Phosphorylation of WAVE Downstream of Mitogen-activated Protein Kinase Signaling*

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WAVE is a Wiskott-Aldrich syndrome protein (WASP)-family protein that functions in membrane-ruffling formation induced by Rac, a Rho family small GTPase. Here we report that WAVE is a phosphoprotein whose phosphorylation increases in response to various external stimuli that activate mitogen-activated protein (MAP) kinase signaling. When Swiss 3T3 cells are stimulated with platelet-derived growth factor, electrophoretic mobility shift occurs to WAVE, which reflects hyperphosphorylation. This is perfectly inhibited by the addition of PD98059, a specific inhibitor of MAP kinase. Indeed, the ectopic expression of an activated mutant of MAP kinase kinase induces WAVE mobility shift. When MAP kinase activation is suppressed by PD98059, the intensity of platelet-derived growth factor-induced membrane ruffling is greatly reduced. In various cancer cell lines, the amount of WAVE mobility shift was found to increase significantly, suggesting the importance of WAVE hyperphosphorylation in the formation of membrane ruffles and oncogenic transformation.

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The abbreviations used are: WAVE, Wiskott-Aldrich syndrome protein; GST, glutathione S-transferase; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; MEK, MAP kinase kinase; PDGF, platelet-derived growth factor; BHK, baby hamster kidney; HA, hemagglutinin; MDCK, Madin-Darby canine kidney.

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Wiskott-Aldrich syndrome protein (WASP)1 has been identified as the gene product whose mutation causes a human hereditary disease, Wiskott-Aldrich syndrome (1). Ectopic expression studies revealed that WASP induces the formation of clusters of actin filaments in a manner dependent on Cdc42, a Rho family small GTPase (2). Soon after the discovery of WASP, we identified a WASP-related molecule in a search of the database search using the verprolin homology domain amino acid sequence and identified WAVE (WASP-family verprolin homologous protein), which was originally isolated in a random cDNA sequencing project as KIAA0269 (8, 9). The carboxy-terminal half of WAVE was structurally similar to both WASP and N-WASP, in that WAVE also possesses the verprolin homology domain and a proline-rich region. Thus, WAVE was thought to be a new member of the WASP family of proteins.

We then demonstrated that WAVE regulates the actin reorganization that is essential for the formation of membrane ruffles induced by Rac, another Rho family member (9). Recently, Machesky and Insall (10) reported that the carboxy-terminal fragment of WAVE (they call the same protein Hs-Scar1) binds to Arp2/3 protein complex and that the ectopic expression of the fragment suppresses the PDGF-induced formation of membrane ruffles. Because PDGF-induced membrane-ruffling formation occurs in a Rac-dependent manner (11), this result also supports the possibility that WAVE functions downstream of Rac.

Thus, all WASP family proteins have been shown to regulate the reorganization of the actin cytoskeleton downstream of Cdc42 or Rac, and Cdc42 has been shown to bind directly to WASP and N-WASP (2, 4, 12). In contrast, we could not detect any direct interaction between WAVE and Rac by conventional far Western blot assay, and the regulation mechanism of WAVE remains unclear.

To understand its regulation mechanism, we have examined here whether WAVE is modified in response to external stimuli that cause membrane ruffling. As a result, we found that WAVE is hyperphosphorylated by various signals that activate a MAP kinase signaling cascade.

**EXPERIMENTAL PROCEDURES**

Cell Culture—All cells used in this study were cultured in Dulbecco’s modified Eagle’s medium (Nissui) supplemented with 10% fetal calf serum. Serum starvation was done in Dulbecco’s modified Eagle’s medium containing 1 mg/ml bovine serum albumin and insulin-transferrin/sodium selenite supplement (Boehringer Mannheim) for 24 h.

Stimulants such as PDGF (used at the final concentration of 10 ng/ml), epidermal growth factor (100 ng/ml), hepatocyte growth factor (100 ng/ml), lysophosphatidic acid (LPA, 200 ng/ml), and bradykinin (100 ng/ml) were purchased from Boehringer Mannheim, Life Technologies, Inc., Calbiochem, Sigma, and Sigma, respectively. MEK inhibitor, PD98059, was purchased from Calbiochem.

**Alkaline Phosphatase Treatment—** WAVE was immunoprecipitated from lysates of Swiss 3T3 cells treated with or without PDGF. The immunoprecipitates, immobilized on protein A-agarose beads (Pierce), were washed in alkaline phosphatase buffer and then mixed with calf intestine alkaline phosphatase buffer. As a negative control, calf intestine alkaline phosphatase pretreated for 30 min at 95 °C was also used.

**[32P]Orthophosphate Labeling—** Swiss 3T3 cells were first serum-starved for 20 h. Culture medium was replaced with phosphate-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing
Hyperphosphorylation of WAVE Downstream of MAP Kinase

Ectopic expression of active MEK induces WAVE mobility shift. WAVE mobilities were assessed before (A) and after (B) MEK activation. A, stimulated with PDGF (P). B, stimulation of Swiss 3T3 cells with bradykinin, PDGF, LPA, and serum. I.B., immunoblot.

Fig. 1. Mobility shift of WAVE in response to various stimuli. A, stimulation with growth factors. Swiss 3T3, A431, and MDCK cells were stimulated for 5 min with PDGF (P), epidermal growth factor (E), and hepatocyte growth factor (H), respectively. The cell lysates were subjected to Western blotting with anti-WAVE antibody. B, stimulation of Swiss 3T3 cells with bradykinin, PDGF, LPA, and serum. I.B., immunoblot.

Fig. 2. Hyperphosphorylation of WAVE in response to PDGF treatment. A, alkaline phosphatase treatment. WAVE proteins were immunoprecipitated from Swiss 3T3 cells stimulated with or without PDGF. The immunoprecipitates were treated with alkaline phosphatase (AP) or heat-inactivated alkaline phosphatase (C). After treatment, they were subjected to Western blotting with anti-WAVE antibody. B, [32P]orthophosphate labeling. Swiss 3T3 cells were labeled with [32P]orthophosphate for 4 h and then stimulated with (P) or without (-) PDGF. WAVE proteins were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis. The results were examined by Western blotting with anti-WAVE antibody and autoradiography. The numbers below the autoradiogram show the intensity of the signals that correlates with the amount of [32P] incorporated into WAVE. I.B., immunoblot.

Results

Electrophoretic Mobility Shift of WAVE in Response to Various Extracellular Stimuli—It is well known that growth factor stimulation induces the formation of membrane ruffles. Thus, we first examined by Western blotting whether WAVE is modified in response to growth factor stimulation. As shown in Fig. 1A, all growth factors tested (PDGF to Swiss 3T3 cells, epidermal growth factor to A431 cells, and hepatocyte growth factor to MDCK cells) induced significant mobility shifts in the WAVE signal. All these growth factors bind and activate receptor-type tyrosine kinases. Thus, we next tested the effect of bradykinin and LPA, which activate G-protein-coupled receptors in Swiss 3T3 cells (19, 20). In these cases, LPA induced a weak but significant mobility shift in the WAVE signal (Fig. 1B, left). Serum stimulation was also found to induce strong mobility shift (Fig. 1B, right).

WAVE Is Hyperphosphorylated in Response to PDGF in Swiss 3T3 Cells—To confirm that these mobility shifts are caused by phosphorylation, we immunoprecipitated WAVE from Swiss 3T3 cells treated with or without PDGF and subjected the precipitates to alkaline phosphatase treatment. As shown in Fig. 2A, this treatment hastened the electrophoretic mobility of WAVE. In addition, WAVE from Swiss 3T3 cells not treated with PDGF also migrated a little faster after alkaline phosphatase treatment. Taken together, these results suggest that WAVE is constitutively phosphorylated and that PDGF treatment induces hyperphosphorylation of WAVE.

We next labeled Swiss 3T3 cells with [32P]orthophosphate. The cell lysates were immunoprecipitated with anti-WAVE antibody and subjected to SDS-polyacrylamide gel electrophoresis. The results indicate that WAVE is indeed a phosphoprotein and becomes hyperphosphorylated in response to PDGF stimulation (Fig. 2B). The phosphorylation level is up-regulated about 2-fold by PDGF treatment.

Hyperphosphorylation of WAVE Occurs Downstream of MAP Kinase Signaling—We next tried to determine what kinase is involved in WAVE hyperphosphorylation in response to PDGF treatment. We first performed Western blot analysis using PY20 and 4G10 anti-phosphotyrosine antibodies to examine whether WAVE is tyrosine-phosphorylated, but we did not obtain any positive signal (data not shown).

The mobility shift occurs in response not only to growth factors but also to LPA and serum, and thus we examined the possible involvement of the MAP kinase pathway. MAP kinase activation can be estimated very easily from the mobility shift in Western blotting. As shown in Fig. 3A, the time course of the activation of MAP kinase correlated well with the mobility shift (hyperphosphorylation) of WAVE. We also tested the effect of PD98059, a specific inhibitor of MEK. When PD98059 was added at a concentration of >50 μM, the activation of MAP kinase was severely suppressed (Fig. 3B), and the mobility shift of WAVE was also suppressed, strongly suggesting that the hyperphosphorylation of WAVE occurs downstream of a MAP kinase signaling cascade.

To further confirm our idea, we ectopically expressed activated mutant MEK (LA-SDSE mutant) in BHK cells. As shown in Fig. 3C, expression of active MEK induced the mobility shift of endogenous WAVE. Under the experimental condition we...
used, the expression rate of active MEK was 30–40% of total cells (estimated from immunofluorescence microscopy), and thus most endogenous WAVE proteins seem to become hyperphosphorylated in cells expressing active MEK. The activated mutant of Ras (RasG12V), an activator of MAP kinase pathway, also induced WAVE mobility shift.

Because WAVE functions downstream of Rac, WAVE hyperphosphorylation may be caused by activation of Rac. Thus, we also expressed an activated mutant of Rac (Rac1G12V). However, in this case, we did not observe any significant mobility shift of WAVE. The activated mutant of Cdc42 (Cdc42G12V) also had no effect. These results strongly suggest that the mobility shift of WAVE is not caused by Rac (or Cdc42) and also imply that nonclassical MAP kinases such as p38 and c-Jun NH₂-terminal kinase are not involved in the mobility shift of WAVE, because Cdc42 and Rac have been shown to activate p38 and c-Jun NH₂-terminal kinase pathways in various cell lines (21, 22).

Suppression of MAP Kinase Cascade Reduces the Intensity of Membrane Ruffling Induced by PDGF—Our results described above indicate that there exists some connection between WAVE and a MAP kinase signaling cascade. It is well known that PDGF treatment induces both the activation of MAP kinase signaling and the formation of membrane ruffles (11, 23), but there have not been any reports on the role of MAP kinase signaling in membrane-ruffling formation induced by PDGF. Thus, we tested whether the suppression of MAP kinase signaling had any effect on the membrane-ruffling formation by using PD98059. Swiss 3T3 cells were stimulated with PDGF in the presence or absence of PD98059 and then were fixed and stained with phalloidin to visualize membrane ruffling. Before the stimulation with PDGF, there was no significant morphological difference between cells treated with or without PD98059 (Fig. 4). PDGF treatment induced the formation of membrane ruffles at 5 min in cells not treated with PD98059. At 30 min, membrane ruffling still persisted. In contrast, the PD98059-treated cells also formed some membrane ruffles (indicated by arrowheads), but they were clearly very weak compared with cells not treated with PD98059. At 240 min, both cells formed many stress fibers, probably through the activation of endogenous Rho as reported previously (20). Although some abnormalities in the arrangement of stress fibers were seen in cells treated with PD98059, the amount of actin filaments composing the stress fibers does not seem to differ significantly from cells not treated with PD98059.

**FIG. 3.** Hyperphosphorylation of WAVE downstream of MAP kinase signaling. A, time course of MAP kinase activation and WAVE hyperphosphorylation. Swiss 3T3 cells were serum-starved and then stimulated with PDGF for 10, 30, and 240 min. The cell lysates were subjected to Western blotting with anti-WAVE, ERK1, and ERK2 antibodies. B, inhibition of WAVE hyperphosphorylation by PD98059. Swiss 3T3 cells were first serum-starved and then pretreated with the indicated concentrations of PD98059 for 2 h. After the treatment, the cells were stimulated with PDGF and then harvested. C, WAVE hyperphosphorylation induced by ectopic expression of active MEK. BHK cells were transfected with plasmids expressing active Cdc42 (Myc-tagged), active Rac (Myc-tagged), active Ras (Myc-tagged), or active MEK (HA-tagged). The cell lysates were subjected to Western blotting with anti-HA, Myc, ERK2, and WAVE antibodies. I.B., immunoblot.

**FIG. 4.** Requirement of MAP kinase signaling in proper formation of membrane ruffles. Swiss 3T3 cells were pretreated with 50 μM PD98059 and then stimulated with PDGF or LPA. After 10, 30, and 240 min (PDGF) or 10 min (LPA), cells were fixed and stained with phalloidin to visualize actin filaments. Weak membrane ruffles formed in PD98059-treated cells are indicated by arrowheads.
As shown in Fig. 3A, most MAP kinases exist in an inactive form at 240 min after PDGF treatment. Thus, we also tested the effect of LPA, which induces both stress fiber formation and MAP kinase activation, at 10 min. In this case, the treatment with PD98059 was found to have no significant effect on stress fiber formation (Fig. 4), suggesting that MAP kinase signaling is specifically involved in the formation of membrane ruffles.

These results suggest that MAP kinase signaling is not essential for the formation of membrane ruffles but plays some permissive role specifically in the full induction of membrane-ruffling formation.

**Hyperphosphorylation Specifically Inhibits the Binding to Grb2/Ash**—We next investigated whether WAVE-binding proteins are changed by PDGF-stimulation. WAVE possesses a proline-rich region that has been shown to bind directly to profilin (9). We examined by GST-profilin pull-down assay whether the association between WAVE and profilin is affected by WAVE hyperphosphorylation. However, we did not see any change in the amount of the precipitated WAVE (Fig. 5A).

The proline-rich region is also known to associate directly with various SH3 domains. We then examined the association with several SH3 domain-containing proteins such as Grb2/Ash adaptor protein, Fyn tyrosine kinase, and the p85 subunit of phosphatidylinositol 3-kinase. As shown in Fig. 5B, we found that only Grb2/Ash bound well to WAVE. In addition, this binding was strongly inhibited when WAVE was hyperphosphorylated by PDGF treatment. Taken together, these results suggest that Grb2/Ash may be a specific binding partner of WAVE, and the binding may be regulated by PDGF treatment. Because Grb2/Ash has been shown to be involved in membrane-ruffling formation (24), complex formation/dissociation between Grb2/Ash and WAVE may be an important step in inducing ruffling formation properly.

**WAVE Mobility Shift in Oncogenically Transformed Cells**—MAP kinase not only regulates growth and differentiation of normal cells but also participates in oncogenic transformation. Thus, we investigated whether there exists any change in the amount or mobility shift status of WAVE in transformed cells.

We performed Western blot analysis against lysates obtained from various transformed cells (and their parental non-transformed cells). As shown in Fig. 6A, there was a greater tendency for WAVE mobility shift to increase in transformed cells (v-Ha-Ras-transformed NIH3T3 cells and v-src-transformed 3Y1 cells) than in parental cells (NIH3T3 cells and 3Y1 cells). In addition, in HT1080 fibrosarcoma cells that are routinely used for metastatic invasion studies, most WAVE proteins existed as a mobility-shifted form. When PD98059 was added to the culture medium, these mobility shifts were clearly suppressed, which correlated well with the suppression of MAP kinase activation (Fig. 6B). These results support our notion described above and strongly suggest that WAVE may be critical for oncogenic transformation downstream of MAP kinase.

**DISCUSSION**

MAP kinase is a protein kinase that is activated by various external stimuli and regulates many fundamental processes such as cell growth and differentiation. The best known function of MAP kinase is to receive signals from Ras and transmit the signal to the nucleus, regulating transcription of specific genes that affect the fate of cells. However, there has been accumulating evidence that MAP kinase not only transmits the signal to the nucleus but also regulates cytoplasmic events such as cell motility (25).

We found in this study that suppression of MAP kinase activation resulted in significant, though not perfect, reduction of membrane ruffling. WAVE is a strong candidate for a downstream target of MAP kinase for proper formation of membrane ruffles, because WAVE is hyperphosphorylated downstream of MAP kinase signaling and has been shown to be a critical regulator of membrane ruffling downstream of Rac (9). The WAVE hyperphosphorylation seems to inhibit specifically the association with Grb2/Ash, although we do not know yet the physiological relevance of this inhibition. It is quite probable that Grb2/Ash recruits WAVE to the activated PDGF receptor through the association between the SH3 domains and the proline-rich region as in the case of Sos (26, 27), a well known

**Fig. 5.** Association with Grb2/Ash is inhibited by WAVE hyperphosphorylation. A, association with profilin. GST-profilin was immobilized on beads and then mixed with lysates of Swiss 3T3 cells treated with (+) or without (−) PDGF. The bound proteins were analyzed by Western blotting with anti-WAVE antibody. B, association with Grb2/Ash. Various indicated GST fusion proteins containing SH3 domains (Grb2/Ash, Fyn, and p85) were immobilized on beads.

**Fig. 6.** Mobility shift of WAVE in oncogenically transformed cells. A, mobility shift of WAVE in oncogenically transformed cells. 3Y1, v-Src-transformed 3Y1 (Src/3Y1), NIH3T3, v-Ha-Ras-transformed NIH3T3 (Ras/NIH3T3), and HT1080 cells were harvested under serum-starved conditions. The cell lysates were subjected to Western blotting with anti-WAVE antibody. B, effect of PD98059 on the WAVE mobility shifts. 50 μM PD98059 was added to the transformed cells (Src/3Y1, Ras/NIH3T3, and HT1080) for 0, 2, and 24 h. The cell lysates were then subjected to Western blotting with anti-WAVE antibody. I.B., immunoblot.
Ras activator. The hyperphosphorylation that follows by MAP kinase pathway may free WAVE from Grb2/Ash, and then WAVE may become fully active in inducing the membrane-ruffling formation. However, it should be noted that the hyperphosphorylation of WAVE is not required for membrane-ruffling formation. As described above, PD98059 treatment could only partially suppress the membrane-ruffling formation. Furthermore, the WAVE hyperphosphorylation is not sufficient for membrane ruffling. Indeed, stimulation of cells with LPA or serum, both of which induce significant WAVE hyperphosphorylation, does not induce membrane ruffling. More directly, we confirmed that expression of active MEK alone did not induce membrane ruffling (data not shown). Thus, we conclude that the Rac pathway is the main route to induction of the membrane-ruffling formation and that the MAP kinase pathway modulates the signaling cascade at some points including WAVE. Now we do not know how WAVE is regulated by Rac. Because WAVE and Rac can form protein complexes when co-expressed in COS 7 cells (9), some adaptor molecule may link between Rac and WAVE. We have performed two-hybrid screening using various parts of WAVE as bait and identified several WAVE-specific binding proteins including known and unknown ones (data not shown), among which we hope the "linking protein" exists.

The important question is what kinase "directly" hyperphosphorylates WAVE. We performed an in vitro kinase assay using activated MAP kinase purified from Xenopus oocyte extracts and found that it indeed phosphorylates the carboxyl-terminal GST fusion fragment of WAVE (data not shown). However, the phosphorylation efficiency was very weak (<1/50 the phosphorylation efficiency of myelin basic protein). More importantly, the full-length WAVE protein immunoprecipitated from Swiss 3T3 cells not treated with PDGF was not phosphorylated at all (data not shown). Thus, it is questionable that MAP kinase itself directly hyperphosphorylates WAVE. Several kinases have been found to be activated downstream of MAP kinases (28), and we think that such a kinase(s) phosphorylates WAVE.

WAVE mobility shift occurs not only in cells stimulated with growth factors but also in oncogenically transformed cells. It has been shown that a MAP kinase cascade is essential for oncogenic transformation by various oncogenes (29). In addition, LA-SDSE mutant MEK, which is constitutively active and can remain in the nucleus, can transform NIH3T3 fibroblasts by itself (19). Rac has also been demonstrated to be involved in transformation. For example, expression of the dominant negative form of Rac can suppress transformation by oncogenic Ras (30). Rac also participates in invasion of carcinoma cells into normal tissues (31). Thus, taken together, this information suggests that both MAP kinase and Rac produce critical signals inducing oncogenic transformation. Considering this, we conclude that WAVE might be a critical regulator of tumorigenesis by integrating two important signals for transformation, MAP kinase and Rac.

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