of SH3 domains bound equally well to WASp-Y291E and WASp-WT (data not shown), suggesting that binding to SH3 domains is not affected by WASp phosphorylation. However, it will be of interest to elucidate the role of SH2 domain/phospho-WASp binding on the regulation of WASp function.

In contrast to the extensive podosome induction induced by PV, we were unable to detect podosome formation following microinjection of WASp. As WASp and N-WASp are essential for podosome formation in various cell-types (20, 50) it is pos-
sible that WASp-Y291E preferentially induces the formation of filopodia over podosomes or that any podosomes formed are transient structures and are dispersed during the 3-h incubation period following microinjection, before fixation.

Hck- and Fgr-deficient macrophages show defects in adherence to fibronectin, motility, and filopodium formation (39, 51), and several Src family kinases play a key role in signal transduction via a number of immunological receptors (52) and integrins (53). Hck is highly expressed in macrophages, and we have observed that Src family kinases, in particular Hck, are more potent than Btk at inducing tyrosine phosphorylation of WASp and are thus implicated in controlling WASp activity and inducing actin polymerization. The finding that a phosphomimicking mutation of WASp stimulates its activity in vitro and in vivo confirms phosphorylation as an important control mechanism and adds another route by which WASP regulation is achieved.

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