MEKK1/MEKK4 are responsible for TRAIL-induced JNK/p38 phosphorylation

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Abstract. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to activate mitogen-activated protein kinases (MAPKs) depending on caspase and mammalian sterile 20-like kinase 1 activations. However, the upstream molecule of MAPKs has not yet been identified. The mitogen-activated protein kinase kinase 1 (MEKK1) and the apoptosis signal-regulating kinase 1 (ASK1) are considered to be possible candidates for the action of MAPKKKs induced by TRAIL and the possibility of reactive oxygen species involvement has also been investigated. We found that MEKK1/MEKK4 as opposed to ASK1, are responsible for TRAIL-induced c-Jun NH_2-terminal kinase (JNK) or p38 activation, and that their catalytic activity is repressed by the caspase-8 inhibitor, suggesting that the caspase-8 activation induced by TRAIL is indispensible for MEKK activation. The 14-3-3 θ was also shown to interact with and to dissociate from MEKK1 by TRAIL treatment, thus implicating the 14-3-3 protein as a negative regulator of MEKK1 activation. Taken together, we show herein that the upstream molecule of the TRAIL-induced MAPK activation is MEKK, as opposed to ASK1, via the mediation of its signal through JNK/p38 in a caspase-8-dependent manner.

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Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase; MEKK1, MEK kinase 1; ASK1, apoptosis signal-regulating kinase 1; SEK, stress-activated protein kinase/extracellular-signal regulated kinase; MLK, mixed lineage kinase; TAK, TGF-ß-activated kinase; ROS, reactive oxygen species; GCK, germinal center kinase; TRAF2, tumor necrosis factor receptor-associated factor 2; Mst1, mammalian sterile 20-like kinase 1

Key words: mitogen-activated protein kinase kinase 1/4, tumor necrosis factor-related apoptosis-inducing ligand, c-Jun NH_2-terminal kinase, p38, 14-3-3

Introduction

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also known as the Apo2 ligand, is a type II transmembrane protein belonging to the TNF superfamily (1,2). The binding of TRAIL to its receptors (DR4/TRAIL-R1 and DR5/TRAIL-R2) results in receptor aggregation and in the recruitment of the Fas-associated death domain (FADD) adaptor proteins, which subsequently induce the formation of the death-inducing signaling complex (DISC) involved in the activation of the caspase-8 initiator (3,4). Activated caspase-8 cleaves Bid and/or caspase-3 and initiates the mitochondrial apoptotic pathway (intrinsic pathway) and/or the caspase cascade (extrinsic pathway), respectively, eventually leading to cell death (5).

Many studies have indicated that TRAIL destroys a variety of tumor cell lines, while leaving normal cells viable, thus suggesting that this protein could function as a specific cancer therapeutic agent (6). Although TRAIL is regarded as a potential anti-cancer agent, a considerable proportion of cancer cells, especially those in certain highly malignant tumors, are resistant to apoptosis induced by TRAIL, and other cancer cells that are initially sensitive to TRAIL-induced apoptosis can become resistant after repeated exposure (acquired resistance) (7). However, the exact mechanisms of acquired TRAIL resistance are still largely unknown.

Previously, we reported that Src, c-Cbl and PI3K are involved in the phosphorylation of Akt during TRAIL treatment, and that these phosphorylations are related to TRAIL-induced acquired resistance (8,9). In addition to the non-apoptotic TRAIL signaling of Akt phosphorylation, TRAIL also induces the activation of mitogen-activated protein kinase (MAPK) kinase pathways in a caspase-8-dependent

manner (10-12). However, the biological roles of c-Jun NH_2-terminal kinase (JNK) and p38 MAPK activations in TRAIL-induced signaling remain uncertain (5). Moreover, multiple mechanisms have been reported, by which JNK or p38 are activated by TRAIL. For example, Varfolomeev *et al* (11) suggested that FADD, caspase-8, RIP1 and TNF receptor-associated factor 2 (TRAF2) are recruited within the primary DISC, leading to the stimulations of JNK and p38, while Liu *et al* (13) concluded that mitogen-activated protein kinase kinase 1 (MEKK1) can be activated via TRAF2 and RIP to activate JNK in the absence of apoptotic conditions. In this study, we demonstrated the occurrence of mammalian sterile 20-like kinase 1 (Mst1)-mediated caspase-8-dependent activation of MAPKs during TRAIL incubation. However, the upstream entities of MAPKs remain unidentified.

The MAPKs are a family of kinases known to transduce external signals to the nucleus in order to determine the fate of the cell (14). Usually, conventional MAPKs consist of three family members, JNK, p38 and the extracellular signal-regulated kinase (ERK), which are involved in different cellular processes, including inflammation, cell proliferation and differentiation, and apoptosis (5). Notwithstanding the TRAIL-induced ERK activation, which is mainly associated with an anti-apoptotic function, the functions of JNK and p38 activation in TRAIL-induced signaling are varied and can be controversial depending on the cell types and cellular contexts involved (15-20). However, the upstream molecules of MAPKs have not yet been identified. It should be noted that, MAPKKKs link a variety of extracellular stimuli to cytoplasmic and nuclear effectors by activating downstream MAPK pathways (21). MEKK1, apoptosis signal-regulating kinase 1 (ASK1), TGF-ß-activated kinase (TAK)1, and mixed lineage kinase (MLK)2, are well-known MAP3 kinases (22). In this study, we clearly demonstrate that MEKK1 and 4 transmit TRAIL-induced signals to JNK or p38 MAPK in a caspase-8-dependent manner.

Materials and methods

Cell culture. The human prostate adenocarcinoma cell line, DU-145, was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 26 mM sodium bicarbonate. The cells were maintained in a humidified environment containing 5% CO_2 and air at 37˚C.

Reagents and antibodies. Polyclonal anti-phospho-ERK, anti-ERK, anti-p38, monoclonal anti-phospho-p38 and anti-caspase-8, were purchased from Cell Signaling (Beverly, MA, USA), and anti-ACTIVE (phosphoT183 and Y185) JNK was purchased from Promega (Madison, WI, USA). Polyclonal anti-JNK1 and 14-3-3 ß, γ, θ and ζ, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-MEKK1 was purchased from Assay Designs (Ann Arbor, MI, USA). Anti-ASK1 was purchased from Millipore (Billerica, MA, USA). Monoclonal anti-poly(ADP-ribose) (PARP) was purchased from Biomol International, L.P. (Plymouth Meeting, PA, USA). Anti-actin antibody was purchased from ICN (Costa Mesa, CA, USA). Caspase-8 inhibitor (Z-IETD-FMK) was purchased from Calbiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Protein extracts and polyacrylamide gel electrophoresis (PAGE). Cells were lysed with 1X Laemmli lysis buffer [2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.002% bromophenol blue and 62.5 mM Tris (pH 6.8)] and boiled for 10 min. Protein content was measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The samples were diluted with 1X lysis buffer, and ß-mercaptoethanol was added until a concentration of 350 mM was achieved. Then, equal amounts of protein were loaded onto 10% SDS polyacrylamide gels. SDS-PAGE analysis was performed according to the procedure of Laemmli using a Hoefer gel apparatus.

Immunoblot analysis. Proteins were separated by SDS-PAGE and were electrophoretically transferred onto nitrocellulose membranes. Each nitrocellulose membrane was blocked with 5% non-fat dry milk in PBS-Tween-20 (0.1%, v/v) at 4˚C overnight. The membrane was then incubated with the primary antibody (diluted according to the manufacturer's instructions) for 2 h. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive proteins were visualized by chemiluminescence (ECL, Amersham, Arlington Heights, IL, USA).

Immune complex kinase assay. For the immune complex kinase assay, DU-145 cells were lysed after TRAIL treatment in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF and protein inhibitor cocktail solution (Sigma-Aldrich). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with mouse anti-MEKK1 or anti-MEKK4 antibodies and protein G agarose (Santa Cruz Biotechnology, Inc.). The beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF and protein inhibitor cocktail solution, washed once more with the kinase buffer solution, and then were subjected to kinase assays. In order to examine the catalytic activity of MEKK1 or 4, GST-tagged JNK1 and stress-activated protein kinase/extracellular signal-regulated kinase (SEK)1 proteins were used as substrates. For the purification of JNK1 or SEK1, the plasmid containing GST-human JNK1 for bacterial fusion protein was constructed into pGEX-4T-1 by inserting the *Hin*dIII/*Xba*I fragment, followed by Klenow treatment with pcDNA3-JNK1. The expression of GST-JNK1 protein was confirmed by Western blotting and purified using glutathione-Sepharose 4B (Amersham Biosciences). GST-SEK1 was purified from ten plates of 293 cells transfected with the pEBG/SEK1 (kindly provided by J.M. Kyriakis, Massachusetts General Hospital, Charlestown, MA), and the purification step was performed as described previously (23). For the measurement of the immune complex activity of MEKK1 or 4, 0.2 μg of GST-SEK1 were first incubated with the immune complexes for 10 min at 30˚C in a final volume of 25 μl of a solution containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, and

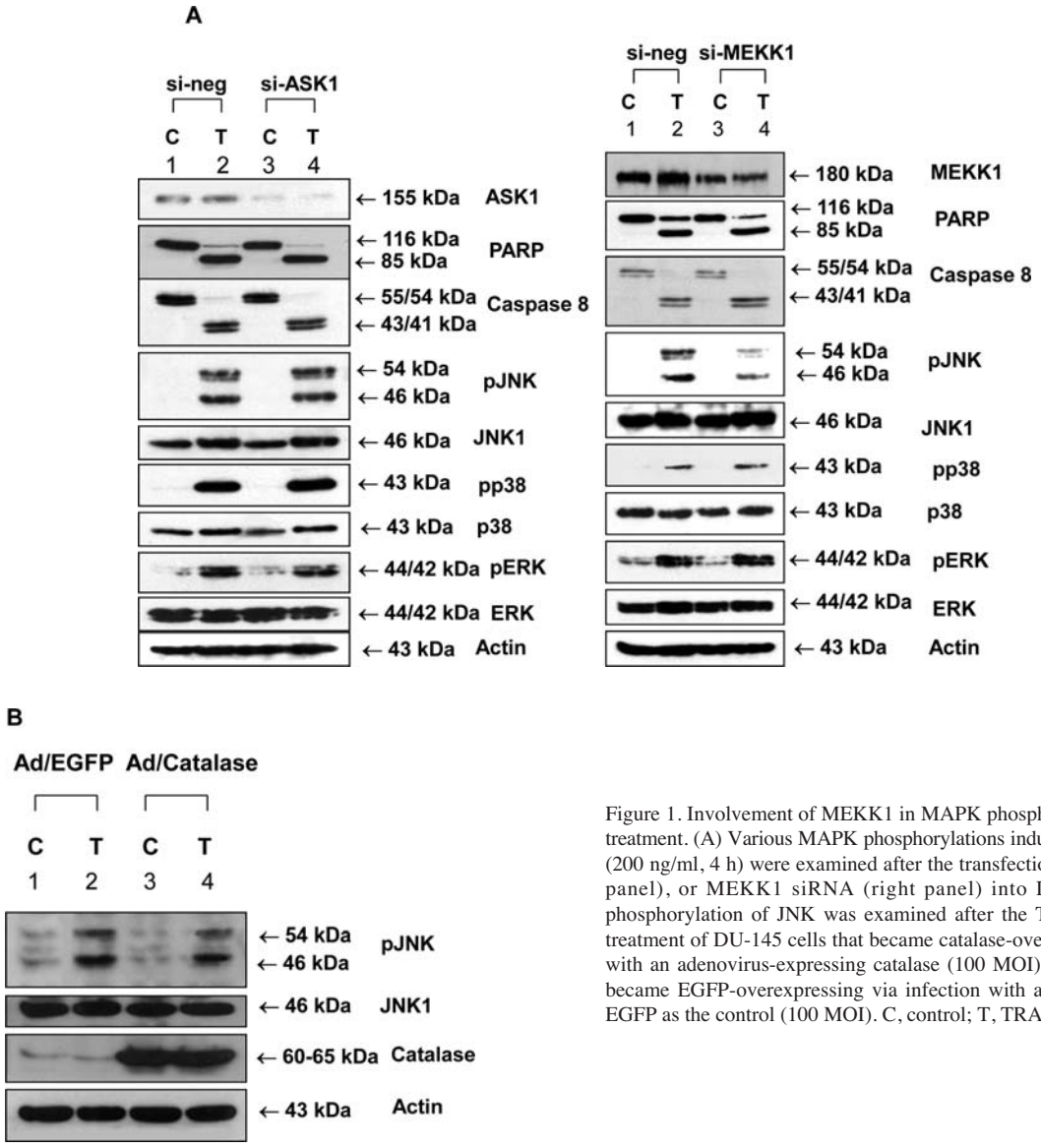


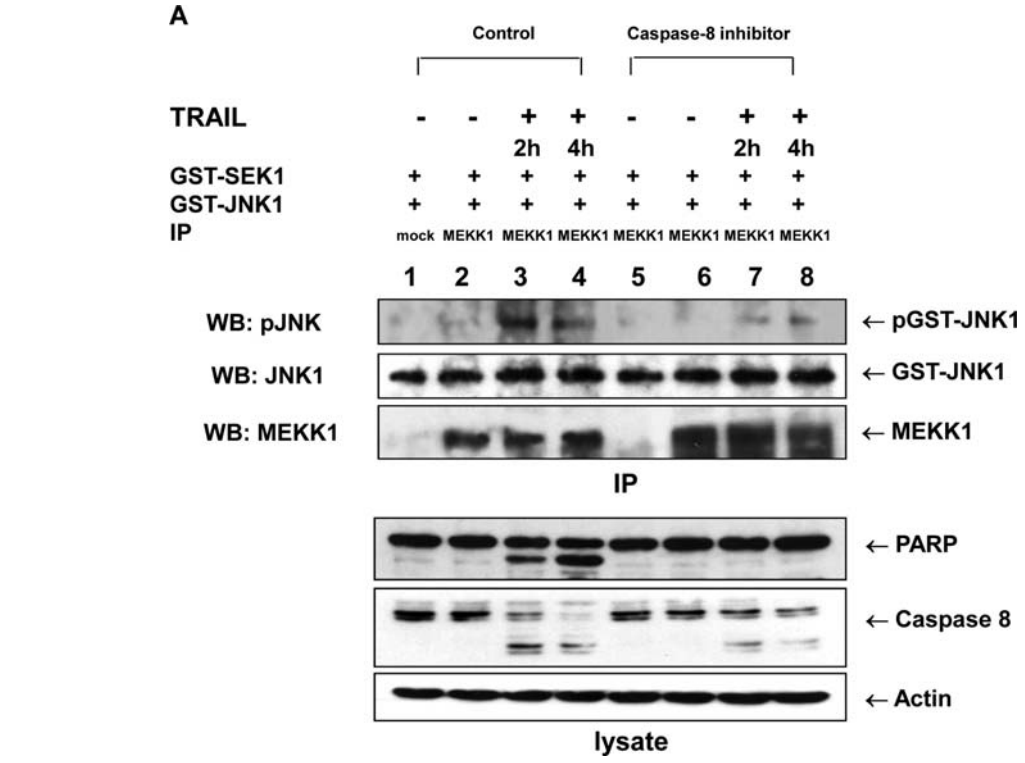
Figure 1. Involvement of MEKK1 in MAPK phosphorylation during TRAIL treatment. (A) Various MAPK phosphorylations induced by TRAIL treatment (200 ng/ml, 4 h) were examined after the transfection of control, ASK1 (left panel), or MEKK1 siRNA (right panel) into DU-145 cells. (B) The phosphorylation of JNK was examined after the TRAIL (200 ng/ml, 4 h) treatment of DU-145 cells that became catalase-overexpressing via infection with an adenovirus-expressing catalase (100 MOI) or of DU-145 cells that became EGFP-overexpressing via infection with an adenovirus-expressing EGFP as the control (100 MOI). C, control; T, TRAIL; si-neg, si-negative.

100 μM ATP and then with 1 μg GST-JNK1 for 10 min at 30˚C. Thereafter, the activated complex was subjected to SDS-PAGE, and the phosphorylated JNK1 was analyzed using rabbit anti-ACTIVE JNK antibody.

RNA interference by siRNA of SEK1. In order to stably express siRNA for the long-term knockdown, *pSilencer* 2.1-U6 hygro vector (Ambion, Inc., Austin, TX) was used for clonal cell lines. The inserts for cloning hairpin siRNA into *pSilencer* were prepared by annealing two oligonucleotides. For human SEK1 siRNA, the top strand sequence was 5-GATCCAC GCAAAGCACTGAAGTTGTTCAAGAGACAACTTCAG TGCTTTGCGTTTTTTTGGAAA-3, and the bottom strand sequence was 5-AGCTTTTCCAAAAAAACGCAAAGC ACTGAAGTTGTCTCTTGAACAACTTCAGTGCTTTGC GTG-3. The annealed insert was cloned into *pSilencer* 2.1-U6 hygro digested with *Bam*HI and *Hin*dIII. The correct structure of *pSilencer* 2.1-U6 hygro-SEK1 was confirmed by nucleotide sequencing. The resulting plasmid, *pSilencer*-SEK1, was transfected into DU-145 cells, and hygromycin B- (250 μg/ml) resistant cell clones were isolated. The interference of SEK1 protein expression was confirmed by immunoblot analysis using anti-SEK1 (Cell Signaling) antibody. For the down-regulation of ASK1 and MEKK1 or 4, their respective siRNAs (Santa Cruz Biotechnology, Inc.) were used. Cells were transfected with each siRNA and incubated for 36 h. The interference of ASK1 and MEKK1 or 4 protein expression was confirmed by immunoblotting using the anti-ASK1, anti-MEKK1, or anti-MEKK4 antibodies, respectively.

Results

MEKK1 as opposed to ASK1 is the upstream molecule of TRAIL-induced JNK activation. We previously observed that TRAIL-induced MAPK activation is dependent on caspase activation, and that Mst1 mediates caspase activation and MAPK activation (12). However, the upstream molecule of MAPKs by TRAIL has not yet been identified. In this study, we show that TRAIL-induced JNK activation is dependent on MEKK1, as opposed to ASK1, and caspase-8 activations. First of all, we examined which MAPKKK is involved in the MAPK activation during TRAIL treatment. For this purpose,



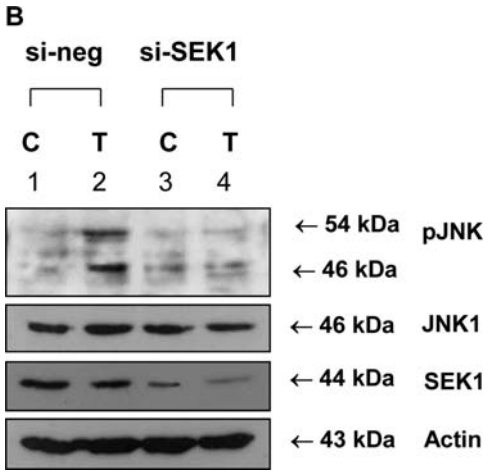


Figure 2. Caspase-8-dependent MEKK1 activation during TRAIL treatment. (A) DU-145 cells were pre-treated with the caspase-8 inhibitor (Z-IETD-FMK 20 μM, 30 min), followed by TRAIL treatment (200 ng/ml) for 2 or 4 h, and were lysed. Cell lysates were immunoprecipitated with anti-MEKK1 antibody. MEKK1 catalytic activity *in vitro* was determined by incubation with GST-SEK1 followed by GST-JNK1 as sequential substrates (upper panels). Phosphorylated-JNK, JNK1, or MEKK1 were detected with anti-ACTIVE JNK, anti-JNK1, or anti-MEKK1 antibodies, respectively. Cell lysates (lower panel) were immunoblotted with anti-PARP, anti-caspase-8, or anti-actin antibodies, respectively. (B) Phosphorylated JNK in the control vector-transfected (si-neg) or *pSilencer* siSEK1 stably-transfected (si-SEK1) single cell clones was determined during TRAIL treatment of DU-145 cells. Lysates containing equal amounts of protein (20 μg) were separated by SDS-PAGE and were immunoblotted with anti-ACTIVE JNK, anti-JNK1, anti-SEK1 or anti-actin antibodies, respectively. C, control; T, TRAIL; si-neg, si-negative.

MEKK1 and ASK1 were chosen and were down-regulated using either MEKK1 or ASK1 siRNA. Then, the phosphorylation of each of the MAPKs was examined. As shown in Fig. 1A and B, ASK1 and reactive oxygen species (ROS) production were not involved in the TRAIL-induced MAPK activation, whereas MEKK1 was closely related to the TRAIL-induced JNK activation.

MEKK1 catalytic activation induced by TRAIL is caspase-8-dependent. We then investigated whether the MEKK1 catalytic activation is really increased by TRAIL and whether this activation is related to caspase-8 activation, as suggested by many controversial studies. For the estimation of MEKK1 catalytic activity, its downstream substrates, SEK1 (MKK4) and JNK1, were used for the consecutive substrates, MEKK1 and SEK1, respectively. Then, immunoprecipitation of MEKK1 after TRAIL treatment was performed using the MEKK1 antibody for an *in vitro* immunocomplex kinase assay. Based upon our previous experience (12), the results were as expected. The increased MEKK1 activity by TRAIL was repressed by caspase-8 activation (Fig. 2A), and SEK1 mediated the MEKK1 signaling cascade through JNK during TRAIL treatment (Fig. 2B).

14-3-3 θ interacts with MEKK1, and its dissociation from MEKK1 by TRAIL treatment is caspase-8-dependent. As reported by Widmann *et al* (24), full-size MEKK1 activated JNK independently of its truncated form (91 kDa) (Fig. 2A and B). MEKK1 (91 kDa) was only observed when TRAIL treatment was used on cells that overexpressed MEKK1 (data not shown). These results do not clearly elucidate the underlying mechanisms of MEKK1 activation. Widmann *et al* (24) suggested that the 14-3-3 proteins, which are a family of serine/threonine-binding proteins that are expressed ubiquitously (25), have anti-apoptotic functions exerted by directly sequestering pro-apoptotic proteins, such as Bad (26,27). We

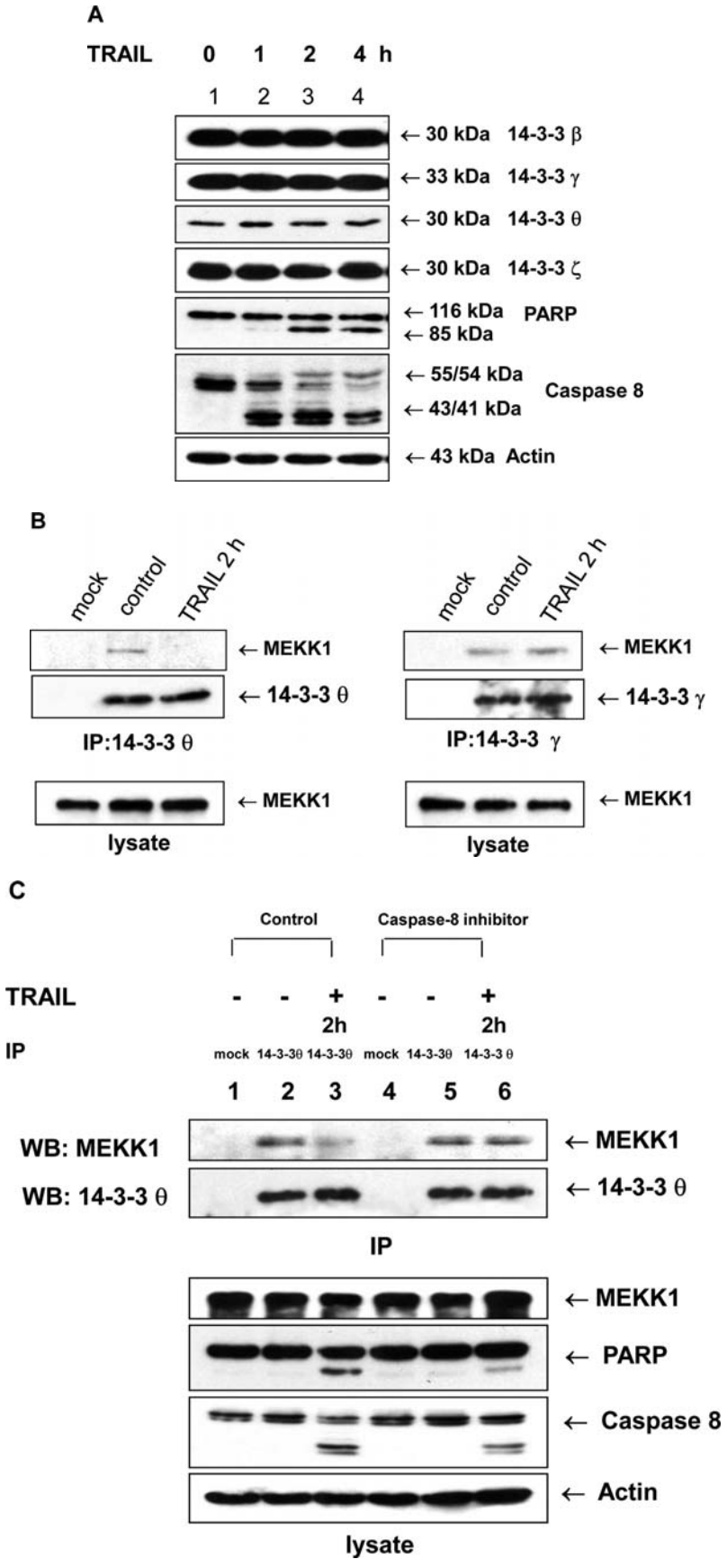


Figure 3. The 14-3-3 isotypes and MEKK1 during TRAIL treatment. (A) Various 14-3-3 isotypes were examined for cleavage by TRAIL (200 ng/ml) after various periods of time. (B) DU-145 cells were treated with TRAIL (200 ng/ml) for 2 h and lysed. Cell lysates were immunoprecipitated with anti-14-3-3 θ (left panel) or 14-3-3 γ (right panel) and immunoblotted with anti-14-3-3 θ/γ or anti-MEKK1 antibodies (upper panel). Cell lysates (lower panel) were immunoblotted with MEKK1 antibody. (C) DU-145 cells were pre-treated with caspase-8 inhibitor (Z-IETD-FMK 20 μM, 30 min), followed by TRAIL treatment (200 ng/ml) for 2 h, and were lysed. Cell lysates were immunoprecipitated with anti-14-3-3 θ antibody and immunoblotted with anti-MEKK1 antibody (upper panel). Cell lysates (lower panel) were immunoblotted with anti-MEKK1, anti-PARP, anti-caspase-8, or anti-actin antibodies, respectively.

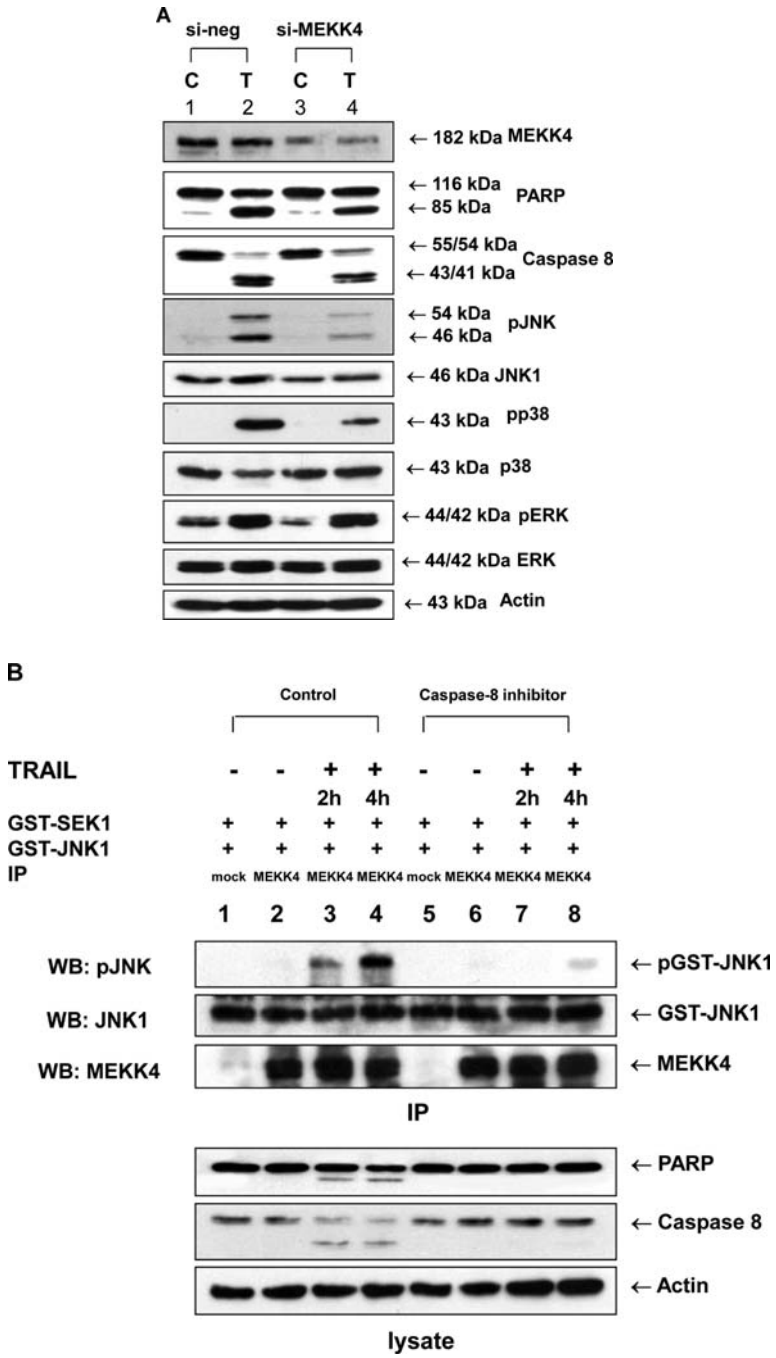


Figure 4. Involvement of MEKK4 in MAPK phosphorylation during TRAIL treatment. (A) MAPK phosphorylation induced by TRAIL treatment (200 ng/ml, 4 h) was examined after the transfection of control or MEKK1 (left) or MEKK4 siRNA (right) into DU-145 cells. (B) DU-145 cells were pre-treated with the caspase-8 inhibitor (Z-IETD-FMK 20 μM, 30 min), followed by TRAIL treatment (200 ng/ml) for 2 or 4 h, and were lysed. Cell lysates were immunoprecipitated with anti-MEKK4 antibody. MEKK4 catalytic activity *in vitro* was determined by incubation with GST-SEK1 followed by GST-JNK1 as sequential substrates (upper panels). Phosphorylated-JNK1, JNK1, or MEKK4 were detected with anti-ACTIVE JNK1, anti-JNK1, or anti-MEKK4 antibodies, respectively. Cell lysates (lower panel) were immunoblotted with anti-PARP, anti-caspase-8, or anti-actin antibodies, respectively. C, control; T, TRAIL; si-neg, si-negative.

examined the same possibility of the sequestration of MEKK1 activity by 14-3-3 and the release of MEKK1 from 14-3-3. First, we examined whether various 14-3-3 isotypes are cleaved by TRAIL treatment and found that there was no cleavage of 14-3-3 (Fig. 3A). Then, we investigated the associations of MEKK1 and various 14-3-3 isotypes. As shown in Fig. 3B, only 14-3-3 θ showed a meaningful change with MEKK1 during TRAIL treatment. In DU-145 cells, the dissociation of MEKK1 from 14-3-3 was inhibited by the caspase-8 inhibitor (Fig. 3C), implying that caspase-8 activity is necessary for the 14-3-3 release and subsequent MEKK1 phosphorylation and activation.

MEKK4 is responsible for p38 as well as JNK activation in TRAIL treatment. As a previous study indicated that MEKK4 can stimulate p38 as well as JNK activity (28), the MEKK4 involvement of p38 activation during TRAIL treatment was examined using MEKK4 siRNA. As shown in Fig. 4A, p38

and JNK phosphorylation were repressed during TRAIL treatment when MEKK4 expression was down-regulated. Additionally, MEKK4 catalytic activity was also affected by caspase-8 activation (Fig. 4B).

Discussion

In this study, we found that the MEKK family is responsible for JNK and p38 phosphorylations during TRAIL treatment. In our previous study, we reported that the UV-induced JNK activation adopted the EGFR-Grb2-MEKK1-SEK1-JNK signaling cascade (29). However, we show here that the MEKK1/4-induced SEK1-JNK/p38 activation by TRAIL is caspase-8-dependent, suggesting that Mst1 could also play a role as an upstream molecule of MEKK1 and 4 phosphorylations (12,30). As has been shown in many studies, MEKK1 itself can be activated in various ways depending on the source of stimulation, e.g., UV light induces MEKK1 activation through EGFR-Grb2, whereas TRAIL induces MEKK1 activation through caspase-8-Mst1. In addition, MEKK1 can be also activated by the caspase-8-independent germinal center kinase (GCK)-MEKK1 signaling pathway mediated by TRAF2 (31). However, we do not and cannot know exactly why there are multiple cellular processes that can lead to the activation of MEKK1. It has been suggested that cells adopt specific signaling schemes to appropriately accommodate the stimulations they experience. In the case of the MAPK activation induced by TRAIL, Mst1 has been known to act as a putative MAPK kinase kinase kinase (32), thus functioning in a positive feedback pathway that amplifies the apoptotic response through MAPK activation.

However, the biological significance of the MAPK activation has only been emphasized in regard to its pro-apoptotic effects, despite being known to have anti-apoptotic effects as well. Varfolomeev *et al* (11) suggested that kinase pathway activation by TRAIL was associated with increased production of the chemokines, IL-8 and MCP-1, and with enhanced macrophage migration for the promotion of chemokine-supported phagocytosis of apoptotic cells. It has also been reported that MCP-1 plays a role in prostate cancer invasion and metastasis (33,34), which suggests that the MAPK activation during TRAIL treatment could serve a number of functions in addition to its role in apoptosis. We have previously reported that cells from DU-145 developed acquired resistance to TRAIL-induced apoptosis after TRAIL treatment and that pAkt and Bcl-xL were involved in the process of acquired resistance (8). Many studies have shown that the ability to resist apoptosis can enhance metas-tasis (35,36). Therefore, in the future, we aim to investigate whether and/or to what extent the MAPK activation induced by TRAIL contributes to acquired TRAIL resistance, which allows for the progression to invasion or metastasis, by measuring MMP activity and MCP-1 and IL-6 levels *in vitro* and/or the incidence and intensity of metastasis *in vivo*.

Acknowledgements

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