c-Abl-mediated Phosphorylation of WAVE3 Is Required for Lamellipodia Formation and Cell Migration

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The activity of the Wiskott-Aldrich syndrome-related WAVE3 protein is critical for the regulation of the Arp2/3-dependent cytoskeleton organization downstream of Rac-GTPase. The Ableson (Abl) non-receptor tyrosine kinase is also involved in the remodeling of actin cytoskeleton in response to extracellular stimuli. Here we show that platelet-derived growth factor stimulation of cultured cells results in WAVE3-Abl interaction and localization to the cell periphery. WAVE3-Abl interaction promotes the tyrosine phosphorylation of WAVE3 by Abl, and STI-571, a specific inhibitor of Abl kinase activity, abrogates the Abl-mediated phosphorylation of WAVE3. We have also shown that Abl targets and phosphorylates four tyrosine residues in WAVE3 and that the Abl-dependent phosphorylation of WAVE3 is critical for the stimulation of lamellipodia formation and cell migration. Our results show that the activation of WAVE3 to promote actin remodeling is enhanced by the c-Abl-mediated tyrosine phosphorylation of WAVE3.

The actin cytoskeleton plays an important role in cell shape and cell motility (1). Members of the Wiskott-Aldrich syndrome protein (WASP) family that include WASP, neuronal-WASP (N-WASP) and WAVE1, -2, and -3 play crucial roles in actin polymerization through the activation of the actin-related protein (Arp) 2/3 complex (2-4). All five members of the WASP family of proteins contain a conserved C terminus verprolin (V), cofilin (C), and acidic (A) region (VCA domain), which binds to the Arp2/3 complex and stimulates its actin polymerization activity (5). The regulation of the activity of both WASP and N-WASP is thought to be achieved by their inclusion in inactive conformations through intramolecular interaction between the GTPase-binding domain and the VCA region (2, 6, 7). The binding of CDC42, or SH3-containing proteins such as WISH and NCK to the proline-rich domain of N-WASP/WASP alleviates their inhibition by disrupting this intramolecular interaction, leading to the activation of N-WASP and WASP (8-11). On the other hand, the activity of the WAVE proteins is believed to be regulated by their inclusion in a protein complex that also contains PIR121/NAP1/Abi/HSPC300, which keeps them in an inactive state (12-16). This inhibition can be lifted by active small GTPases such as GTP-Rac (13), leading to the translocation of the complex to sites of active actin polymerization (14, 16).

Phosphorylation of the WASP/WAVE proteins has been shown to regulate their activity (17-21). Phosphorylation of N-WASP/WASP proteins by non-receptor tyrosine kinases, such as ACK1 enhances the ability of WASP to stimulate actin polymerization (22), whereas growth factor stimulation of cultured cells results in hyper-phosphorylation of the WAVE proteins as well as a delay in their mobility in SDS-PAGE (23). WAVE1 was shown to be phosphorylated by the cyclin-dependent kinase 5 (24), whereas phosphorylation of WAVE2 has recently been shown to involve the non-receptor tyrosine kinase c-Abl (25), resulting in both cases in the stimulation of membrane ruffling and cell spreading. Whether the activity of WAVE3 is regulated in a similar manner is not known. The Abl-tyrosine kinase has also been implicated in the remodeling of actin cytoskeleton, because cells lacking Abl expression also show a severe deficiency in membrane ruffling in response to growth factors (26). c-Abl has been found to regulate cell motility and neurite extension and to localize to specific actin structures, such as focal adhesions, and lamellipodia (27-29). Abl-mediated tyrosine phosphorylation of WAVE2 was found to greatly enhance cell migration and adhesion, and both Abl and WAVE2 localize to the cell periphery upon growth factor stimulation (30).

In the present study, we determined whether the activity of WAVE3 is regulated at the phosphorylation level downstream of c-Abl tyrosine kinase. We show that phosphorylation of WAVE3 is stimulated upon treatment with PDGF, and that both WAVE3-Abl interaction and Abl-kinase activity are required for the Abl-mediated phosphorylation of WAVE3. We also show that four tyrosine residues, which map to different domains of the WAVE3 protein, are targeted for phosphorylation by Abl. We determined that Abi1 is not required for mediating phosphorylation of WAVE3 by Abl, contrary to WAVE2, although Abi1 could be found in the same immunocomplex as WAVE3. We finally show that loss of WAVE3 phosphorylation results in inhibition of lamellipodia formation and cell migra-
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EXPERIMENTAL PROCEDURES

Materials—The antibodies used in this study were: rabbit anti-human WAVE3/Scar from Upstate Biotechnology (Charlottesville, VA); mouse anti-human p85 and Living Colors Rabbit anti-GFP from BD Biosciences (San Diego, CA); rabbit anti-Abl from Cell Signaling Inc. (Beverly, MA); mouse anti-GFP from Clontech (Palo Alto, CA); mouse anti HA tag (6E2) and anti-Myc tag antibodies from Cell Signaling Inc. The secondary antibodies used were donkey anti-rabbit and goat anti-mouse from Jackson ImmunoResearch (West Grove, PA). PDGF was obtained from Sigma. Gel electrophoresis reagents were from Bio-Rad.

Cell Culture—Human MDA-MB-231 adenocarcinoma cells American Type Culture Collection (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 units of penicillin/ml, 100 µg of streptomycin/ml, and 2 mM-glutamine. For stimulation with PDGF, the cells were first subjected to serum starvation for 24 h in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum, and then treated with 5 ng/ml recombinant PDGF. Wild-type mouse embryonic fibroblasts (MEFs) and the Abl/Arg double knock-out MEFs (27) were kindly provided by Alexis Gautreau (Institut Curie, Paris France). The HA tag in the HA-WAVE2 construct was replaced by a Myc tag using the T-A cloning kit (Stratagene), following the manufacturer's instruction for the full-length WAVE3 and the WAVE3 lacking the Kozak sequence and the open reading frame of the EGFP (GFP). The vectors expressing the wild-type c-Abi, the constitutively active (P131L), or the catalytically inert (K290R), forms of c-Abi were kindly provided by Dr. Anthony Koleske (Yale University, New Haven, CT) and maintained in culture as described above.

Plasmid Construction and Transfections—WAVE3 constructs were generated using the PCR from the template WAVE3 cDNA IMAGE clone 4838122 (ATCC). In the 5' primer for the full-length WAVE3 and the WAVE3 lacking the VCA domain (ΔVCA), the ATG sequence for the first methionine was replaced by a TTG. Amplified fragments were subcloned into the pCR2-1 vector using the T-A cloning kit (Invitrogen). The subcloned fragments were inserted into EcoRI-digested pEGFP-C1–1 vector (Clontech), in-frame with the 3' terminus of EGFP (GFP). The vectors expressing the wild-type c-Abi, the constitutively active (P131L), or the catalytically inert (K290R), forms of c-Abi were kindly provided by Dr. Ann Marie Pendergast (Duke University Medical Center, Durham, NC). The vectors expressing either HA-tagged Abi1 or HA-tagged WAVE2 fusion proteins were kind gifts from Dr. Alexis Gautreau (Institut Curie, Paris France). The HA tag in the HA-WAVE2 construct was replaced by a Myc tag using standard cloning procedures. All constructs were sequence-verified using a 3100 Genetic Analyzer (ABI Prism). The EGFP-recombinant vectors were used for either transient or stable transfections using standard protocols.

Site-directed Mutagenesis—The cDNA fragment that contains the Kozak sequence and the open reading frame of the wild-type WAVE3 transcript was PCR-amplified using WAVE3 cDNA IMAGE clone 4838122, as a template, and subcloned into pCDNA3.1 mammalian expression vector (Invitrogen). The correct sequence and orientation were verified by sequencing. We used the QuikChange® site-directed mutagenesis kit (Stratagene), following the manufacturer's instruction to generate the WAVE3 phospho-mutant variants, where the tyrosine residues were replaced by a phenylalanine (TAT to TCT, or TAC to TCC), by introducing a single nucleotide change at the second position of the tyrosine codon. Sequences of the primers used for mutagenesis are available upon request.

Three-dimensional Migration Assay—Three-dimensional migration was assayed using collagen IV-coated inserts (8-µm pore size, BD Falcon) in 6-well plates. Cells were serum-starved for 24 h, and 2 ml of Dulbecco's modified Eagle's medium containing 2 × 10^5 cells was added to the upper chamber. The lower chamber contained 3 ml of Dulbecco's modified Eagle's medium. Where required, PDGF (5 ng/ml) was added to the lower chamber. The inserts were incubated overnight at 37 °C, where after the non-migrating cells on the upper surface of the membrane were removed with a cotton swab. The cells that were able to migrate through the pores on the lower surface of the membrane were fixed and stained with Diff-Quick (American Scientific Products, McGaw Park, IL) and photographed using an inverted Leica DMIRB microscope fitted with a charge-coupled device camera (Leica Microsystems, Germany), and counted.

Immunofluorescence Microscopy—Cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 20 min in phosphate-buffered saline (PBS) at room temperature and washed with PBS. The cells were then permeabilized in 0.2% Triton X-100 in PBS for 15 min, washed again with PBS, and incubated in the blocking solution containing 5% bovine serum albumin (Sigma) in PBS for 2 h at room temperature. Primary as well as secondary antibodies were diluted at the recommended concentration in 5% bovine serum albumin in PBS. Cells were incubated with the primary antibody for 1 h, washed with PBS, and then incubated with the secondary antibody for 1 h. Actin filaments (F-actin) were stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) in PBS. The coverslips were mounted on object slides using Vectashield mounting medium containing 4´,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescence images were captured using a Nikon TE2000-E inverted microscope.

Immunoprecipitation and Immunoblotting—For endogenous co-immunoprecipitation, cells were grown in 10-cm diameter Petri dishes and washed twice with ice-cold PBS. Immediately, ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and a mixture of proteases inhibitors) was added. The cells were rapidly scraped off the plates, and the crude lysates were transferred to prechilled Eppendorf tubes and centrifuged at 15,000 × g for 20 min at 4 °C. Cleared lysates were incubated with rabbit anti-WAVE3 antibody or rabbit anti-serum for 1 h, followed by incubation with protein G-Sepharose beads (Amersham Biosciences) for 45 min at 4 °C on a rotary wheel. Beads were then washed twice with lysis buffer. Precipitates were resolved by SDS-PAGE and analyzed by immunoblotting. For immunoprecipitation using EGFP fusion proteins the cleared cell lysates were immediately mixed with 100 µg of either EGFP-, EGFP-WAVE3-, or EGFP-WAVE3-truncated fusion proteins, and the tubes were incubated on a rotary wheel overnight at 4 °C. The next morning the protein mixtures were incubated with the anti-EGFP antibody, followed by incubation with protein G-Sepharose beads as described above. Beads were then washed twice
with lysis buffer. Precipitates were resolved by SDS-PAGE and analyzed by immunoblotting. For immunoblotting cellular lysates containing equivalent amounts of total protein (50 μg) were treated with SDS sample buffer and resolved on a 10% SDS-polyacrylamide gel, followed by transfer to nitrocellulose or Immobilon-P (polyvinylidene difluoride) membranes using Bio-Rad gel and transfer apparatus. Membranes were incubated in 5% whole milk (or bovine serum albumin) for 1 h at room temperature, washed with PBS, followed by incubation with the primary antibody (as specified) overnight at 4 °C. Membranes were then washed and incubated in the appropriate secondary antibody at room temperature for 1 h, and the signals were developed using Western Lights Chemiluminescence Detection kit (PerkinElmer Life Sciences).

RESULTS

Abl Kinase Activity Is Required for WAVE3 Tyrosine Phosphorylation—Our previous study suggested that WAVE3 phosphorylation is required for WAVE3-p85 interaction, and for the PDGF regulation of WAVE3-mediated regulation of lamellipodia formation and cell migration, downstream of phosphatidylinositol 3-kinase (3). To investigate the effect of PDGF on WAVE3 phosphorylation, we treated cultured MDA-MB-231 cells with 5 ng/ml PDGF for various amounts of time and analyzed the phosphorylation levels of endogenous WAVE3 protein (Fig. 1A). PDGF stimulation of MDA-MB-231 cells resulted in a time-dependent increase in the levels of WAVE3 phosphorylation, which could be detected as early as 10 min after stimulation, and reached maximum levels after 30 min. Westphal and colleagues (32) showed that c-Abl interacts with the WAVE protein complex. Furthermore, c-Abl tyrosine kinase activity was found to be required for the phosphorylation of WAVE2, a close relative of WAVE3 (30). Next, we investigated whether WAVE3 and Abl interact endogenously in MDA-MB-231 cells. Co-immunoprecipitation analyses of protein lysates from PDGF-stimulated MDA-MB-231 cells determined that endogenous WAVE3 and c-Abl proteins are present in the same immunocomplex (Fig. 1B), suggesting that Abl might be involved in WAVE3 phosphorylation. We also used immunostaining analyses to confirm the localization of WAVE3 and Abl to membrane ruffles at the edge of PDGF-stimulated MDA-MB-231 cells (Fig. 1C). Immunostaining of MDA-MB-231 cells with WAVE3 antibody showed co-localization of endogenous WAVE3 with membrane ruffles at the edge of MDA-MB-231 cells (Fig. 1C, left panel). Similarly, immunostaining with Abl antibody showed that endogenous Abl also co-localizes with membrane ruffles of MDA-MB-231 cells (Fig. 1C, middle panel), suggesting a possible co-localization of Abl and WAVE3 in vivo as well. Co-staining of MDA-MB-231 cells with both anti-WAVE3 and anti-Abl antibodies (Fig. 1C, right panel), showed co-localization of both endogenous WAVE3 and Abl proteins to cell edges. To determine whether Abl kinase activity is required for WAVE3 phosphorylation, we analyzed the effect of STI-571 (Gleevec), a specific inhibitor of Abl kinase activity, on WAVE3 phosphorylation. Although PDGF-stimulated cells showed relatively strong levels of WAVE3 phosphorylation, pre-treatment of MDA-MB-231 cells with STI-571 resulted in a dramatic reduction in the phosphorylation levels of WAVE3 (Fig. 1D). The low levels of phosphorylated WAVE3 that could be detected without PDGF stimulation where also reduced to undetectable levels as a result of the treatment with STI-571 (Fig. 1D). These results,
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Next, we analyzed the Abl-mediated phosphorylation of WAVE3 in COS7 cells by performing anti-WAVE3 immunoprecipitation reactions from COS7 cells overexpressing WAVE3 alone or both WAVE3 and Abl. When immunoprecipitated proteins were analyzed by SDS-PAGE and anti-phosphotyrosine immunoblotting, phosphorylation of WAVE3 occurred only in the presence of Abl (Fig. 2B). Wild-type or constitutively active (P131L) forms of Abl kinase, but not the catalytically inert (K290R) Abl kinase, were able to mediate phosphorylation of WAVE3 (Fig. 2C), suggesting that not only the presence of Abl protein, but also an active Abl kinase is required for WAVE3 tyrosine phosphorylation. Similarly, in COS7 cells, inhibition of Abl kinase activity with STI-571 results in inhibition of WAVE3 phosphorylation (Fig. 2C).

**Abi1 Is Not Required for Abl-mediated Phosphorylation of WAVE3**—WAVE3 along with WAVE1 and WAVE2 are thought to be regulated by their inclusion in a protein complex also containing the Abelson (Abl) interactor-1 (Abi1) adaptor protein (33). On the other hand, it has been shown that Abl-mediated phosphorylation of WAVE2 is mediated by Abi1 in an Abl-Abi1-WAVE2 complex (30). To test whether Abi1 is required for coupling of Abl to WAVE3 to promote Abl-mediated phosphorylation of WAVE3, we analyzed WAVE3 phosphorylation by Abl both in the presence and absence of Abi1. Although Abi1 could be detected in the same immunocomplex that contains WAVE3 or WAVE2 (Fig. 3, A and B, respectively), indicating a possible interaction between the two proteins, the presence of Abi1, however, has no effect on WAVE3 phosphorylation, because phosphorylation of WAVE3 could be achieved even in the absence of Abi1 (Fig. 3A). No WAVE2

therefore, demonstrate the requirement of Abl tyrosine kinase activity for WAVE3 phosphorylation. To further confirm the requirement of Abl activity for WAVE3 phosphorylation, we analyzed the phosphorylation status of ectopically expressed WAVE3 in both wild-type MEFs, and MEFs derived from mice where both Abl and the Abl-related-gene (Arg) were targeted for double knock-out (Abl\(^{-/-}\)/Arg\(^{-/-}\)). Phosphorylated WAVE3 could be detected only in the wild-type MEFs, whereas no WAVE3 phosphorylation could be detected in the MEFs where Abl activity was lacking (Fig. 2A, left panel). Phosphorylation of WAVE3 could, however, be restored when Abl activity was rescued in the (Abl\(^{-/-}\)/Arg\(^{-/-}\)) MEFs (Fig. 2A, right panel).

![FIGURE 2. Abl-mediated phosphorylation of exogenous WAVE3.](image1)

![FIGURE 3. Abi1 is not required for the Abl-mediated phosphorylation of WAVE3.](image2)
phosphorylation could, however, be achieved in the absence of Abi1 (Fig. 3B), confirming the requirement of Abi1 for the Abl-mediated phosphorylation of WAVE2. Possible endogenous levels of Abi1 in COS7 cells could not explain the detection of phosphorylated WAVE3, in the absence of exogenous Abi1. If that were the case, then this endogenous Abi1 would have been...
able to mediate WAVE2 phosphorylation in the absence of exogenous Abi1.

Four WAVE3 Tyrosine Residues Are Targeted for Abl Phosphorylation—To determine the location of potential tyrosine residues that are phosphorylated by Abl, truncated forms of WAVE3, tagged with GFP (Fig. 4A), were expressed together with Abl in COS7 cells, and their phosphorylation was examined. Immunoblotting with anti-tyrosine antibody of anti-GFP immunoprecipitates of proteins from cells expressing the different WAVE3 mutants shows that WAVE3 is phosphorylated as a full-length protein or when amino acids 91–440 are present, therefore, indicating the presence of target tyrosine residue(s) in this region of the WAVE3 protein (Fig. 4B). The presence of additional target tyrosine residues in the VCA domain cannot, however, be excluded. The only region of WAVE3 that could be excluded is the N-terminal 90-amino acid region, which contains the Scar Homology domain.

To identify which of the one or more WAVE3 tyrosine residues are targeted for phosphorylation by Abl, WAVE3-mutant cDNAs in which each of the 15 tyrosine residues were individually replaced with phenylalanine (Tyr to Phe) and expressed in COS7 cells together with Abl, and the phosphorylation status of the mutant proteins was determined. Immunoblotting with anti-phospho-tyrosine antibody of anti-WAVE3 proteins precipitates from these cells (Fig. 4C) shows that mutation of individual tyrosine residues did not result in a loss of WAVE3 phosphorylation by Abl, clearly suggesting that more than a single tyrosine in the WAVE3 protein is phosphorylated by Abl. Next, we generated a series of WAVE3 mutant cDNAs in which all 15 tyrosine residues were sequentially mutated (Table 1), and their phosphorylation status was examined as described above. Complete loss of WAVE3 phosphorylation could be achieved only when all 15 tyrosine residues were mutated (Fig. 4D and E), which is a clear indication that at least tyrosine residue 486 (Tyr-486), along with other tyrosines are targeted for phosphorylation by Abl.

Analysis of the WAVE3 amino acid sequence using the Simple Modular Architecture Research Tool (SMART) identified a single potential target for tyrosine kinase phosphorylation at amino acid 151 (Tyr-151). Mutation of Tyr-151 residue did not, however, completely abrogate phosphorylation of WAVE3 by Abl (Fig. 4, C and F). The SCANSITE program, on the other hand, identified four tyrosine residues (Tyr-151, Tyr-248, Tyr-337, and Tyr-486) as possible targets for phosphorylation by Abl. We then sequentially mutated these four tyrosine residues and analyzed WAVE3 phosphorylation in constructs lacking one, two, three, or all four tyrosine residues. Only when all four tyrosine residues were mutated, the Abl-mediated phosphorylation of WAVE3 is completely abrogated (Fig. 5A). To further confirm that these 4 tyrosine residues are indeed phosphorylated by Abl, we used the WAVE3 construct where all 15 tyrosine residues were mutated as a template to restore each of these 4 mutated residues back to tyrosine. WAVE3 phosphorylation could be rescued when each of the these four residues are the only tyrosine residues to be present in the WAVE3 protein (Fig.

### Table 1

| Description of the WAVE3 expression vector carrying the sequential tyrosine residues substitutions |
|---------------------------------------------------------------|
| W3-Mut1: Y63F |
| W3-Mut2: Y63F, Y125F |
| W3-Mut3: Y63F, Y125F, Y140F |
| W3-Mut4: Y63F, Y125F, Y140F, Y151F |
| W3-Mut5: Y63F, Y125F, Y151F, Y156F |
| W3-Mut6: Y63F, Y125F, Y156F |
| W3-Mut7: Y63F, Y125F, Y156F, Y210F, Y225F, Y248F, Y250F |
| W3-Mut8: Y63F, Y125F, Y156F, Y210F, Y225F, Y248F, Y250F, Y267F |
| W3-Mut9: Y63F, Y125F, Y156F, Y210F, Y225F, Y248F, Y250F, Y267F, Y286F |
| W3-Mut10: Y63F, Y125F, Y156F, Y210F, Y225F, Y248F, Y250F, Y267F, Y286F, Y327F |
| W3-Mut11: Y63F, Y125F, Y156F, Y210F, Y225F, Y248F, Y250F, Y267F, Y286F, Y327F, Y337F |
| W3-Mut all: Y63F, Y125F, Y156F, Y210F, Y225F, Y248F, Y250F, Y267F, Y286F, Y327F, Y337F, Y386F |

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**FIGURE 5.** Four WAVE3 tyrosine residues are phosphorylated by Abl. A, protein lysates from COS7 cells co-transfected with Abl and WAVE3 expression plasmids carrying the indicated Tyr to Phe substitutions, were immunoprecipitated with anti-WAVE3 antibody and subjected to immunoblotting with anti-phospho-tyrosine (a). WAVE3 phosphorylation is lost only when all four tyrosines are mutated. The presence of equal amounts of WAVE3 and Abl proteins in the cell lysates was confirmed by immunoblotting with anti-WAVE3 and anti-Abl antibodies (b and b, respectively). B, protein lysates from COS7 cells co-transfected with Abl and GFP or GFP-WAVE3 expression plasmids carrying the indicated Phe to Tyr reversions, were immunoprecipitated with rabbit anti-GFP antibody and subjected to immunoblotting with anti-phospho-tyrosine (a). WAVE3 phosphorylation is restored after each of the four residues was reverted back to tyrosine. The presence of equal amounts of GFP fusion proteins in the cell lysates was confirmed by immunoblotting with mouse anti-GFP and anti-Abl antibody (b). The ~100-kDa band that is detected in the lysates (panel b) is a nonspecific band linked to the use of the rabbit anti-GFP antibody.
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FIGURE 6. Loss of WAVE3 phosphorylation inhibits lamellipodia formation and cell migration. A, representative photomicrographs of rhodamine-phalloidin-stained COS7 cells transfected with the indicated expression plasmids. The arrowhead indicates lamellipodia. Scale bar, 20 μm (a). Student t test p values are shown on the graphs. In b the immunoblots show the expressed proteins. B, migration of COS7 cells, transfected with the indicated phosphorylation, migration of COS7 cells is reduced by ~4-fold compared with COS7 overexpressing wild-type WAVE3 (a). Each point represents the average value from three independent experiments (error bars ± S.D.). Student t test p values are shown on the graphs. In b the immunoblots show the expressed proteins.

5B), strongly supporting that tyrosine residues 151, 248, 337, and 486 are indeed phosphorylated by Abl. Restoration of residues 63 or 156 back to tyrosine, did not however, result in the restoration of WAVE3 phosphorylation (see the supplemental data), further supporting that the Abl-mediated phosphorylation of WAVE3 is specific to the 4 tyrosine residues mentioned above.

WAVE3 Phosphorylation by Abl Stimulates Lamellipodia Formation—We have previously shown that WAVE3 expression is required for lamellipodia formation in migrating cells (3). Next we determined whether phosphorylation of WAVE3 would affect the formation of lamellipodia in COS7 cells. Co-immunoprecipitation analyses (Fig. 4F) provided evidence on the presence of WAVE3 and Abl in the same immunocomplex, in vitro, confirming the results of the immunostaining analyses (Fig. 1C), where co-localization of WAVE3 and Abl in vivo is shown. These results clearly show that both WAVE3 and Abl are recruited to sites of active actin polymerization, where phosphorylation of WAVE3 by Abl may activate the process of lamellipodia formation. This hypothesis was further tested by analyzing the effect of WAVE3 phosphorylation on the formation of lamellipodia in COS7 cells. Co-expression of active Abl (P131L) and phospho-mutant WAVE3 (pMut) resulted in an ~25% decrease in the number of cells with lamellipodia, compared with the control cells co-expressing Abl and wild-type WAVE3 (WT) (Fig. 6A). On the other hand, co-expression of phospho-mutant WAVE3 and the kinase-dead Abl (K290R) resulted in >4-fold reduction in the number of cells with lamellipodia (Fig. 6A). These results clearly suggest that both an active Abl and a phosphorylated WAVE3 are required for the stimulation of lamellipodia formation.

WAVE3 Phosphorylation by Abl Enhances Cell Migration in Vitro—We have shown that WAVE3 expression is required for lamellipodia formation, cell migration, and invasion, in vitro (3, 31). Next, we determined the effect of WAVE3 phosphorylation on the migration of COS7 cells in vitro. Expression of wild-type WAVE3 resulted in a >3-fold increase in the number of migrating cells compared with expression of empty vector of Abl alone (Fig. 6B). The number of migrating cells was further increased to >4-fold when both wild-type WAVE3 and Abl co-expressed, whereas expression of phospho-mutant WAVE3 alone or with Abl did not lead to a significant change in the number of migrating cells, clearly suggesting that phosphorylation of WAVE3 is required of cell migration.

DISCUSSION

The WAVE proteins are known to regulate the remodeling of actin cytoskeleton downstream of Rho GTPases (2, 4, 7, 10, 34). Rac GTPase has especially been shown to act upstream of the WAVE proteins to promote their actin polymerization activity, which is crucial for the regulation of lamellipodia and filopodia formation (2, 4, 7, 10, 34). In this study, we identified a pathway of WAVE3 activation that promotes lamellipodia formation and cell migration, which might be separate from the Rac pathway. We showed that phosphorylation of WAVE3 by c-Abl tyrosine kinase plays an important role in the remodeling of the actin cytoskeleton and cell migration. Our results show that stimulation of cultured MDA-MB-231 cells with growth factors, such as PDGF, significantly increases the phosphorylation of WAVE3 at tyrosine residues 151, 248, 337, and 486.
levels of the WAVE3 protein. We also found that PDGF stimulation of MDA-MB-231 cells promotes the interaction between c-Abl kinase and WAVE3, and this interaction results in the phosphorylation of WAVE3 at tyrosine residues. Furthermore, we found that WAVE3 and c-Abl co-localize at the cell periphery, where active actin polymerization occurs, in response to extracellular stimuli. Treatment of MDA-MB-231 cells with STI-571, a specific inhibitor of c-Abl kinase activity, or expression of a kinase-dead Abl, inhibited the c-Abl-mediated phosphorylation of WAVE3, without abrogating their interaction, but resulted in a significant decrease in cell migration, clearly suggesting that the c-Abl-mediated phosphorylation of WAVE3 might be critical for the activity of WAVE3 in promoting cell migration. The interaction between c-Abl and the other members of the WAVE family of proteins has already been reported to involve the SH3 domain of c-Abl and the proline-rich domain of the WAVE proteins (25, 32, 35). The WAVE3-Abl interaction is more likely to involve the same region in WAVE3, because the proline-rich domain is very conserved among the WAVE proteins (5). Although this interaction might provide a novel mechanism for c-Abl regulation, it could also solely contribute to the Abl-mediated phosphorylation of WAVE3.

c-Abl phosphorylates four tyrosine residues (Tyr-151, Tyr-248, Tyr-337, and Tyr-486) that are scattered throughout the functional domains of the WAVE3 protein, the Wiskott homology domain, the basic domain, the proline-rich domain, and the VCA domain, respectively. Complete loss of WAVE3 phosphorylation occurs only when all four tyrosine residues are mutated. Tyrosine 151 is conserved among all three WAVE proteins, both in human and mouse (5). Furthermore, Tyr-150 in WAVE2, which is the equivalent of Tyr-151 in WAVE3, is also targeted for phosphorylation by c-Abl (30). Whether Tyr-150 is also phosphorylated in WAVE1 by c-Abl, has, however, not been determined. Tyr-337 is specific to WAVE3, whereas Tyr-486 is conserved in both WAVE1 and WAVE3, but not in WAVE2 (5). Phosphorylation of different tyrosine residues in the different WAVE proteins might account for the specific and independent roles of the WAVE proteins in the regulation of actin polymerization and cytoskeleton remodeling. Leng and colleagues (30) reported that Tyr-150 is the only tyrosine residue to be phosphorylated in WAVE2 by c-Abl. Phosphorylation of WAVE2 by c-Abl was also shown to stimulate membrane ruffling and cell spreading, suggesting that WAVE2 might be an effector of the c-Abl kinase to regulate cytoskeletal remodeling in vivo (25). Here we show that the c-Abl-mediated phosphorylation of WAVE3 also results in the stimulation of lamellipodia formation and cell migration, which clearly supports the hypothesis that WAVE3 is also a downstream effector of c-Abl. Interestingly, because c-Abl can bind all WAVE isoforms, it is possible that it might exert its effect to regulate the specific functions of the different WAVE isoforms in the regulation of actin cytoskeleton.

Ectopic expression of Abi1 showed that Abi1 might be required for the Abl activity to promote Abl-mediated phosphorylation of WAVE2 (30), which was shown to be necessary to link WAVE2 with actin polymerization and cytoskeleton remodeling at the cell periphery (25). Abi1 is also present in the macromolecular complex that includes WAVE3, a protein complex that is believed to regulate the activity of the WAVE proteins (14, 36). Our results show that both WAVE3 and Abi1 can be detected in the same immunocomplex, confirming the previously published findings (33). We found, however, that unlike WAVE2, Abi1 is not required for the Abl-mediated phosphorylation of WAVE3, because phosphorylation of WAVE3 by Abi is not inhibited or even diminished in the absence of Abi1. Abi1 along with the other WAVE protein complex might be critical for maintaining the WAVE proteins in a constitutively inactive state (14, 35). Our study could not, however, demonstrate that Abi1 is a critical intermediate that connects WAVE3 and Abl and promotes Abl-mediated phosphorylation of WAVE3.

In our previous study, we showed that stimulation of MDA-MB-231 cells with PDGF results in an increase in cell motility and lamellipodia formation downstream of phosphatidylinositol 3-kinase (3). We have also shown that the phosphatidylinositol 3-kinase-mediated activation of WAVE3 required the binding of the constitutive subunit of PIK3 kinase (p85) to a phosphorylated WAVE3 at tyrosine residues (3), suggesting that tyrosine phosphorylation of WAVE3 might increase the activity of WAVE3 to promote cell motility and lamellipodia formation. Here, we show that tyrosine phosphorylation levels of WAVE3 are indeed increased after PDGF stimulation of cultured cells. More importantly, we show that phosphorylation of WAVE3 requires c-Abl kinase activity, supporting the hypothesis that c-Abl phosphorylation of WAVE3 at tyrosine residues might be required to link WAVE3 to the PI3K complex, thus allowing for the PDGF-mediated activation of WAVE3. Our study has, therefore, provided a new mechanism by which WAVE3, and probably other members of the WAVE family of proteins, is linked to the PDGF-induced cytoskeleton remodeling downstream of PI3K.

We have probed further into the biological significance of WAVE3 phosphorylation by c-Abl and found that the lack of WAVE3 phosphorylation results in a dramatic inhibition of lamellipodia formation and cell migration, even in the presence of extracellular stimuli, such as PDGF. It is not clear, however, which of the four tyrosine residues is (are) the most critical for lamellipodia formation and cell migration. More studies are warranted to investigate the specific role of each tyrosine, or the combination of two or more tyrosine residues, in the activity of WAVE3. We have also shown that increased levels of the WAVE3 protein are associated with more aggressive and metastatic stages of human breast cancer (31, 36). Another aspect worth investigating is whether increased levels of WAVE3 phosphorylation in breast cancer are also associated with the more aggressive phenotype in these types of tumors.

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