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WAVE2, N-WASP, and Mena Facilitate Cell Invasion Via Phosphatidylinositol 3-Kinase-Dependent Local Accumulation of Actin Filaments

Kazuhide Takahashi* and Katsuo Suzuki

Molecular Cell Biology Division, Kanagawa Cancer Center Research Institute, Yokohama 241-0815, Japan

ABSTRACT

Cell migration is accomplished by the formation of cellular protrusions such as lamellipodia and filopodia. These protrusions result from actin filament (F-actin) rearrangement at the cell cortex by WASP/WAVE family proteins and Drosophila enabled (Ena)/vasodilator-stimulated factor proteins. However, the role of each of these actin cytoskeletal regulatory proteins in the regulation of three-dimensional cell invasion remains to be clarified. We found that platelet-derived growth factor (PDGF) induces invasion of MDA-MB-231 human breast cancer cells through invasion chamber membrane pores. This invasion was accompanied by intensive F-actin accumulation at the sites of cell infiltration. After PDGF stimulation, WAVE2, N-WASP, and a mammalian Ena (Mena) colocalized with F-actin at the sites of cell infiltration in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. Depletion of WAVE2, N-WASP, or Mena by RNA interference (RNAi) abrogated both cell invasion and intensive F-actin accumulation at the invasion site. These results indicate that by mediating intensive F-actin accumulation at the sites of cell infiltration, WAVE2, N-WASP, and Mena are crucial for PI3K-dependent cell invasion induced by PDGF. J. Cell. Biochem. 112: 3421–3429, 2011.

KEY WORDS: CELL INVASION; PI3K; WAVE2; N-WASP; MENA; PDGF

Cell migration is dependent upon the rearrangement of actin filaments (F-actin) at the cell cortex [Mitchison and Cramer, 1996]. F-actin rearrangement is accompanied by the formation of cellular protrusions such as lamellipodia and filopodia [Svitkina and Borisy, 1999]. The formation of these cellular protrusions is regulated by the Rho family of small GTPases, including Rac, Cdc42, and Rho [Hall, 1998], which act through the WASP/WAVE family of actin cytoskeletal regulatory proteins [Miki et al., 1998; Machesky et al., 1999; Suetsugu et al., 1999; Higgs and Pollard, 2000]. N-WASP, a member of WASP family proteins [Derry et al., 1994; Miki et al., 1996], is thought to be necessary for lamellipodia formation [Lorenz et al., 2004]. WAVE family proteins, including WAVE1, WAVE2, and WAVE3 [Derry et al., 1994; Miki et al., 1998; Suetsugu et al., 1999], induce not only lamellipodia [Takenawa and Miki, 2001; Takahashi and Suzuki, 2008, 2009], but also filopodia [Nakagawa et al., 2003], indicating that the signaling pathways that lead to formation of lamellipodia and filopodia may be neither entirely parallel nor independent.

Drosophila enabled (Ena)/vasodilator stimulated factor proteins (VASP), including mammalian Ena (Mena) [Gertler et al., 1996], are localized to the tips of lamellipodia [Rottner et al., 1999; Nakagawa et al., 2001] and filopodia [Krugmann et al., 2001; Homem and Peifer, 2009]. These proteins antagonize F-actin capping and reduce the density of F-actin branches [Bear et al., 2002; Pasic et al., 2008] through the association with and clustering of F-actin barbed ends [Applewhite et al., 2007]. Despite the current understanding of the process, some theories regarding the roles of Ena/VASP in the formation of cellular protrusions remain controversial [Rottner et al., 1999; Bear et al., 2000; Krugmann et al., 2001; Nakagawa et al., 2001; Moeller et al., 2004; Homem and Peifer, 2009].

Hepatocyte growth factor (HGF) induces lamellipodia formation and cell migration through N-WASP and WAVE2 [Kawamura et al., 2004; Takahashi and Suzuki, 2008; Takahashi and Suzuki, 2009]. HGF also induces three-dimensional cell invasion through pores in the basement membrane matrix-coated invasion chamber membrane; however, HGF-induced cell invasion is far weaker than invasion induced by serum [Suzuki and Takahashi, 2008]. This result suggests the involvement of a potent invasion-inducing factor in serum. Platelet-derived growth factor (PDGF), for instance, is identified as a serum growth factor for fibroblasts, smooth muscle cells, and glial cells [Kohler and Lipton, 1974; Westermark and Wasteson, 1976]. Via its receptor PDGFR, PDGF induces a variety of cellular responses, including cell migration, in several cell types [Suetsugu et al., 2003; Sossey-Alaoui et al., 2005]. However, little is

*Correspondence to: Kazuhide Takahashi, Molecular Cell Biology Division, Kanagawa Cancer Center Research Institute, Yokohama 241-0815, Japan. E-mail: ktaka@gancen.asahi.yokohama.jp
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known about the role played by PDGF in the induction of cell invasion.

In this study, we use an invasion chamber membrane coated with the basement membrane matrix to investigate whether PDGF can induce three-dimensional invasion of MDA-MB-231 human breast cancer cells. We also investigate the role of WASP/WAVE and Ena/VASP family proteins, which are considered necessary for the formation of two-dimensional cellular protrusions, in PDGF-induced invasion.

MATERIALS AND METHODS

CELL CULTURE

MDA-MB-231 human breast cancer cells (European Collection of Cell Culture, Wiltshire, UK) were maintained as described previously [Takahashi and Suzuki, 2008, 2009]. The cells were serum starved in a low-serum medium containing 0.1% fetal bovine serum (FBS) for 16 h before PDGF stimulation.

CELL INVASION ASSAY

A three-dimensional cell invasion assay was performed, using an invasion chamber (24-well, 8-μm pore size; BD Biosciences, Bedford, MA) as described previously [Suzuki and Takahashi, 2008]. Briefly, cells (1×10^4) were seeded on chamber insert membranes and incubated for 6 h toward low-serum medium or low-serum medium containing 50 ng/ml recombinant human PDGF-AB or PDGF-BB (Peprotech, London, UK) in lower chambers together with or without the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin [Powis et al., 1994] (Echelon Biosciences, Salt Lake City, UT) or LY294002 [Vlahos et al., 1994] (Calbiochem, La Jolla, CA) at the indicated concentrations. To quantify invasion, cells that invaded through the pores and spread over the bottom surface of chamber insert membrane in 3 mm × 3 mm area were counted after staining with Giemsa solution [Suzuki and Takahashi, 2008]. Statistical analyses of the difference between the mean (SD) values of triplicate experiments were carried out by unpaired Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

ANTIBODIES

Anti-WAVE2, anti-N-WASP (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Mena (BD Biosciences) antibodies were used for immunofluorescence experiments. Anti-WAVE2 (Millipore, Temecula, CA), anti-N-WASP (Cell Signaling, Beverly, MA), anti-Mena (BD Biosciences), and anti-β-actin (Sigma–Aldrich, St. Louis, MO) antibodies were used in immunoblotting analysis as described previously [Takahashi and Suzuki, 2008, 2009].

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Cells adhered to the top and bottom surfaces of chamber insert membranes after invasion assay were fixed in 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 3% bovine serum albumin. The cells were incubated with rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA) and primary antibodies, followed by a secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Cells were observed using a confocal microscope (LSM510; Carl Zeiss Microimaging, Jena, Germany), equipped with Ar and He/Ne lasers with excitation filters of 488 and 543 nm, respectively. Fluorescence images were scanned using Plan-Neofluar objective (40×, 0.75) at 512 × 512 bits per pixed resolution and a pinhole of 104 with band pass filters of 505–530 and 560 nm, respectively. All horizontal and vertical images were obtained from corresponding single images at the positions indicated as the horizontal lines in the vertical and horizontal sections, respectively. The images were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

RNA INTERFERENCE ASSAY

To achieve RNA interference (RNAi), cells seeded on plastic culture dishes were incubated for 48 h with 50 nM of control small interfering RNA (siRNA) or siRNA against WAVE2 (WASF2), N-WASP (WASL), or Mena (ENAH) (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen). The following target sequences were used in this study: WAVE2: 5′-AAAGGTATTTGCCTGAGTAAAGAGC-3′; N-WASP: 5′-GAAATGTGTGACTATGTCTTT-3′; Mena: 5′-GAGTCCA-GACGGAAGGACTTGACTA-3′. To assess the efficiency of siRNA against protein expression, total cell lysates or WAVE2 immunoprecipitates in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer were prepared, and immunoblotting was carried out as previously described [Takahashi and Suzuki, 2008, 2009].

RESULTS

PDGF INDUCES THREE-DIMENSIONAL INVASION OF MDA-MB-231 HUMAN BREAST CANCER CELLS

Human PDGF was originally identified as a dimer of two different polypeptide chains (A and B), which binds to one of two types of the receptor PDGFR. As PDGF-AB is found in human platelets and PDGF-BB is found in many cancers [Andrae et al., 2008], we used both forms to examine their role in inducing invasion of MDA-MB-231 cells. After incubation toward low-serum medium or medium containing PDGF-AB or PDGF-BB, cells that had infiltrated membrane pores and adhered to the bottom surface of the membrane were stained with Giemsa solution and quantified (Fig. 1a). Compared to control cultures, the number of invaded cells was increased by PDGF-AB and PDGF-BB to more than 2-fold ($^{**}P < 0.01$) or 1.9-fold ($^{*}P < 0.05$), respectively (Fig. 1b). Thereafter, we used PDGF-AB to induce MDA-MB-231 cell invasion.

PDGF INDUCES INTENSIVE F-ACTIN ACCUMULATION AND COLOCALIZATION OF WAVE2, N-WASP, AND MENA WITH F-ACTIN AT THE SITES OF CELL INFILTRATION

To determine whether PDGF-induced cell invasion is accompanied by rearrangement of F-actin via WAVE family proteins, cells on chamber insert membrane were stained with phalloidin and anti-WAVE2 antibody. WAVE2 staining was distributed over the central region of the cytoplasm surrounded by peripheral phalloidin staining in cells that spread over the top and bottom surfaces of chamber insert membrane (Fig. 2a, X–Y section). The X–Z vertical view revealed that WAVE2 colocalized with F-actin to the round cell body on the membrane pores and to the columnar structure within

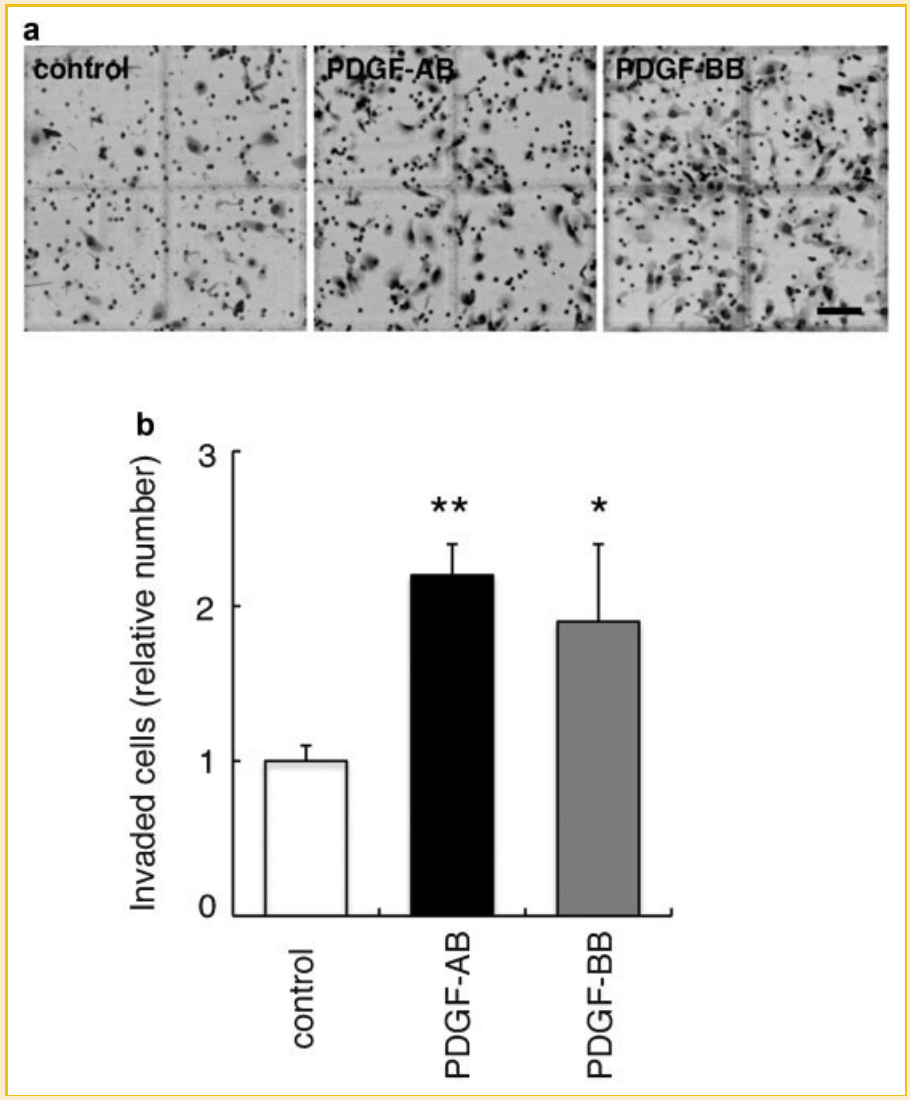

Fig. 1. PDGF induces three-dimensional invasion of MDA-MB-231 cells. a: After incubation toward low-serum medium (control) or low-serum medium containing PDGF-AB or PDGF-BB for 6 h, cells were stained with Giemsa solution. Scale bar, 100 μm. b: The number of invaded cells relative to control cells was determined; the mean (SD) values of triplicate assays are given. $^{**}P < 0.01$; $^{*}P < 0.05$, by Student's *t*-test.

the pores at the rear of invading cells (Fig. 2a, arrowhead in the X–Z section). When cells were stained with anti-N-WASP antibody and phalloidin, N-WASP that was co-distributed with F-actin throughout the cytoplasm of cells that spread over chamber insert membrane (Fig. 2b, X–Y section) was strongly concentrated with F-actin to the columnar structure within the pore at the rear of invaded cells (Fig. 2b, arrowhead in the Y–Z section). Dual staining of Mena and F-actin revealed that Mena colocalized with F-actin throughout the cytoplasm of cells that spread over the top surface of chamber insert membrane (Fig. 2c, X–Y section in left panels) with intensive colocalization at the tips of cellular protrusions within the membrane pore (Fig. 2c, arrowhead in the Y–Z section of left panels) or at the rear of invaded cells in the columnar structure within the membrane pore (Fig. 2c, arrowhead in the Y–Z section of right panels).

PDGF-INDUCED CELL INVASION AND INTENSIVE F-ACTIN ACCUMULATION REQUIRE WAVE2, N-WASP, AND MENA

Colocalization of WAVE2, N-WASP, or Mena with F-actin at the tips of cell protrusions and at the sites of cell infiltration (Fig. 2) suggested the involvement of WAVE2, N-WASP, and Mena in PDGF-induced cell invasion and F-actin accumulation. To confirm this, WAVE2, N-WASP, and Mena were independently depleted by siRNA. When WAVE2 or N-WASP expression was suppressed (Fig. 3a), the number of invaded cells after PDGF incubation was significantly reduced to 63% ($^{*}P < 0.01$) or 43% ($^{**}P < 0.001$), respectively, of the level in cells transfected with control siRNA (Fig. 3b). Phalloidin staining revealed that control siRNA-transfected cells that infiltrated through the membrane pores exhibited intensive F-actin accumulation in the columnar structures of the cells (Fig. 3c, arrowhead in the X–Z section of left panels). In

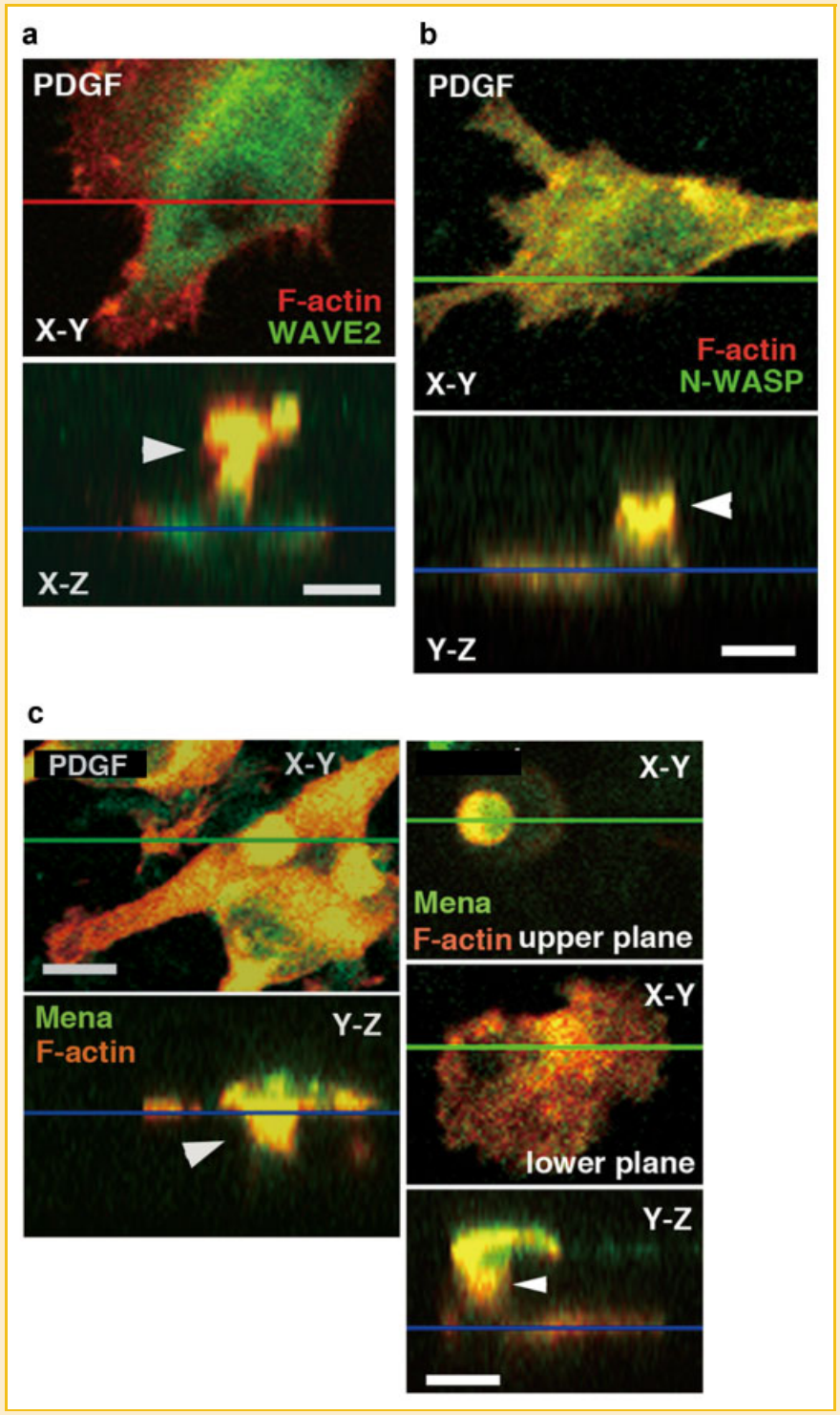

Fig. 2. PDGF induces colocalization of WAVE2, N-WASP, or Mena with F-actin at the sites of cell infiltration. After incubation toward PDGF, cells were stained with phalloidin (red) and (a) anti-WAVE2 (green), (b) anti-N-WASP (green), or (c) anti-Mena antibody (green). Merged images in the X–Z or Y–Z sections (arrowheads) indicate colocalization of (a) WAVE2, (b) N-WASP, or (c) Mena with F-actin at the tips of cellular protrusions or in the columnar structures at the rear of cells. The horizontal lines in the X–Y sections and in the X–Z or Y–Z sections represent the positions shown as vertical single views in the X–Z or Y–Z sections and as horizontal single views in the X–Y sections, respectively. Some merged images are overexposed in order to show other fainter images shown in green or red. Scale bars, 10 μm.

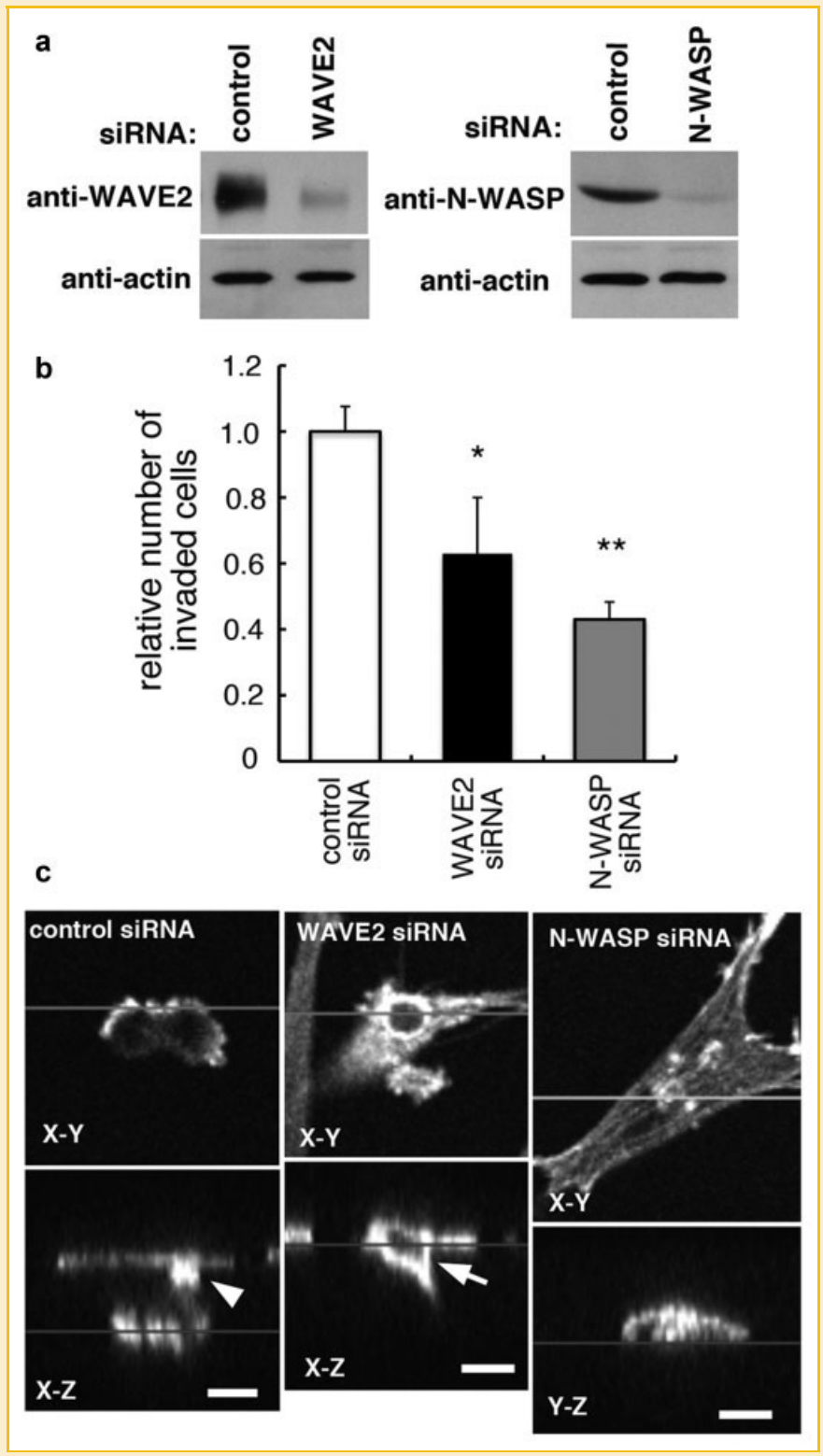

Fig. 3. Cell invasion and intensive F-actin accumulation induced by PDGF require WAVE2 and N-WASP. a: Whole lysates or WAVE2 immunoprecipitates from cells transfected with control, WAVE2, or N-WASP siRNA were immunoblotted with antibodies to β-actin, WAVE2, and N-WASP. b: Cells transfected with control, WAVE2, or N-WASP siRNA were incubated toward PDGF and stained with Giemsa solution. The number of invaded cells relative to control siRNA-transfected cells was determined, and the mean (SD) values of triplicate assays are given. $^{**}P < 0.001$; $^{*}P < 0.01$. c: Cells transfected with control, WAVE2, or N-WASP siRNA were incubated toward PDGF and stained with phalloidin. The arrowhead and arrow in the X–Z sections indicate intensive F-actin accumulation and incomplete F-actin accumulation, respectively. Scale bars, 10 μm.

contrast, WAVE2-depleted cells that did not infiltrate through the pores on the chamber membrane formed short cellular protrusions into the pores with minimal accumulation of F-actin (Fig. 3c, arrow in the X–Z section of middle panels). The effect of N-WASP depletion on F-actin accumulation was more severe; that is, most cells that failed to infiltrate through the pores on the membrane had no cellular protrusions containing intensive F-actin accumulation and remained spread over the top surface of chamber insert membrane (Fig. 3c, right panels).

When Mena expression was depleted by siRNA, no significant effect on the expression of WAVE2 or N-WASP was observed (Fig. 4a). Nor did Mena depletion cause any significant effect on the frequency of invasion in serum-starved cells (Fig. 4b). However, the relative number of invaded cells after PDGF treatment was significantly reduced to 26% of that observed in control cells ($^{*}P < 0.01$) (Fig. 4b). In cells transfected with control siRNA, intensive F-actin accumulation at the tail tip of the invaded cell was obvious after PDGF stimulation (Fig. 4c, arrowhead in the Y–Z section of left panels). In contrast, most Mena-deficient cells, having short cellular protrusions with actin-rich tips, barely infiltrated the membrane pores and remained spread on chamber insert membrane (Fig. 4c, arrow in the X–Z section of right panels).

PDGF-INDUCED CELL INVASION, INTENSIVE F-ACTIN ACCUMULATION, AND COLOCALIZATION OF WAVE2, N-WASP, AND MENA WITH F-ACTIN AT THE SITES OF CELL INFILTRATION REQUIRE PI3K ACTIVITY

The relative number of invaded cells was significantly increased by PDGF to 4.6-fold above that in control cells ($P < 0.03$) (Fig. 5a). The PDGF signal is transmitted to PI3K [Hawkins et al., 1995], an

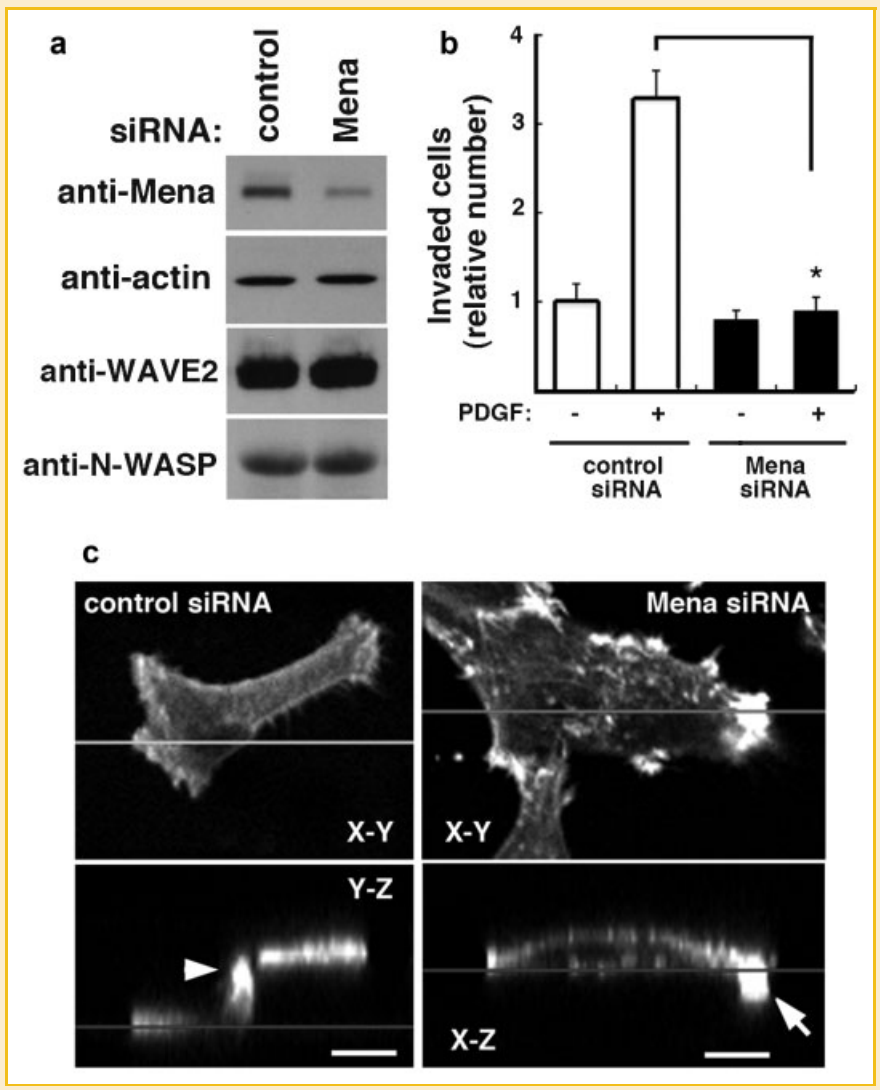

Fig. 4. Cell invasion and intensive F-actin accumulation induced by PDGF require Mena. a: Whole lysates from cells after transfection with control or Mena siRNA were immunoblotted with antibodies against β-actin, Mena, WAVE2, and N-WASP. b: Cells transfected with control or Mena siRNA were incubated toward PDGF and stained with Giemsa solution. The number of invaded cells relative to control siRNA-transfected cells was determined; the mean (SD) values of triplicate assays are given. $^{*}P < 0.01$. c: Cells transfected with control or Mena siRNA were incubated toward PDGF and stained with phalloidin. The arrowhead and arrow in the X–Z or Y–Z sections indicate intensive and incomplete F-actin accumulation, respectively. Scale bars, 10 μm.

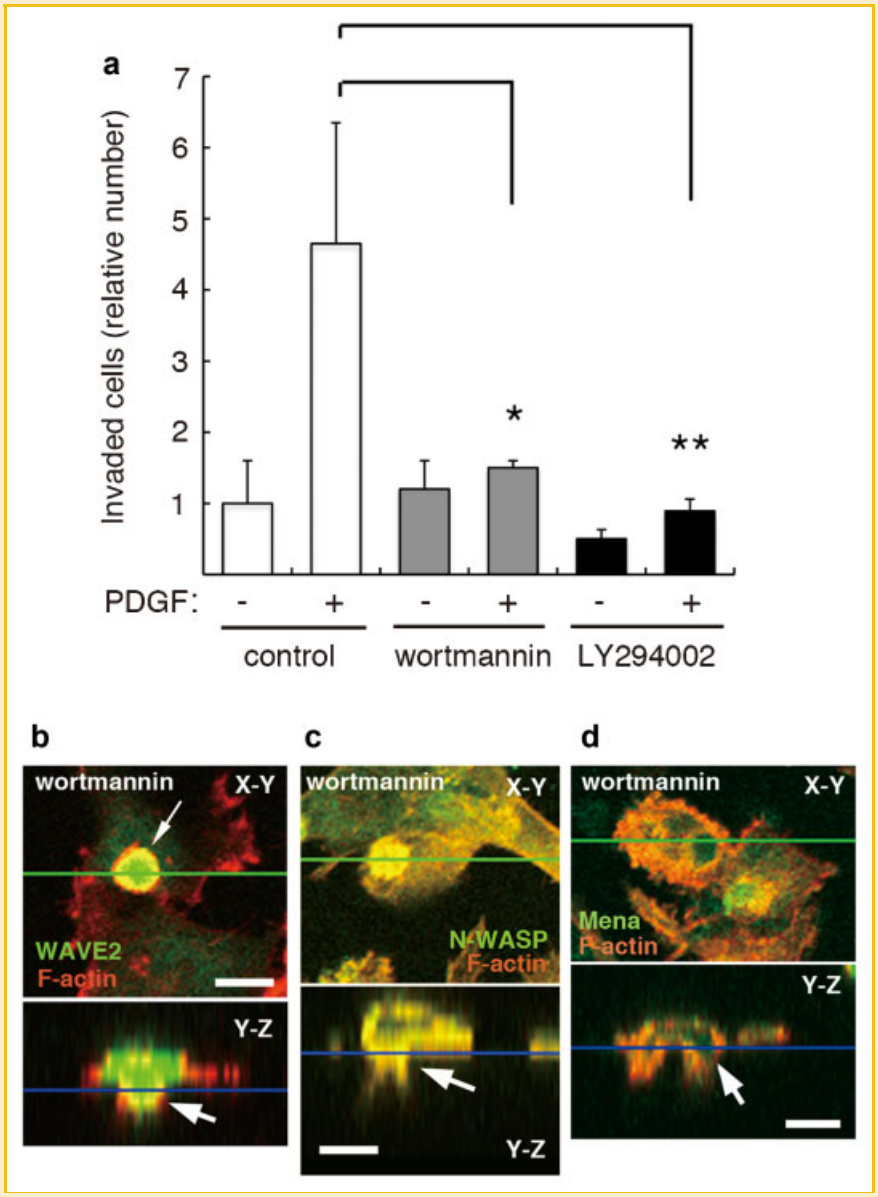

Fig. 5. Cell invasion, intensive F-actin accumulation, and colocalization of WAVE2, N-WASP, and Mena with F-actin at the sites of cell infiltration induced by PDGF require PI3K activity. a: After incubation toward low-serum medium (−) or PDGF (+) in the absence (control) or presence of 20 nM wortmannin or 20 μM LY294002, cells were stained with Giemsa solution. The number of invaded cells relative to control cells was determined, and the mean (SD) values of triplicate assays are given. $^{*}P < 0.03$; $^{**}P < 0.02$. Cells incubated toward PDGF with 20 nM wortmannin were stained with phalloidin (red) and (b) anti-WAVE2 (green), (c) anti-N-WASP (green), or (d) anti-Mena antibody (green). Arrows indicate the partial and incomplete colocalization of F-actin with WAVE2, N-WASP, or Mena at the tips of short cellular protrusions in the membrane pores. Scale bars, 10 μm.

upstream signaling molecule of WASP/WAVE family proteins. To examine whether PI3K regulates PDGF-induced cell invasion, an invasion assay was carried out using either the PI3K inhibitors wortmannin [Powis et al., 1994] or LY294002 [Vlahos et al., 1994]. Neither 20 nM wortmannin nor 20 μM LY294002 significantly affected the frequency of invasion in serum-starved cells (Fig. 5a). However, both wortmannin and LY294002 significantly reduced the PDGF-induced increase to 32% ($^{*}P < 0.03$) or 19% ($^{**}P < 0.02$), respectively (Fig. 5a).

To determine whether colocalization of WAVE2, N-WASP, or Mena with F-actin requires PI3K, an invasion assay was carried out in the presence of 20 nM wortmannin. Most cells failed to infiltrate the membrane pores, most likely due to the effects of wortmannin. In these cells, WAVE2 was distributed in the central region of the cytoplasm and accumulated within the pores; F-actin accumulation was greatly reduced compared to control (Fig. 5b, arrows in left panels). Cell staining revealed that N-WASP and F-actin partially colocalized and were evenly distributed over the cytoplasm of most cells (Fig. 5c, X–Y section), which hardly infiltrated through the membrane pores (Fig. 5c, arrow in the Y–Z section), in the presence of wortmannin. Dual staining with anti-Mena antibody and phalloidin revealed that cells that did not infiltrate the membrane

pores but instead settled over chamber insert membrane when wortmannin was present (Fig. 5d, X–Y section), exhibited short cellular protrusions; however, Mena and F-actin colocalization was incomplete (Fig. 5d, arrow in the Y–Z section).

DISCUSSION

Invasion assays showed that both PDGF-AB and PDGF-BB significantly increase the frequency of MDA-MB-231 cell invasion. In addition, it is clear that PDGF more strongly induces MDA-MB-231 cell invasion than does HGF [Suzuki and Takahashi, 2008].

Phalloidin staining revealed that PDGF (PDGF-AB) induced intensive F-actin accumulation at the tips of cellular protrusions and at the sites of cell infiltration. The intensive F-actin accumulation at these areas seems to provide the driving force for cell migration toward PDGF and for infiltration of the cell nuclei into the membrane pores of chamber insert. The process of cancer cell invasion and metastasis involves intravasation and extravasation of the cell nuclei into and out of blood vessels in vivo [Chambers et al., 2002]. Alternatively, in culture systems, cell invasion involves cell penetration between endothelial [Kramer and Nicolson, 1979] and mesothelial cells [Akedo et al., 1986]. The invasion chamber assays used in this study quantify a cell's ability to infiltrate narrow gaps, similar to those experienced in vivo or in culture. This infiltration of cells through gaps appears to require intensive F-actin accumulation in the area of invasion.

Rearrangement of F-actin is regulated by WASP/WAVE family proteins [Miki et al., 1998; Machesky et al., 1999; Suetsugu et al., 1999]; meanwhile, PDGF-induced lamellipodia formation is regulated by WAVE family proteins [Suetsugu et al., 2003; Sossey-Alaoui et al., 2005]. In this study, immunostaining revealed that both WAVE2 and N-WASP colocalized with F-actin at the sites of cell infiltration in response to PDGF, suggesting the involvement of N-WASP and WAVE2 in PDGF-induced intensive F-actin accumulation. The necessity of WAVE2 and N-WASP for cell invasion, as well as for intensive F-actin accumulation, was clearly established by RNAi assays. Theories regarding the roles of Ena/VASP family proteins, including Mena [Gertler et al., 1996], in the formation of cellular protrusions are controversial [Rottner et al., 1999; Bear et al., 2000; Krugmann et al., 2001; Nakagawa et al., 2001; Moeller et al., 2004; Homem and Peifer, 2009]. In this study, immunostaining demonstrated that, after PDGF stimulation, Mena colocalized with F-actin at the sites of cell infiltration. This suggests that Mena plays a crucial role in PDGF-induced cell invasion and intensive F-actin accumulation. This notion is supported by RNAi assay, which used siRNA to knock down Mena expression. Although Mena, a member of Ena/VASP family proteins [Gertler et al., 1996], is thought to act in reducing F-actin branch density by antagonizing F-actin capping [Bear et al., 2002; Pasic et al., 2008], this study suggests that Mena also responds to PDGF signaling by increasing F-actin accumulation at infiltration sites, thereby leading to cell invasion. Taken together, all WAVE2, N-WASP, and Mena are crucial for cell invasion to infiltrate narrow gaps through intensive accumulation of F-actin. With respect to the individual function of WAVE2, N-WASP, and Mena in inducing cell invasion, WAVE2 is suggested to induce F-actin nucleation, followed by F-actin elongation mediated by Mena [Nakagawa et al., 2001] through clustering of F-actin barbed ends [Applewhite et al., 2007]. WAVE2 links to Mena [Nakagawa et al., 2003] via IRSp53 [Krugmann et al., 2001; Takahashi and Suzuki, 2010]. N-WASP is thought to be necessary for lamellipodia formation [Lorenz et al., 2004] and to play a role in the bundling of F-actin to form microspikes in lamellipodia [Nakagawa et al., 2001]. Therefore, it is suggested that the process of cell invasion through the basement membrane matrix and narrow gaps by intensive accumulation of F-actin at the sites of cell infiltration involves the nucleation, elongation, and bundling of F-actin, which are mediated by WAVE2, Mena, and N-WASP, respectively.

The PDGF signal is transmitted to PI3K [Hawkins et al., 1995], an upstream signaling molecule of WASP/WAVE family proteins. Pharmacological analyses using PI3K inhibitors demonstrated significant inhibition of both PDGF-induced cell invasion and intensive F-actin accumulation, suggesting the necessity of PI3K activity for these events. Taken together, it can be concluded that the PDGF signal leads to PI3K-mediated activation of WAVE2, N-WASP, and Mena, which in turn leads to cell invasion through F-actin rearrangement and accumulation at the sites of cell infiltration.

In conclusion, PDGF can induce MDA-MB-231 cell invasion via PI3K activity. During the process of cell invasion, WAVE2, N-WASP, and Mena may function in a binary fashion to promote intensive F-actin accumulation at the tips of cell protrusions, cell migration toward PDGF, and intensive F-actin accumulation at the rear of infiltrating cells. This likely serves to push the cells into narrow gaps. Further clarification of the mechanisms by which WAVE2, N-WASP, and Mena cooperatively rearrange F-actin at the sites of cell infiltration should lead to an increased understanding of the regulation of three-dimensional cell invasion.

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