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Tumor Suppressor WWOX Interacts with MEK2 and Activates ERK Pathway

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WW domain-containing oxidoreductase, WWOX, possesses two WW domains at the N-terminus and a short-chain alcohol dehydrogenase/reductase domain, suggesting a role in steroid metabolism. However, many of studies with this protein are focused on its role as a putative tumor suppressor and a proapoptotic protein. It has been reported that WWOX expression is downregulated in various cancer types.[1] In addition, loss of WWOX expression resulted in resistance to apoptosis induced by tumor necrosis factor, staurosporine, UV, and p53 overexpression Furthermore, ectopically over-expressed WWOX promoted apoptosis synergistically with p53.[2] These reports suggest that WWOX plays an essential role in stress stimuli-induced apoptosis.

There have been extensive studies to understand molecular mechanisms how WWOX regulates many physiological and pathological states. WWOX is involved in the Wnt-catenin pathway through physical interaction with the Dvl proteins,[3] JNK signaling through interaction with JNK and c-Jun,[2,4] osteoblast differentiation through interaction with RUNX2,[5] and apoptosis through interaction with p53/p73.[6,7]

The extracellular signal-regulated kinase (ERK) signaling is one of three major mitogen-activated protein kinase pathways.[8] It is involved in the control of cell proliferation, migration, cell division, and differentiation. ERK serves as a phosphorylation substrate for MAP kinase kinases (MAP2Ks) such as MEK1/2 which are activated in turn by phosphorylation via MAP kinase kinase kinases including Raf.

In this study, WWOX is identified as a novel regulator in ERK signaling through interaction with MEK2. To investigate whether WWOX interacts with MEK2 or ERK in cells, WWOX was immunoprecipitated by *anti*-FLAG M2 agarose from cell lysates and the immunoprecipitates were immunoblotted with *anti*-HA antibody after human embryonic kidney (HEK) 293 cells were transiently co-transfected with FLAG-tagged WWOX and HA-tagged MEK2 or HA-tagged ERK expression plasmids (Fig. 1). MEK2 was co-immunoprecipitated with WWOX whereas ERK was not, suggesting that WWOX interacts with MEK2 but not with ERK in cells.

Since WWOX interacts with MEK2, it was investigated whether WWOX regulates the kinase activity of MEK2 that phosphorylates ERK in cells. HEK 293 cells were transiently transfected with FLAG-WWOX and HA-MEK2 expression plasmids. After 48 h, the endogenous levels of phospho-ERK were determined by immunoblotting analysis (Fig. 2). Levels of phospho-ERK were significantly enhanced

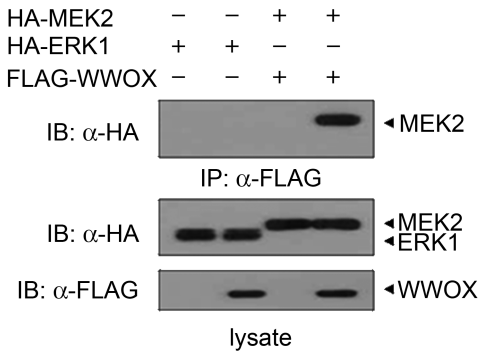

Figure 1. WWOX interacts with MEK2. HEK 293 cells were transfected using HA-MEK2, HA-ERK, or FLAG-WWOX expression plasmids. After 48 h of transfection, cell lysates were prepared and then immunoprecipitated with *anti*-FLAG M2-agarose. Immunoprecipitates were subjected to immunoblot analysis with an *anti*-HA antibody as described in the Experimental Section.

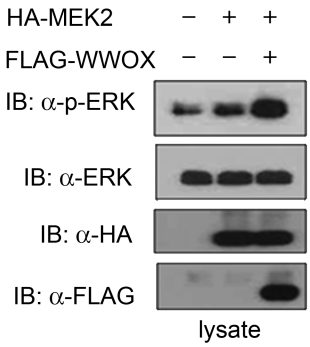

Figure 2. WWOX induces MEK2-mediated ERK phosphorylation. After transfection into HEK 293 cells, cells were lysed with lysis buffer and immunoblotted by appropriate antibodies.

when WWOX and MEK2 were co-expressed while over-expression of MEK2 alone enhanced phospho-ERK level slightly, suggesting that WWOX activates ERK signaling through activation of MEK2 by WWOX.

To further confirm that WWOX positively regulates ERK signaling, *in vitro* kinase assays were performed using GST-Elk as a substrate of ERK (Fig. 3(a)). Cell lysates prepared from HEK 293 cells transfected with WWOX, MEK2, and ERK expression plasmids were immunoprecipitated with *anti*-HA agarose beads to pull-down HA-ERK. The immunoprecipitates were incubated with GST-Elk in the presence of [γ-^{32}P] ATP. Elk phosphorylation levels were apparently enhanced when WWOX was co-expressed along with MEK2

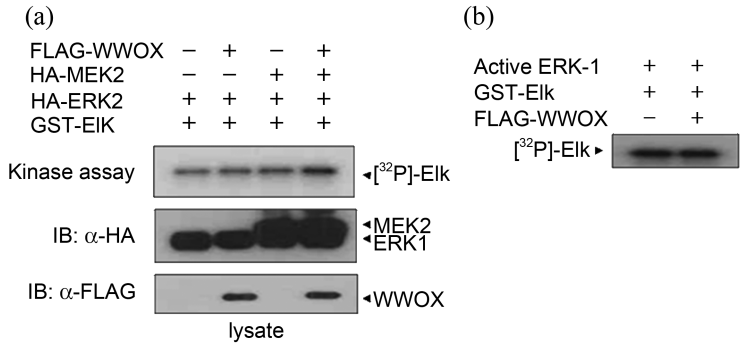

Figure 3. WWOX enhances MEK2-mediated ERK activity. (a) Expression plasmids were transfected into HEK 293 cells. After 48 h of transfection, cells were lysed and immunoprecipitated with *anti*-HA agarose. ERK activities were determined by *in vitro* kinase assays. (b) FLAG-WWOX was immunoprecipitated from transfected HEK 293 cell lysates and incubated with active ERK for *in vitro* kinase assays using GST-Elk as a substrate.

and ERK. Figure 3(b) shows that WWOX does not enhance ERK-mediated Elk phosphorylation *in vitro*, confirming that WWOX is not a direct regulator of ERK.

In conclusion, the results of this study suggest that WWOX acts as a positive regulator of ERK signaling *via* activating MEK2 kinase activity. ERK signaling is known to be positively involved in cell proliferation and cell division. However, previous studies showed that MEK1 and MEK2 might have opposite roles in cell survival. MEK1/ERK signal induces proliferation, whereas the MEK2/ERK induces growth arrest at the G_1/S boundary.[9] Therefore, the results from this study might suggest that WWOX enhances MEK2 activity to induce growth arrest.

Experimental Section

Cell Culture and Transfection. Human embryonic kidney (HEK) 293 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad California) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin in the presence of 5% CO_2. For transient transfection, 1.4×10^6 cells were plated in each 60-mm cell culture plate, grown overnight, and transfected with DNA using LipofectAMINE (Invitrogen).

Plasmid Constructs. HA-MEK2, HA-ERK1, and FLAG-WWOX for expression in mammalian cells were constructed by polymerase chain reaction (PCR), followed by cloning into the pcDNA3.1/Zeo plasmid (Invitrogen). GST-Elk was constructed in the pGEX-6P-1 (Amersham Biosciences, Little Chalfont, UK) plasmid for protein expressions in *Escherichia coli*.

Reagents and Antibodies. Polyclonal *anti*-ERK, and *anti*-phospho-ERK (Thr-202/Tyr-204) antibodies were purchased from Cell Signaling Technology (Danvers, MA). *Anti*-HA antibody and *anti*-HA agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The *anti*-FLAG M2 antibody and *anti*-FLAG M2 agarose beads were purchased from Sigma-Aldrich (St. Louis, MO). Active ERK1 protein was obtained from Dr. Dae Gwin Jeong (Korea Research Institute of Bioscience and Biotechnology).

Immunoblotting Analysis. After HEK 293 cells were transiently transfected with FLAG- or HA-tagged expression plasmids for 48 h, cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/mL aprotinin for 30 min at 4 °C. Cleared cell lysates from centrifugation were resuspended with protein sample buffer, boiled at 100 °C for 5 min, subjected to SDS-PAGE, and subsequently transferred onto nitrocellulose membrane. Immunoblotting was carried out as previously described.[10]

***In vitro* Binding Assays.** Cells were co-transfected with HA-MEK2 and FLAG-WWOX expression plasmids. After 48 h of transfection, cells were washed twice with phosphate-buffered saline (PBS) and extracted in the lysis buffer. Cell extracts were clarified by centrifugation, and the supernatants were incubated with *anti*-FLAG M2-agarose for 5 h at 4 °C with rotation. After binding, the beads were collected by centrifugation at 6,000 rpm for 2 min and washed five times with lysis buffer. The bound proteins were eluted with the SDS-PAGE sample buffer, separated by SDS-PAGE, and then immunoblotted with an *anti*-HA antibody. The protein bands were visualized using the ECL detection system (Pierce, Rockford, IL, USA).

***In vitro* Kinase Assays.** For the immune complex kinase assay, HEK 293 cells were transfected with appropriate expression plasmids. After 48 h of transfection, cells were lysed in the lysis buffer. Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with *anti*-HA agarose beads for 16 h at 4 °C. The beads were washed once with lysis buffer, twice with a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, and 1 mM PMSF, and then once with a solution containing 20 mM Tris-HCl (pH 7.5) and 20 mM $MgCl_2$. The beads were then re-suspended in 20 μL of kinase reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol) containing 20 μM ATP and 0.3 μCi of [γ-^{32}P] ATP with 1 μg of GST-Elk for 30 min at 30 °C. The products of kinase reactions were separated by

SDS-PAGE. The gels were dried and exposed to film.

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