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Effect of WAVE2 Phosphorylation on Activation of the Arp2/3 Complex

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Members of the family of WASP-family Verprolin homologous proteins (WAVEs) activate the Arp2/3 complex to induce actin polymerization. The WAVE family comprises three proteins, namely, WAVE1, WAVE2 and WAVE3. Among them, WAVE2 is crucial for activation of the Arp2/3 complex for the formation of branched actin filaments in lamellipodia. Activation of mitogen-activated protein (MAP) kinase signalling results in the phosphorylation of the WAVE family proteins; however, which of the three WAVE proteins is phosphorylated is unclear. We found that *in vitro* WAVE2 is directly phosphorylated by a MAP kinase, i.e. extracellular signal-regulated kinase (ERK) 2. The proline-rich region and the verprolin, cofilin and acidic (VCA) region of WAVE2 were phosphorylated. Interestingly, the phosphorylated VCA region had a higher affinity for the Arp2/3 complex. However, the phosphorylation of the VCA region resulted in reduced induction of Arp2/3-mediated actin polymerization *in vitro*. The role of the phosphorylation of the proline-rich region was not determined.

Key words: Arp2/3 complex, lamellipodia, MAP kinase, vinculin, WAVE2.

Abbreviations: Arp2/3, actin-related protein 2/3; MAP kinase, mitogen-activated protein kinase; WAVE2, WASP-family Verprolin homologous protein 2; WT, wild type.

INTRODUCTION

A lamellipodium is a structure that is observed at the leading edge of motile cells and is rich in branched actin filaments (*1*). This structure is also formed when cells are stimulated with growth factors or when they express the constitutively active form of the small GTPase Rac (*2*). In lamellipodia, branched actin filaments are formed by the activated Arp2/3 complex (*1, 2*). WAVE2 is a member of the WAVE family of proteins, a subfamily of the WASP family of proteins (*3*). WAVE2 directly activates the Arp2/3 complex in lamellipodia, thus generating the branched actin filaments. WASP family proteins including WAVE2 are characterized by the presence of a C-terminal verprolin, cofilin and acidic (VCA) region. The VCA region binds to both actin monomers (through the V region) and the Arp2/3 complex (through the CA region), thus stimulating actin polymerization (*1, 2*).

Induction of actin polymerization and the subsequent formation of branched filaments are essential but not sufficient for lamellipodia formation. A lamellipodium must be attached to the substratum through adhesion machinery including integrin, vinculin, paxillin, etc. (*4*). The structure is unstable without adhesion, and as a result, the protrusive structure collapses. The Arp2/3 complex is reported to directly and transiently associate with vinculin when cells receive lamellipodia-inducing stimuli (*5*).

Mitogen-activated protein (MAP) kinase signalling is involved in a variety of essential phenomena for cell viability, growth and differentiation; it functions through nuclear translocation of specific target genes and the regulation of their transcription. However, MAP kinases are also reported to function in the cytosol for cell motility and cell-substratum adhesion through the regulation of the adhesion protein assembly (*6–8*). Treatment of cells with the MAP kinase-ERK kinase (MEK) inhibitor U0126 (*9*) is reported to reduce adhesion disassembly (*10*).

WAVE proteins are phosphorylated by various stimulations (*11*). The tyrosine at position 150 in WAVE2 is phosphorylated by Abl tyrosine kinase (*12*). However, serine/threonine phosphorylation in WAVE2 has not been defined.

MATERIALS AND METHODS

Cell Culture and Transfection—Mouse embryonic fibroblasts (MEFs) were obtained and cultured as described previously (*13*). They were transfected with various plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Materials—The MEK inhibitor U0126 was purchased from Promega (Madison, WI, USA) (Cat. No. V1121). Platelet-derived growth factor (PDGF) was purchased from Diaclone Research (Stansford, CT, USA) (Cat. No. IM-03B).

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STI-571 was a generous gift from Novartis Pharma AG (Basel, Switzerland).

Antibodies—Anti-FLAG monoclonal antibody M2 (Cat. No. F3165) and anti-vinculin monoclonal antibody (Cat. No. V9131) were purchased from Sigma (St Louis, MD, USA). Anti-phospho-extrocellular signal-regulated kinase ½ (ERK1/2) monoclonal antibody (Cat. No. 9106) was purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal anti-WAVE2 antibody was prepared as described previously (*14*).

Plasmids and Site-directed Mutagenesis—WAVE2 cDNA was subcloned into the mammalian expression vector pCMV-HA Clontech Laboratories (Mountain View, CA, USA). In order to obtain glutathione S-transferase (GST)-tagged proteins, a fragment (amino acids 180–241) of WAVE2 was introduced into the pGEX vector Amersham Biosciences (Buckinghamshire, England). GST fusion protein encompassing WAVE homology domain (WHD) and the basic region (WHD+basic), and GST-fusion VCA fragment were expressed in *Escherichia coli* and purified as described previously (*3, 15*). The GST fusion proteins of full-length WAVE2 and amino acids 171–402 of WAVE2 were expressed in the Sf9 cells by using a baculovirus system and the pFASTBAC vector (Invitrogen), as described previously (*16*). Amino acid-substituted mutants of WAVE2 were constructed using a QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mutations were confirmed by DNA sequencing.

Actin Polymerization Assay—Actin and the Arp2/3 complex were purified as described previously (*17*). A pyrene-actin assay was performed as described (*18*). The final concentrations of the Arp2/3 complex, actin monomer and pyrenyl-actin were 60 nM, 2 μM and 0.2 μM, respectively.

In Vivo Cell Labelling—MEFs were serum-starved overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% BSA. These cells were further incubated with phosphate-free DMEM (Invitrogen). Then, [^{32}P]orthophosphate (200 μCi/ml; Amersham Biosciences) was added to the medium. After 2 h of incubation, the cells were harvested immediately or after stimulation with PDGF (20 ng/ml) for 10 min. Cell lysates were immunoprecipitated using anti-FLAG antibody, and the immunoprecipitated proteins were analysed by Sodium dodacyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), western blotting and autoradiography.

In Vitro Kinase Assay—Full-length human ERK2 was cloned into pFAST-HTa (Invitrogen) and expressed in Sf9 cells. His-tagged ERK2 was purified using Ni-NTA agarose (Qiagen). GST, GST fusion WAVE proteins and myelin basic protein (MBP) were phosphorylated in kinase buffer (20 mM Tris–HCl (pH 7.5), 75 mM KCl, 10 mM $MgCl_2$ and 10 μM ATP). After addition of 10 μCi of [γ-^{32}P] ATP, the reaction mixture was incubated at 30°C for 30 min. The reaction was terminated by the addition of SDS–PAGE sample buffer containing 50 mM Tris (pH 6.8), 2% SDS, 6% 2-mercaptoethanol, 10% glycerol and 0.5 mg/ml bromophenol blue. The phosphorylated proteins were separated by SDS–PAGE and visualized by autoradiography.

RESULTS AND DISCUSSION

Phosphorylation of WAVE2 by MEK-ERK signalling—Using the pan-WAVE antibody that recognizes all three WAVEs, we previously showed that WAVE proteins are phosphorylated on PDGF stimulation, and their mobility shift is inhibited by treatment with PD98059, a MEK1/2-specific inhibitor (*11*). First, we identified the isoform of WAVE proteins that were phosphorylated by PDGF stimulation in MEFs; this was performed by western blotting using antibodies specific to WAVE1, WAVE2 and WAVE3. In the MEFs, the mobility shift of WAVE2 was observed, but no mobility shift of WAVE1 or WAVE3 was observed on PDGF treatment that caused ERK activation (Fig. 1A). The mobility shift was inhibited when the cells were treated with U0126, a more potent MEK1/2-specific inhibitor than PD98059 (Fig. 1A) (*9*). Treatment of the cells with inhibitors to Src family kinases (PP2), PI-3 kinases (LY294002 and Wortmannin), Rho-associated kinase (ROCK; Y27632), tyrosine kinase or epidermal growth factor (EGF) receptor (genistein) and myosin-light-chain kinase (MLCK; ML-7) did not affect the mobility shift of WAVE2 (Fig. 1B). Treatment of the cells with Abl inhibitor (STI-571) reduced the mobility shift; the ERK activity was also reduced, as judged by western blotting with anti-phospho-ERK antibody (Fig. 1B). Expression of the constitutively active form of MEK (LA-SDSE MEK) also induced the band shift of WAVE2 (Fig. 1C).

To confirm that phosphorylation of WAVE2 actually occurs downstream of the MEK-ERK cascade, WAVE2-knockout MEFs expressing FLAG-tagged WAVE2 were incubated with [^{32}P]orthophosphate and were then stimulated with PDGF for 10 min. An autoradiogram indicated that PDGF treatment induced the incorporation of phosphate into WAVE2, while only serum starvation did not (Fig. 1D). U0126 treatment suppressed the phosphorylation, confirming that WAVE2 is phosphorylated downstream of the MEK-ERK cascade.

Phosphorylation of WAVE2 by ERK In Vitro—To examine whether or not ERK directly phosphorylates WAVE2, we performed an *in vitro* kinase assay using ERK2 and GST-tagged WAVE2 purified from Sf9 cells (Fig. 2). The ERK2 protein purified from Sf9 cells was active *in vitro*, as revealed by the autophosphorylation of ERK2 (Fig. 2B). The GST-tagged WAVE2 protein was weakly phosphorylated without the ERK2 protein, and addition of the ERK2 protein markedly increased the phosphorylation of the WAVE2 protein (Fig. 2B).

To identify the ERK2-phosphorylated region of WAVE2, the phosphorylation of various WAVE2 fragments (Fig. 2A) was examined. The VCA domain was efficiently phosphorylated by purified ERK2, as was the proline-rich region (Fig. 2B). In contrast, the middle region (amino acids 180–241), N-terminal region, including WHD, and the basic region were not phosphorylated (Fig. 2B). These results suggested that WAVE2 has multiple phosphorylation sites for ERK2.

Phosphorylation Site of WAVE2 In Vitro—The consensus sequence of substrates for ERK1 and

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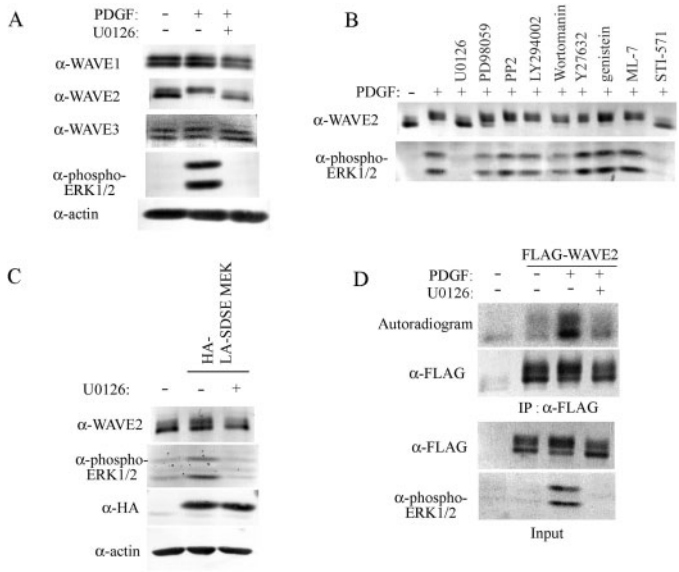

Fig. 1. **Phosphorylation of WAVE2.** (A) WT MEFs were stimulated with PDGF in the presence or absence of U0126, and whole-cell lysates were subjected to SDS–PAGE and western blot analysis. (B) WT MEFs were stimulated in the presence of various kinase inhibitors: U0126, 20 μM; PD98059, 50 μM; PP2, 10 μM; LY294002, 5 μM; Wortmannin, 100 μM; Y27632, 10 μM; genistein, 10 μg/ml; ML-7, 20 μM and STI-571, 10 μM. (C) NIH3T3 fibroblasts were transfected with HA-tagged constitutively active (LA-SDSE) MEK or control vector. Whole-cell lysates were analysed by western blot. (D) WAVE2-knockout MEFs were transfected with a retrovirus expressing FLAG-tagged WAVE2 and were incubated with [^{32}P]orthophosphate. After PDGF stimulation for 10 min, FLAG-tagged WAVE2 was immunoprecipitated and analysed by western blot and autoradiography.

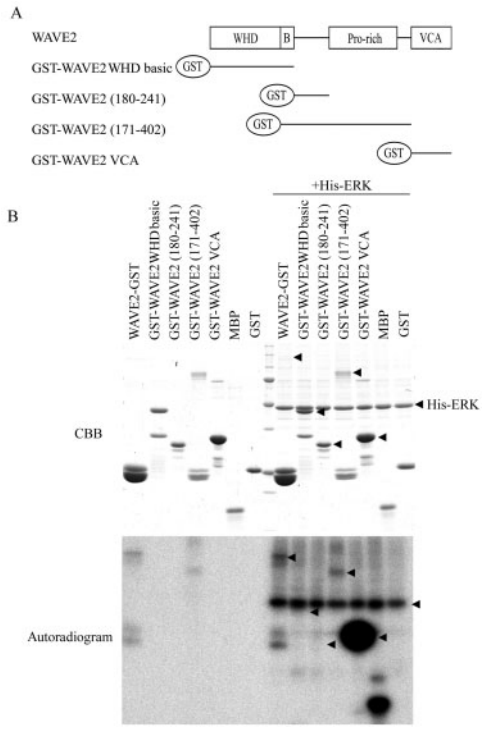

Fig. 2. ***In vitro*** **phosphorylation of WAVE2 by ERK2.** (A) Schematic diagram of recombinant protein used in the *in vitro* kinase assay. (B) GST fusion proteins of WAVE2 WHD-basic amino acids 180–241, amino acids 171–402 and VCA were incubated with purified ERK2 for 30 min, and the proteins were analysed by SDS–PAGE followed by autoradiography. Arrowheads indicate the recombinant WAVE2 fragment and ERK proteins.

ERK2 protein kinases is reported to be -Pro-Xn-Ser/Thr-Pro- (where Xn is a neutral or basic amino acid and $n = 1$ or 2) (*19*). The proline-rich region of WAVE2 contains two serine residues and one threonine residue matching this consensus (Fig. 3A). To examine whether these residues were phosphorylated by ERK2, we substituted these three serine/threonine residues with alanine. An *in vitro* kinase assay revealed that the phosphorylation of this fragment of WAVE2 (amino acids 171–402) that has these three mutations was greatly reduced compared with that in the wild type (WT) fragment (Fig. 3B). These results indicated that ERK2 phosphorylates these residues.

The VCA region of WAVE2 does not contain the consensus sequence of the substrates of ERK2 kinase. Any serine residues of VCA are not surrounded by any proline residues (Fig. 3A). To examine whether the residues phosphorylated by ERK2 were conserved among proteins of the WASP and WAVE families, we performed an *in vitro* kinase assay with purified ERK and the VCA regions of N-WASP, WAVE1, WAVE2 and WAVE3. All VCA regions of the WAVEs were phosphorylated by ERK2, but the VCA region of N-WASP was not (Fig. 3C). These results indicated that ERK2 phosphorylates conserved residues in the VCA regions of WAVEs.

There are eight serine/threonine residues that are conserved among WAVE isoforms. Next, we substituted these residues of WAVE2 with alanine and performed the *in vitro* kinase assay. S434A/S438A, T471A/S474A and S488A/S489A mutants were phosphorylated as strongly as the WT VCA domain (Fig. 3, D and E). In contrast, phosphorylation of the S482A/S484A mutant was markedly reduced (Fig. 3, D and E).

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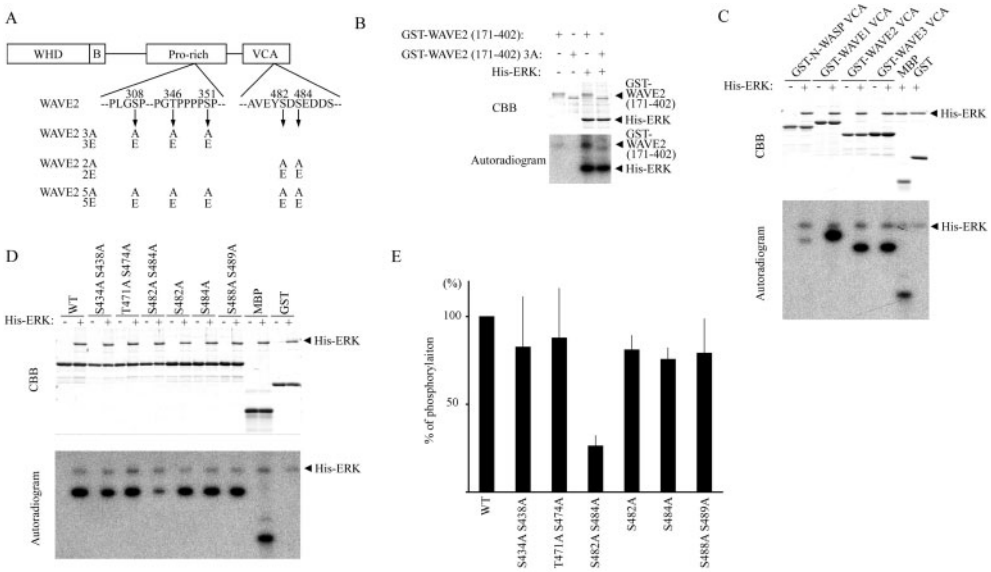

Fig. 3. **Phosphorylation site of WAVE2.** (A) Schematic diagram of phosphorylation site mutants. (B) WT or the 3A mutant of a GST fusion WAVE2 protein fragment (amino acids 171–402) was incubated with purified ERK2 *in vitro*, and phosphorylation was examined. (C) GST fusion VCA proteins of N-WASP, WAVE1, WAVE2 and WAVE3 were incubated with purified ERK2 *in vitro*, and phosphorylation was examined. (D) GST-VCA proteins of WAVE2 with alanine substitutions for serine were incubated with purified ERK2 *in vitro*, and phosphorylation was examined. (E) Relative incorporation of [^{32}P] in (D) was measured by densitometry and expressed as the percentage incorporated in WT VCA.

To determine whether Ser482, Ser484, or both were phosphorylated by ERK2, we examined the phosphorylation in the VCA regions of the S482A and S484A mutants. Phosphorylation of the S482A and S484A mutants was reduced but not completely suppressed, indicating that both Ser482 and Ser484 are phosphorylated by ERK2 *in vitro*.

The above data indicated that ERK phosphorylated the non-consensus site of the VCA region. As shown in Fig. 3A, the amino acid sequence of the phosphorylation site is AVEYSD*S*EDD. The Na+/K+ transporter is phosphorylated at serine 11. This serine is present in the amino acid sequence AAV*S*EHGD (*20*). Therefore, it is suggested that ERK can phosphorylate the serine residues adjacent to the acidic and hydrophobic residues.

Phosphorylation Site of WAVE2 In Vivo—To confirm that the Ser308, Ser351, Ser482, Ser484 and Thr346 residues are phosphorylated *in vivo*, we substituted these residues in full-length WAVE2 with alanine to suppress the phosphorylation, or with glutamate to mimic the phosphorylation (Fig. 3A). S308A/T346A/S351A, S482A/S484A, S308A/T346A/S351A/S482A/S484A, S308E/T346E/S351E, S482E/S484E and S308E/T346E/S351E/S482E/S484E were named the 3A, 2A, 5A, 3E, 2E and 5E mutants, respectively. FLAG-tagged WT WAVE2 or these mutants were expressed in WAVE2-knockout MEFs. As expected, on autoradiographic examination, the 3A and 2A mutants were less phosphorylated, and the phosphorylation of the 5A mutant was markedly reduced than WT WAVE2 (Fig. 4A).

Interestingly, the band shifts of the 3A and 5A mutants were suppressed, but that of the 2A mutant

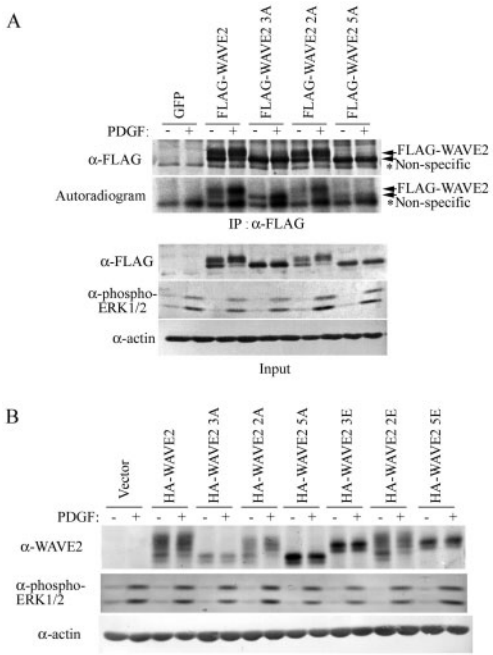

Fig. 4. **Phosphorylation sites of WAVE2 in cells.** (A) WAVE2-knockout MEFs were transfected with a retrovirus expressing FLAG-tagged WT WAVE2, 2A WAVE2, 3A WAVE2, or 5A WAVE2 and were incubated with [^{32}P] orthophosphate. After 2 hours of incubation, the MEFs were stimulated with PDGF for 10 min. Then, FLAG-tagged WAVE2 was immunoprecipitated and subjected to western blot analysis. Arrows indicate the band corresponding to the FLAG-tagged WAVE2, and ∗ indicates non-specifically immunoprecipitated phosphorylated protein. (B) WAVE2-knockout MEFs were transfected with WT or mutant WAVE2 expression vectors and then stimulated with PDGF. Whole-cell lysates were subjected to western blotting.

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was not (Fig. 4). Band shifts of 3E and 5E were observed consistently in cell lysates without PDGF treatment, but that of the 2E mutant was not (Fig. 4B). The band shifts of the 3E and 5E mutants were considerably stronger than that of WT WAVE2, suggesting that the latter was not completely phosphorylated on PDGF stimulation.

Suppression of Arp2/3-complex-mediated Actin Polymerization by Phosphorylation of the VCA Region—The VCA regions of proteins from the WASP family activate the actin-nucleating activity of the Arp2/3 complex. To examine the influence of phosphorylation on the activation of the Arp2/3 complex, we performed a pyrene-actin assay in which an increase in the intensity of pyrene fluorescence indicates the formation of actin filaments, i.e. actin polymerization. The purified VCA region of WAVE2 was phosphorylated by ERK2 *in vitro* for the indicated period (Fig. 5A). The phosphorylation of the VCA region was proportional to the incubation period (Fig. 5A). After the phosphorylation reaction, the affinities of the WAVE2 VCA region to the Arp2/3 complex and actin monomer were examined by pull-down assays. Purified Arp2/3 complex had a higher affinity to the phosphorylated VCA region than to the unphosphorylated VCA region (Fig. 5B). The affinity of the Arp2/3 complex to the VCA region of the 2A (S482A/S484A) mutant remained unchanged after phosphorylation. In contrast, the affinity of the VCA region to the actin monomer was not affected by the phosphorylation (Fig. 5C).

In order to examine the effect of phosphorylation on VCA-Arp2/3-induced actin polymerization, the phosphorylation reaction mixture was added to the actin polymerization assay. In the absence of ERK, the activation of the Arp2/3 complex by the VCA region of the 2A (S482A/S484A) mutant was similar to that by the WT VCA region. ERK-mediated phosphorylation of the WT VCA region reduced its ability to activate the Arp2/3 complex in a phosphorylation-dependent manner, whereas phosphorylation of the VCA region of the 2A mutant did not (Fig. 6, A and B).

These results indicate that phosphorylation of the VCA region attenuates Arp2/3 activation. However, it is unclear how the increased affinity of the VCA region to the Arp2/3 complex results in reduced Arp2/3 activation. The affinity to the Arp2/3 complex is not an important factor in the activation of Arp2/3-mediated actin polymerization. The △a mutant of N-WASP with a partial deletion in the acidic region had a reduced affinity to the Arp2/3 complex compared with the VCA fragment; however, it showed similar Arp2/3 activation as the VCA fragment (*17*).

Possible Role of WAVE2 Phosphorylation in Lamellipodia Formation—Because WAVE2 is involved in lamellipodia formation, we next examined whether phosphorylation affects PDGF-induced lamellipodia formation. WAVE2-knockout MEFs are defective in lamellipodia formation, and this defect can be restored by ectopically expressing WT WAVE2 (*13*). WT or WAVE2 mutants were ectopically expressed in WAVE2-knockout MEFs, and PDGF-induced lamellipodia formation in these cells was examined (Fig. 7).

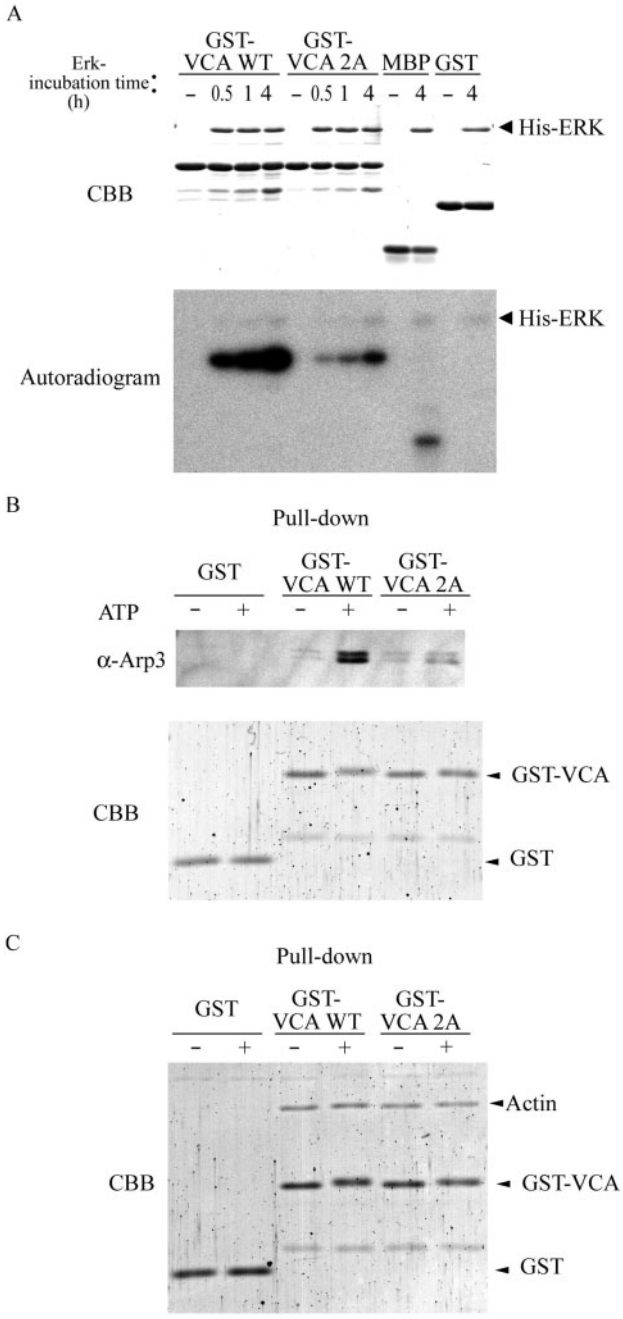

Fig. 5. **Effect of phosphorylation on VCA–Arp2/3 complex interaction.** (A) WT or 2A mutant of the GST fusion VCA fragment (GST-VCA) of the WAVE2 protein was incubated with purified ERK2 for the indicated time in the presence of [γ-^{32}P]ATP. The proteins were then analysed by SDS–PAGE and autoradiography. (B, C) Phosphorylated GST-VCA was prepared by incubation with purified ERK2 for 4 h. Then, the GST-VCA protein and phosphorylated GST-VCA were immobilized and incubated with purified Arp2/3 complex (B) or actin monomer (C). After washing, the bound proteins were analysed by western blotting.

Restoration of lamellipodia formation by WAVE2-knockout MEFs could be achieved by the 2A, 3A and 5A mutants of WAVE2 (Fig. 7). Restoration by the 5A mutant was slightly more efficient than that by

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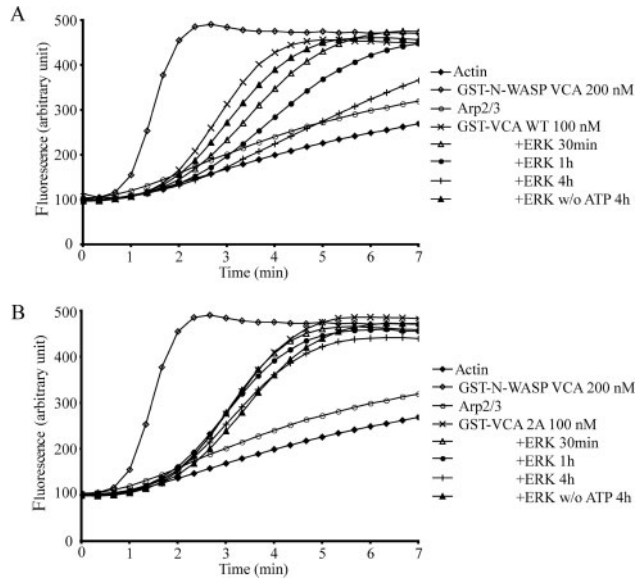

Fig. 6. **Effect of VCA phosphorylation on Arp2/3 complex activation.** GST-VCA (A) or GST-VCA 2A (B) that underwent the phosphorylation reaction was incubated with purified Arp2/3 complex and pyrene-labelled actin. Phosphorylated GST-VCA was prepared as shown in Fig. 5A. Actin polymerization was monitored based on pyrene fluorescence. The phosphorylation reaction was performed without ATP to serve as a control.

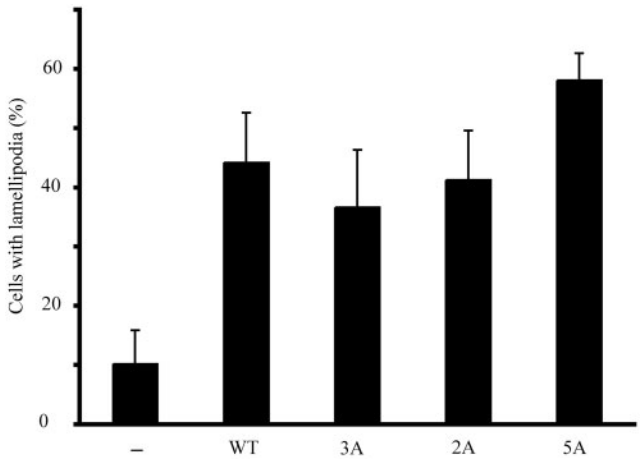

Fig. 7. **Effect of WAVE2 phosphorylation on lamellipodia formation.** WAVE2-knockout MEFs were transfected with HA-tagged WAVE2 and WAVE2 mutants and then seeded onto glass coverslips. These MEFs were stimulated with PDGF for 40 min, and the percentage of cells possessing lamellipodia was examined.

WT WAVE2, but the difference in the restoration between WT and the 5A mutant was not statistically significant (Fig. 7).

Despite the reduced activity of phosphorylated WAVE2 in Arp2/3 activation, the difference between the lamellipodia of cells expressing WT WAVE2 and those of cells expressing mutant WAVE2 was not statistically significant under the current experimental conditions. Furthermore, the role of proline phosphorylation was not determined in this study. Thus, the physiological role of the phosphorylation of WAVE2 is not yet clear.

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