Regulated Downstream of Phosphatidylinositol 3-Kinase*

WAVE3-mediated Cell Migration and Lamellipodia Formation Are Regulated Downstream of Phosphatidylinositol 3-Kinase*

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WAVE3 is a member of the WASP/WAVE family of protein effectors of actin reorganization and cell movement. The precise role of WAVE3 in cell migration and its regulation, however, have not been elucidated. Here we show that endogenous WAVE3 was found to be concentrated in the lamellipodia at the leading edge of migrating MDA-MB-231 cells. Platelet-derived growth factor (PDGF) treatment induced lamellipodia formation as well as two-dimensional migration of cells in the wound-closure assay and chemotactic migration toward PDGF in three-dimensional migration chambers. Knockdown of WAVE3 expression by RNA interference prevented the PDGF-induced lamellipodia formation and cell migration. Treatment of cells with LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K), also abrogated the PDGF-induced lamellipodia formation and cell migration, suggesting that PI3K may be required for WAVE3 activity. WAVE3 and the PI3K regulatory subunit, p85, were found to interact in a yeast two-hybrid screen, which was confirmed through co-immunoprecipitation. The WAVE3-p85 interaction was mediated by the N-terminal region of WAVE3 and the C-terminal SH2 domain of p85. These results imply that the WAVE3-mediated migration in MDA-MB-231 cells via lamellipodia formation is activated downstream of PI3K and induced by PDGF. The findings of the WAVE3-p85 partnership also suggest a potential regulatory role for p85 in WAVE3-dependent actin-cytoskeleton reorganization and cell migration.

Remodeling of the actin cytoskeleton plays a critical role in altering both cellular morphology and motility (1) by controlling a range of cellular events, including immune defense, embryonic development, and neuronal outgrowth (2, 3). In response to extracellular signals such as growth factors, the WASP family of proteins, which contains WASP and N-WASP (4), and the WAVE family of proteins, which contains WAVE1, -2, and -3 (5–7), activate the Arp2/3 complex, leading to the regulation of the WAVE proteins, and WAVE3 in particular, has not been well elucidated. The best characterized WAVE protein partner is IRSp53, which has been shown to be an essential intermediate between Rac and WAVE2 in the regulation of membrane ruffling (13). In addition to the involvement of WAVE3 in actin polymerization and cytoskeleton organization (9, 24), we have shown that WAVE3 may also be associated with the development of low grade neuroblastoma (25, 26).

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1 The abbreviations used are: VCA, verprolin-homology, cofilin-homology, acidic domain; PDGF, platelet-derived growth factor; WAVE3, WASP family verprolin-homologous protein 3; RNAi, RNA interference; WASP, Wiscott-Aldrich syndrome; N-WASP, neural WASP; Arp2/3, actin-related proteins; BD, basic domain; AD, activating domain; co-IP, co-immunoprecipitation; GST, glutathione S-transferase; GFP, green fluorescent protein; EGFP, enhanced GFP; PI3K, phosphatidylinositol 3-kinase; siRNA, small interference RNA; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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Platelet-derived growth factor (PDGF) induces a variety of cellular responses, including proliferation, migration, invasion, and cell survival, in several cell types via its receptor (27, 28). Although PDGF has clearly been shown to modulate WAVE1 and WAVE2-mediated cell motility through activation of PI3K and the production of phosphatidylinositol 3,4,5-triphosphate, which in turn stimulates the formation of lamellipodia (19, 29–31), the role of PDGF in WAVE3-mediated actin reorganization and cell motility has not been demonstrated. We used the human MDA-MB-231 adenocarcinoma cells to investigate whether PDGF is involved in WAVE3-mediated actin reorganization and cell motility. Here we show that PDGF treatment of MDA-MB-231 cells induced the formation of lamellipodia at the edges of migrating cells, and the accumulation of WAVE3 in these lamellipodia. WAVE3 has also been shown to act downstream of PI3K and is involved in cell migration and the formation of lamellipodia induced by PDGF. We also show that this WAVE3 activity might be regulated through interaction with p85, the regulatory subunit of PI3K.

**EXPERIMENTAL PROCEDURES**

**Materials**

The WAVE3 siRNA oligonucleotide, 5'-UAGUACUCUUGGCGC-UAC-3', and the control siRNA, 5'-UCUCAAGACAAAGUGGUA-3', sense strand containing 3'-overhangs of two deoxythymidines, were purchased from Dharmacon and annealed according to the manufacturer's instructions. The control siRNA was initially designed to target the ZNF198 gene transcript but failed to affect the expression levels of ZNF198 in all the cell lines we tested. SuperScript II Reverse Transcriptase Kits and Tq polymerase were obtained from Invitrogen. PCR primers were synthesized by Integrated DNA Technologies (Corvallis, OR). The primer sequences assessing glyceraldehyde-phosphate dehydrogenase were 5'-GAAGGAGGATGCTGAGT-3' for the forward primer and 5'-GAAGATTGCGTGGATGAT-3' for the reverse primer, for p85, 5'-TACCCCCGTCTGACTCCCT-3' for the forward primer and 5'-TCATCCCTCTTGGAAATTG-3' for the reverse primer. Primers for WAVE1, WAVE2, and WAVE3 were as previously reported (6). The antibodies used in this study were as follows: human WAVE3/Scar and GST obtained from Upstate Biotechnology (Charlottesville, VA) and human PI3K p85 obtained from BD Biosciences (San Diego, CA). Gel electrophoresis reagents were from Bio-Rad (Hercules, CA).

**Methods**

**Cell Culture**—Human MDA-MB-231 adenocarcinoma cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. For stimulation with PDGF, the cells were first subjected to serum starvation for 24 h in DMEM without serum. Cells were then incubated with 50 ng/ml PDGF for 1 h, followed by incubation with 20% fetal bovine serum for 20 min at 37°C. The cells were then washed twice with PBS. Immediately, cells were lysed in 0.5% Triton X-100 in PBS for 20 min at room temperature and washed again with PBS, and the cell lysates were centrifuged (25 000 × g) for 10 min at 4°C, followed by incubation with protein G-Sepharose beads (Amersham Biosciences) for 45 min at 4°C on a rotary wheel overnight at 4°C. The next morning the protein mixtures were incubated with the anti-WAVE3 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 immunoprecipitation using GST fusion proteins the cleared cell lysates were immediately mixed with 100 μg of either GST, GST-p85, or GST-containing proteins and the tubes were rotated on a rotary wheel overnight at 4°C. Precipitates were resolved by SDS-PAGE and analyzed by immunoblotting. For immunoprecipitation using GST fusion proteins the cleared cell lysates were immediately mixed with 100 μg of either GST, GST-p85, or GST-containing proteins and the tubes were rotated on a rotary wheel overnight at 4°C. The next morning the protein mixtures were incubated with the anti-WAVE3 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 immunoprecipitation using GST fusion proteins the cleared cell lysates were immediately mixed with 100 μg of either GST, GST-p85, or GST-containing proteins and the tubes were rotated on a rotary wheel overnight at 4°C. The next morning the protein mixtures were incubated with the anti-WAVE3 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 immunoprecipitation using GST fusion proteins the cleared cell lysates were immediately mixed with 100 μg of either GST, GST-p85, or GST-containing proteins and the tubes were rotated on a rotary wheel overnight at 4°C. The next morning the protein mixtures were incubated with the anti-WAVE3 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.

**siRNA Treatment**—An siRNA oligonucleotide targeting the human WAVE3 mRNA at nucleotide position 93–111 from the start codon was designed. Cells were cultured in DMEM supplemented with 4.5 g/liter glucose, 10% fetal bovine serum, and 2 mM glutamine. For transient transfections, 2 × 10^5 cells were plated in 6-well plates in DMEM containing 0.5% bovine serum albumin (BSA) in PBS for 4 h at room temperature. Primary as well as secondary antibodies were diluted at the same 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 DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA). Fluorescent images were captured using a Nikon TE2000-E inverted microscope.

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Fig. 1. Suppression of PDGF-induced lamellipodia formation and cell migration following siRNA targeting of WAVE3. RT-PCR analysis (A) of MDA-MB-231 cells shows reduced levels of the WAVE3 mRNA in the cells transfected with anti-WAVE3 siRNA (siWAVE3) compared with either the cells transfected with the control si-RNA (si-control), nontransfected cells, or cells transfected with Oligofectamine alone (Oligo). Expression levels of WAVE1, WAVE2, p85, and the control glyceraldehyde-3-phosphate dehydrogenase did not show any changes. B, Western blot analysis of MDA-MB-231 cells shows reduced levels of the WAVE3 protein in the cells transfected with anti-WAVE3 siRNA (siWAVE3) compared with either cells transfected with the control si-RNA (si-control), nontransfected cells, or cells transfected with Oligofectamine alone (Oligo). p85 levels show equivalent protein loading. C, photomicrographs of rhodamine-phalloidin-stained cells transfected with either Oligofectamine alone (left), with siWAVE3 (center), or with the control siRNA (right) show the loss of lamellipodia and increased stress fiber formation in siWAVE3-transfected cells, but not in the cells transfected with Oligofectamine alone or with the control siRNA. Scale bar, 20 μm. Quantitation of the ratio of cells with lamellipodia in the siWAVE3-transfected and control groups are shown in D. The values shown represent the mean ± S.D. of triplicate experiments. In E three-dimensional migration chamber assay shows reduced PDGF-induced migration in cells transfected with siWAVE3. Magnification, ×200. Quantitation (F) shows a 80% reduction in migration capacity in cells transfected with siWAVE3. Retardation of PDGF-induced migration by siWAVE3 was also demonstrated using the wound-closure assay (G). Cells transfected with Oligofectamine alone or with the control siRNA readily closed the wound over 24 h, whereas siWAVE3-transfected cells could not. Magnification, ×200.

RESULTS

The WASP and WAVE proteins have been shown to play essential roles in the regulation of actin reorganization and in the formation of filopodia and lamellipodia, respectively, leading to cell migration in several cell types (7, 29, 31–33). WAVE1 and WAVE2 are required for PDGF-induced lamellipodia formation and cell migration (19, 29, 30). The involvement of WAVE3 in the PDGF-induced cell migration has, however, not been determined. We, therefore, addressed whether WAVE3 is also involved in PDGF-induced lamellipodia formation and cell migration in MDA-MB-231 cells.

WAVE3 Is Required for Cell Migration—To investigate whether WAVE3 plays a role in cell migration, we targeted the WAVE3 transcript in MDA-MB-231 cells using short interfering RNAs (siRNA). Untreated cells show high endogenous levels of WAVE3, and siRNA treatment resulted in >90% knockdown of the mRNA (Fig. 1A). The expression levels of the closely related WAVE1 and WAVE2 genes were not affected by the siRNA treatment, indicating that, within this family of genes, the siRNA we used is specific for WAVE3. Treatment with WAVE3 siRNA resulted in a comparable reduction in protein levels (Fig. 1B). Because WAVE3 is predicted to be involved in actin remodeling, we next determined whether reduction in WAVE3 protein levels affect the formation of lamellipodia, which are necessary for cell movement and migration. ~30% of cells treated with Oligofectamine alone or with the control siRNA (si-control) formed lamellipodia after being induced with PDGF (Fig. 1, C and D). The number of cells with PDGF-induced lamellipodia was, however, reduced to <10% after transfection with siRNA against WAVE3 (Fig. 1, C and D). Because actin polymerization has also been shown to be involved in cell migration, we investigated the migration ability of MDA-MB-231 cells toward PDGF, after treatment with siRNA using the three-dimensional migration chamber assay. As shown in Fig. 1E, both untreated cells and the cells treated with the control siRNA are easily attracted toward PDGF and pass freely through the collagen-treated barrier, whereas siRNA-treated cells show a marked reduction in their ability to migrate toward PDGF (Fig. 1, E and F). We also determined 

PI3K Regulates WAVE3-mediated Cell Migration
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Fig. 2. Lamellipodia formation and migration of MDA-MB-231 cells is induced by PDGF. In A MDA-MB-231 cells were serum-starved for 24 h and then stimulated with 50 ng/ml PDGF for 30 min. Rhodamine-phalloidin stained lamellipodia (arrows; scale bar, 20 μm), which were absent in the control (left) are induced by PDGF (right)—3-fold (B). The values represent the means ± S.D. of triplicate experiments. In C the wound-closure assay (magnification, ×100) of serum-starved cells shows a vastly increased ability of the PDGF-treated cells to close the gap. In D a three-dimensional migration chamber assay shows that serum-starved cells have a greatly increased ability to pass through the chamber in the presence of PDGF (right), compared with untreated cells (left), which is ~6-fold greater (E). These values were derived from the analysis of cell numbers in ten microscopic fields in each experiment.

PDGF Induces Lamellipodia Formation and Migration of MDA-MB-231 Cells—The role of PDGF in cell migration has already been determined in several cell types (3, 31, 34). To establish a link between PDGF and cell migration in our system, we investigated the effect of PDGF treatment on the ability of MDA-MB-231 cells to form lamellipodia and migrate toward PDGF. When MDA-MB-231 cells were serum-starved for 24 h, the majority of cells showed elongated and flattened shapes, and only ~10% of cells formed lamellipodia (Fig. 2, A and B). Treatment of these cells with 50 ng/ml PDGF for 30 min induced lamellipodia formation and a polarized shape in more than 30% of cells (Fig. 2, A and B). We next examined the effect of PDGF treatment on cell motility using the wound-closure assay. PDGF-stimulated cells migrated into the wounded area and were able to close the wound within 24 h, whereas, in the serum-starved cells, the wound remained open after 24 h (Fig. 2C). As an alternative measure of cell motility, we also examined the effect of PDGF on the ability of cells to migrate in a three-dimensional migration chamber assay. We observed an increase of ~6-fold in the number of cells that were able to migrate toward PDGF, compared with the control cells not treated with PDGF (Fig. 2, D and E). Thus, PDGF is also capable of stimulating the migration of MDA-MB-231 cells, in both nondirectional (wound-closure) and directional (three-dimensional migration chamber) assays, probably by inducing lamellipodia formation at the leading edges.

WAVE3 Co-localizes with the Actin Structures in the Lamellipodia—Immunoblotting with a specific WAVE3 antibody showed that WAVE3 protein is present in both the serum-starved and PDGF-induced cells (Fig. 3A). WAVE3 is usually predominantly expressed in normal brain tissue (6, 26, 35). However, we have shown that WAVE3 is also expressed in several advanced stage neuroblastoma tumors and cell lines as well as in metastatic breast cancer cell lines, including MDA-MB-231 cells (26), which may account for the relationship between overexpression of WAVE3 and the metastatic phenotype.

We next investigated whether treatment of MDA-MB-231 cells with PDGF affects the intracellular distribution of the WAVE3 protein. Immunocytochemistry analyses showed that WAVE3 was largely concentrated in the nucleus and the cytoplasm of the untreated cells (Fig. 3B). WAVE3 protein is present in both the nucleus, WAVE3 was also concentrated in the lamellipodia and co-localized with the actin structures in the lamellipodia (Fig. 3B). Additionally, the unstained cells showed elongated and flattened shapes and numerous stable actin fibers (Fig. 3B). On the other hand, cells induced with PDGF were able to form lamellipodia at the leading edges of migrating cells (Fig. 3B), and, in addition to the diffuse distribution of WAVE3 in the cytoplasm and the nucleus, WAVE3 was also concentrated in the lamellipodia and co-localized with the actin structures in the lamellipodia (Fig. 3B).

Interaction between WAVE3 and p85 May Be Required for WAVE3-mediated Lamellipodia Formation and Cell Migration—Analysis of the WAVE1 and WAVE2 proteins demonstrates that their function is mediated through specific protein-protein interactions (12, 13, 16, 17). To investigate whether the same is true for WAVE3 we used the yeast two-hybrid system.
to identify potential binding partners for WAVE3. As a result, p85, the regulatory subunit of the PI3K, was identified as a potential WAVE3-interacting protein. This interaction between WAVE3 and p85 was confirmed by co-immunoprecipitation (IP) of WAVE3 and p85 from lysates of MDA-MB-231 cells. The presence of both the WAVE3 (Fig. 4A) and p85 (Fig. 4A) proteins in the lysate was confirmed by immunoblotting using anti-WAVE3 and anti-p85 antibodies. Using the anti-p85 antibody for IP and the anti-WAVE3 antibody for Western blot analysis, a 66-kDa band, consistent with the size of the WAVE3 protein, was detected (Fig. 4A). The rabbit pre-immune IgG alone was not able to precipitate the WAVE3-p85 complex, thus demonstrating the specificity of the co-IP.

To identify which particular domain of p85 mediates the WAVE3-p85 interaction, a series of truncated fragments of p85, fused to GST, were used in co-IPs from protein extracts of MDA-MB-231 cells. Immunoprecipitation of WAVE3 with the anti-WAVE3 antibody resulted in the co-IP of the C-terminal Src homology 2 domain of p85 (p85CSH2), but did not co-IP either the Src homology 3 (p85SH3), or the N-terminal Src homology 2 (p85-NSH2) domains, or GST alone (Fig. 4B). These results clearly indicate that p85 interacts directly with WAVE3 through the C-terminal SH2 domain.

A series of WAVE3-truncated fragments fused to the C terminus of either EGFP or the GAL4 DNA binding domain (GAL4BD) were generated to determine which particular domain of WAVE3 was involved in the interaction with p85. HEK-293 cells were transfected with either the GFP-tagged WAVE3 constructs or the pEGFP-C1 vector alone, and clones were selected that showed stable expression of the fusion proteins. These clones were used for IP using an anti-p85 antibody, which resulted in the co-IP of both the full-length WAVE3 protein (W3F) and the WAVE3 construct lacking the VCA domain (W3ΔVCA), but not GFP (Fig. 4C). These results indicate that the VCA domain of WAVE3 is not required for the interaction with p85.

The WAVE3 domain that binds to p85 was further refined using the β-galactosidase assay with yeast clones co-transformed with the GAL4BD-WAVE3 constructs and the AD-p85 construct. As expected, those clones containing W3F or W3ΔVCA constructs generated β-galactosidase activity indicating an interaction with AD-p85 (Fig. 4D), further confirming the observations in the co-IP assays. On the other hand, yeast clones containing either the DNA BD domain alone, or other truncated forms of WAVE3, did not yield blue colonies (Fig. 4D), indicating that these WAVE3 domains are not directly responsible for the interaction with p85.

**PI3K Activity May Be Required for the Regulation of WAVE3-mediated Lamellipodia Formation and Cell Migration**—The direct interaction between WAVE3 and the regulatory subunit
of PI3K strongly supports the involvement of PI3K in the regulation of WAVE3-mediated cell migration and lamellipodia formation. To investigate whether PI3K is involved in PDGF-induced lamellipodia formation and cell migration in MDA-MB-231 cells, as it has been shown in a variety of cell types (1, 30, 36–40), MDA-MB-231 cells were treated with the PI3K inhibitor LY294002. Only 10% of the serum-starved cells were able to form lamellipodia (Fig. 5, A and B), whereas treatment of the cells with 50 ng/ml PDGF for 30 min resulted in the formation of lamellipodia in >30% of cells (Fig. 5, A and B). Addition of LY294002 to cells at the concentration of 30 μM, together with PDGF, suppressed the formation of lamellipodia in >90% of cells (Fig. 5, A and B). Similarly, PDGF treatment resulted in a ~6-fold increase in the number of cells able to migrate through the collagen-coated membrane (Fig. 5C), and treatment with LY294002 inhibited cell migration to levels comparable with the untreated cells (Fig. 5C). These results show that inhibition of PI3K activity interferes with the PDGF-induced lamellipodia formation and cell migration. Because we have also shown that WAVE3 is required for PDGF-mediated lamellipodia formation and cell migration (Fig. 1), these results also support an essential role for PI3K in WAVE3-mediated lamellipodia formation and cell migration by recruiting WAVE3 to the edges of polarized cells. Together, the results presented here demonstrate that WAVE3 is essential for PDGF-induced lamellipodia formation and migration of MDA-MB-231 cells, that PI3K activity is required for this WAVE3-mediated process, and that a direct interaction between WAVE3 and p85 may be required for WAVE3 activity.

**DISCUSSION**

The ability of a cell to migrate is a critical component for both normal cellular processes as well as for tumorigenesis (1, 41, 42). WAVE3 is a member of the WASP/WAVE family of proteins, which mediate cellular motility through the Rac and Cdc42 pathways (12, 13, 17, 43, 44). WASP and N-WASP are activated downstream of Cdc42 and phosphatidylinositol 4,5-biphosphate to induce filopodia formation (43, 44), whereas activation of Rac leads to the formation of lamellipodia and cell migration by the WAVE proteins (12, 13). Although much is known about the pathways leading to the activation of WASP, N-WASP, WAVE1, and WAVE2, relatively little is known about the involvement of WAVE3 and its regulation in the processes of actin reorganization and cell migration. Furthermore, several studies have recently reported the involvement of PDGF in the N-WASP- and WAVE2-mediated lamellipodia formation and cell migration (19, 29, 30). Thus, we investigated whether WAVE3 has a role in the formation of lamellipodia and cell migration in MDA-MB-231 cells in response to PDGF.

As a first step toward understanding the involvement of WAVE3 in actin reorganization that leads to cell motility, we determined whether WAVE3 is associated with actin struc-
PI3K Regulates WAVE3-mediated Cell Migration

Sequence analysis of WAVE3 identified only a single potential target for tyrosine kinase phosphorylation at amino acid 151 (Tyr-151). Furthermore, this tyrosine residue is conserved in all three WAVE proteins, and a recent study has shown that, in WAVE2, it is the only tyrosine residue to undergo phosphorylation by abelson tyrosine kinase (48), further supporting our findings. WAVE3 Tyr-151 is involved in mediating the interaction between WAVE3 and p85.

We suggest two possible functional consequences of the direct interaction between p85 and WAVE3 that is described here. First, the interaction may positively regulate actin cytoskeletal remodeling by providing a necessary link between Rac and WAVE3, with the resulting activation of the Arp2/3 complex and the reorganization of the cytoskeleton. The product of PI3K activity, phosphatidylinositol 3,4,5-triphosphate, was shown to be required for the regulation of Rac activity (49). The activation of Rac is also mediated by a direct p85-Rac interaction (50, 51). We have found that p85 binds to WAVE3 through the C-terminal SH2 domain. This association is direct, as indicated by the one-to-one interaction in the yeast two-hybrid assay, and occurs both in vitro and in vivo, as suggested by the co-IP assays. WAVE3 and p85 also co-localize to sites of active actin polymerization in the cell membrane. These results suggest that p85 may be an essential intermediate between Rac and WAVE3 in the regulation of the actin-cytoskeleton, much in the same way IRSp53 funnels signals from Rac to WAVE1 and WAVE2 proteins (13). Alternatively, the interaction between WAVE3 and p85 may play a role in negatively regulating the activity of WAVE3. A direct interaction between p85 and Abi1 has recently been suggested to be necessary for Abi1-dependent Rac activation, which leads to actin reorganization (52). Interestingly, Abi1, a component in a multiprotein complex, was shown to negatively regulate WAVE1- and WAVE2-mediated actin polymerization and reorganization (12, 16, 17). The demonstration that WAVE3 and p85 interact also suggests that p85, in association with an inhibitory multiprotein complex that may include Abi1 as well as other proteins, may regulate the activity of WAVE3 by sequestering it in an inactive form until appropriate extracellular stimuli are received. It is possible, however, that both of these possibilities are part of the same regulatory pathway.

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PI3K Regulates WAVE3-mediated Cell Migration

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