WWOX Induces MEK2-Mediated Cell Death in a p53 Independent Manner

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WW domain-containing oxidoreductase WWOX that contains two WW domains in the N-terminal, which are involved in protein-protein interactions, and an alcohol dehydrogenase (ADH) domain is a putative tumor suppressor and a proapoptotic protein. It has been reported that WWOX gene is located at the common fragile site FRA16D in chromosome 16q23.3-24.1 and its expression is down-regulated in various cancer types.\(^1,2\) Furthermore, aberrantly spliced WWOX mRNA forms that were not detected in normal cells were detected in cancer cell lines and primary breast tumors.\(^3\) In addition, loss of WWOX expression resulted in resistance to apoptosis induced by tumor necrosis factor, staurosporine, UV, and p53 overexpression whereas overexpressed WWOX promoted apoptosis synergistically with p53.\(^4\)

WWOX is known to participate in several physiological functions through interaction with several regulators, such as Dvl proteins for the regulation of Wnt-catenin pathway,\(^5\) JNK and c-Jun for JNK signaling,\(^4,6\) RUNX2 for osteoblast differentiation\(^7,\) and p53/p73 for apoptosis.\(^8,9\)

Previous report showed that WWOX is a novel regulator in extracellular signal-regulated kinase (ERK) signaling through interaction with MEK2.\(^10\) ERK signaling is important for the control of cell proliferation, migration, cell division, and differentiation.\(^11\) ERK is phosphorylated and activated by MAP kinase kinases (MAP2Ks) such as MEK1/2. However, ERK activated by either MEK1 or MEK2 might have opposite roles in cell survival. MEK1/ERK signal enhances cell proliferation, whereas the MEK2/ERK induces growth arrest at the G1/S boundary.\(^12\)

Since the previous study showed that WWOX interacts with MEK2 and activates ERK pathway\(^10\) and WWOX is a putative tumor suppressor, it implicates that the association of WWOX with MEK2 may have a negative role in cell growth.

In this study, it was investigated whether WWOX regulates MEK2-mediated cell growth. Since ERK activity is increased by WWOX,\(^10\) cell viability assays were carried out to test whether WWOX regulates MEK2-induced proliferation of HEK 293 cells. HEK 293 cells were transiently co-transfected with FLAG-tagged WWOX wild-type (WT) and HA-tagged MEK2 expression plasmids. To enhance MEK2 activity and susceptibility to apoptosis, cells were treated with 0.5 mM H\(_2\)O\(_2\) for 16 h prior to assay. WWOX-induced cell death was significantly increased in the presence of MEK2 while MEK2 alone did not induce cell death (Fig. 1), indicating that WWOX enhances MEK2-mediated cell death.

H\(_2\)O\(_2\) decreases the cellular threshold for cell death induction by inducing pro-apoptotic events. The threshold concentration of H\(_2\)O\(_2\) which affects WWOX/MEK2-mediated cell viability was investigated. HEK 293 cells co-transfected with FLAG-WWOX WT and HA-MEK2 expression plasmids were treated with various concentrations of H\(_2\)O\(_2\) for 16 h prior to viability assays. Cells transfected with WWOX or MEK2 alone did not show any change of cell viability in the absence of H\(_2\)O\(_2\) and those treated with 1 mM were severely decreased in cell viability (Fig. 2(a)). At 0.5 mM H\(_2\)O\(_2\), cells transfected with both WWOX and MEK2 showed significantly decreased level of cell viability. These data suggest that WWOX is involved in cell death.

Since p53 is a key regulator of apoptosis induced by WWOX,\(^9\) human colon epithelial HCT116 and HCT116 p53\(^{−/−}\) cells were used for cell viability assays to test whether p53 is critical in WWOX/MEK2-mediated cell death (Fig. 2(b)). The results implicate that p53 is dispensable for
WWOX/MEK2-mediated cell death, suggesting that MEK2 activated by WWOX enhances death signals other than p53 pathway.

WWOX possesses an alcohol dehydrogenase (ADH) domain in the central region of the protein. It was investigated whether ADH domain is involved in the regulation of MEK2 activation. An alternatively spliced form of WVOX, v4, was used for this investigation since the isoform has deletion of most ADH domain (Fig. 3(a)). The v4 form of WVOX was used for binding assays with MEK2 and in vitro kinase assays using GST-Elk as a substrate of ERK that is a downstream factor of MEK2 (Fig. 3(b) and 3(c)). The results show that the v4 isoform interacts with MEK2 but does not activate MEK2 activity, when compared to WVOX wild-type.

In conclusion, the results of this study suggest that WVOX acts as a positive regulator of cell death via activating MEK2 kinase activity. HEK 293 cells overexpressing both WVOX and MEK2 are susceptible to cell death at 0.5 mM of H$_2$O$_2$. It is also shown that p53 is not involved in WVOX/MEK2-induced cell death. Furthermore, the ADH domain of WVOX is indispensable for activation of MEK2.
Experimental Section

Cell Culture and Transfection. Human colon epithelial HCT116, HCT116 p53−/−, and 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% CO2. For transient transfection, 1.4 × 10⁶ 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, FLAG-WWOX WT, and FLAG-WWOX v4 (173-352) 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. Anti-HA antibody and anti-FLAG antibody and anti-HA agarose beads 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).

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. 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) buffer and lysed in a 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 MgCl2. The beads were then resuspended in 20 µL of kinase reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol) containing 20 µM ATP and 0.3 µCi of [γ-32P] 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.

Cell Viability Assay. Cell viability was determined using a CCK-8 cell viability assay kit (DOJINDO Laboratories, Japan). For transfected HEK 293 cells, cells (5 × 10⁵ cells/well) were cultured in DMEM medium supplemented with 5% FBS and incubated for 32 h in a 96-well plate. Cells were treated with 0.5 mM H2O2 for 16 h prior to assay. For HCT116 cells, cells were treated with 0.5 mM H2O2 for 8 h prior to assay. 10 µL of cell viability assay kit solution was added to each well of the plate. After incubation for 1 h at 37 °C in the dark, absorbances were measured at 450 nm using a multiwell plate reader.

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References