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

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Abstract

Previously, TRAIL has been shown to activate mitogen-activated protein kinases (MAPKs) depending on caspase and Mst1 activations. However, the upstream molecule of the MAPKs has not yet been clearly elucidated. MEKK1 and ASK1 as possible candidates for the action of MAPKKKs induced by TRAIL and the possibility of ROS involvement were investigated. MEKK1/MEKK4 rather than ASK1 are responsible for TRAL-induced JNK or p38 activation, and their catalytic activity were repressed by caspase 8 inhibitor suggesting caspase 8 activation induced by TRAIL was indispensible for MEKK activation. 14-3-3 θ was also shown to interact with and to dissociate from MEKK1 by TRAIL treatment, which implicates the possibility of 14-3-3 protein as a negative regulator of MEKK1 activation. Taken together, here we show that the upstream molecule of TRAIL-induced MAPKs activation is MEK kinase (MEKK), rather than ASK1, through mediation of its signal to JNK/p38 in a caspase 8-dependent manner.

Keywords

MEKK1/4; TRAIL; JNK; p38; 14-3-3

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also known as 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 recruitment of the FADD (Fas-associated death domain) 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

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pathway) and/or the caspase cascade (extrinsic pathway), respectively, eventually leading to cell death (5).

Many reports indicate that TRAIL kills a variety of tumor cell lines, while leaving normal cells viable, which suggests that this protein may function as a specific cancer therapeutic agent (6). Although TRAIL is regarded as a potential anticancer agent, a considerable proportion of cancer cells, especially some highly malignant tumors, are resistant to apoptosis induction by TRAIL, and some 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.

Recently, we reported that Src, c-Cbl, and PI3K are involved in the phosphorylation of Akt during TRAIL treatment, and 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 MAPK kinase pathways in a caspase 8-dependent manner (10–12). However, the biological roles of JNK and p38 MAPK activations in TRAIL-induced signaling are uncertain (5). Moreover, multiple mechanisms by which JNK or p38 are activated by TRAIL have been reported. For example, Varfolmeev et al. (11) suggested that FADD, caspase-8, RIP1, and TRAF2 are recruited within the primary death-inducing signaling complex (DISC), leading to the stimulations of JNK and p38, while Liu et al. (13) concluded that MEKK1 could be activated via TRAF2 and RIP to activate JNK in the absence of apoptotic conditions. We demonstrated the occurrence of Mst1-mediated caspase 8-dependent activation of mitogen-activated protein kinases (MAPKs) during TRAIL incubation, but the upstream entities of the MAPKs remain unidentified.

The MAPKs are a family of kinases that transduce external signals to the nucleus in order to decide the fate of the cell (14). Usually, conventional MAPKs consist of three family members, JNK, p38, and 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 the MAPKs have not yet been classified. Actually, MAPKKKs link a variety of extracellular stimuli to cytoplasmic and nuclear effectors by activating downstream MAPK pathways (21). MEKK1, ASK1, TAK1, and MLK2 are well-known MAP3 kinases (22). In this study, we clearly demonstrate that MEKK1 and MEKK4 transmit TRAIL-induced signals to JNK or p38 MAPK in caspase 8-dependent manners.

Materials and Methods

Cell culture

A human prostate adenocarcinoma cell line, DU-145, was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (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 phosphoY185) JNK was purchased from Promega (Madison, WI, USA). Polyclonal anti-JNK1 and 14-3-3 β, 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-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

Cells were lysed with 1× Laemmli lysis buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.002% bromophenol blue, 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 1× lysis buffer, and β-mercaptoethanol was added until a concentration of 350 mM was achieved. Then, equal amounts of protein were loaded onto 10% sodium dodecyl sulfate (SDS) polyacrylamide gels. SDS-polyacrylamide gel electrophoresis (PAGE) analysis was performed according to the procedure of Laemmli using a Hoefer gel apparatus.

Immunoblot analysis

Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Each nitrocellulose membrane was blocked with 5% nonfat 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 hr. 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 using centrifugation, and the supernatants were immunoprecipitated with mouse anti-MEKK1 or anti-MEKK4 antibody 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. To examine the catalytic activity of MEKK1 or MEKK4, GST-tagged JNK1 and SEK1 proteins were used as substrates. For the purification of JKN1 or SEK1, the plasmid containing GST-human JNK1 for bacterial fusion protein was constructed in pGEX-4T-1 by inserting the *Hind*III/*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). To measure the immune complex activity of MEKK1 or MEKK4, 0.2 μg GST-SEK1 was 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 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

To stably express siRNA for the long-term knockdown, p*Silencer* 2.1-U6 hygro vector (Ambion, Inc., Austin, TX) was used for clonal cell lines. The inserts for hairpin siRNA into p*Silencer* were prepared by annealing two oligonucleotides. For human SEK1 siRNA, the top strand sequence was 5_-GATCCACGCAAAGCACTGAAGTTGTTCAAGAGACAACTTCAGTGCTTTGCGTTTTTTTGGAAA-3, and the bottom strand sequence was 5-AGCTTTTCCAAAAAAACGCAAAGCACTGAAGTTGTCTCTTGAACAACTTCAGTGCTTTGCGTG-3. The annealed insert was cloned into p*Silencer* 2.1-U6 hygro digested with BamHI and HindIII. The correct structure of p*Silencer* 2.1-U6 hygro-SEK1 was confirmed by nucleotide sequencing. The resultant plasmid, p*Silencer*-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 using anti-SEK1 (Cell Signaling) antibody. To down regulate ASK1, MEKK1 or MEKK4, their respective siRNAs (Santa Cruz Biotechnology, Inc.) were used. Cells were transfected with each siRNA and incubated for 36 hr. The interference of ASK1, MEKK1, or MEKK4 protein expression was confirmed by immunoblotting using the anti-ASK1 antibody, anti-MEKK1 antibody, or anti-MEKK4 antibody, respectively.

Results

MEKK1 rather than ASK1 is the upstream molecule of TRAIL-induced JNK activation

We previously observed that TRAIL-induced MAPKs activation is dependent on caspase activation, and that Mst1 (mammalian sterile 20-like kinase 1) mediates caspase activation and MAPKs activation (12). However, the upstream molecule of MAPKs by TRAIL has not yet been clarified. Here, we show that TRAIL-induced JNK activation is dependent on MEKK1, rather than ASK1, and caspase 8 activations. First of all, we examined which MAPKKK is involved in MAPKs activation during TRAIL treatment. For this purpose, MEKK1 and ASK1 were chosen and down regulated using siRNA of MEKK1 or ASK1; then, the phosphorylation of each of the MAPKs was examined. As shown in figures 1A and 1B, ASK1 and ROS production were not involved in TRAIL-induced MAPKs activation, whereas MEKK1 was closely related to the TRAIL-induced JNK activation.

MEKK1 catalytic activation induced by TRAIL is caspase 8-dependent

Next, we investigated whether the MEKK1 catalytic activation is really increased by TRAIL and whether the activation is related to caspase 8 activation, as suggested by many controversial reports. 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. As expected based upon our previous experience (12), increased MEKK1 activity by TRAIL was repressed by caspase 8 activation (Fig. 2A), and SEK1 mediated the MEKK1 signaling cascade to 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 mentioned by Widmann et al. (24), full-size MEKK1 activated JNK independent of its truncated form (91 kDa) (Figs. 2A and 2B); 91 kDa of MEKK1 was only observed when TRAIL was treated to 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 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 examined the same possibility of sequestration of MEKK1 activity by 14-3-3 and 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 figure 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 caspase 8 inhibitor (Fig. 3C), implying that caspase 8 activity is necessary for 14-3-3 release and subsequent MEKK1 phosphorylation and activation.

MEKK4 is responsible for p38 activation as well as JNK activation in TRAIL treatment

From the report indicating that MEKK4 can stimulate p38 as well as JNK activity (28), MEKK4 involvement of p38 activation during TRAIL treatment was examined using siRNA of MEKK4. As shown in figure 4A, p38 and JNK phosphorylation were repressed during TRAIL treatment when MEKK4 expression was downregulated.. 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 paper, we reported that UV-induced JNK activation adopted EGFR-Grb2-MEKK1-SEK1-JNK (29); however, here, we show that MEKK1/4-induced SEK1-JNK/p38 activation by TRAIL is caspase 8-dependent, suggesting that Mst1 might also play a role as an upstream molecule of MEKK1 and MEKK4 phosphorylations (12,30). As shown in many reports, 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 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 MAPKs 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 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. Interestingly, it has been reported that MCP-1 also plays a role in prostate cancer invasion and metastasis (33,34), which suggests that MAPK activation during TRAIL treatment may serve several functions in addition to its role in apoptosis. We have already published the findings 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). Also, several lines of evidence show that the ability to resist apoptosis may enhance metastasis (35,36). Therefore, in our next study, we will investigate whether and/or how much MAPKs activation induced by TRAIL contributes to acquired TRAIL resistance, which allows for progression to invasion or metastasis, by measuring MMP activity and MCP1 and IL-6 levels *in vitro* and/or incidence and intensity of metastasis *in vivo*.

Acknowledgments

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Abbreviations used in this paper

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
SEK1	stress-activated protein kinase/extracellular-signal regulated kinase
MLK	mixed lineage kinase
TAK1	TGF-β activated-kinase
ROS	reactive oxygen species
GCK	germinal center kinase
TRAF2	TNF receptor-associated factor 2
Mst1	mammalian sterile 20-like kinase 1

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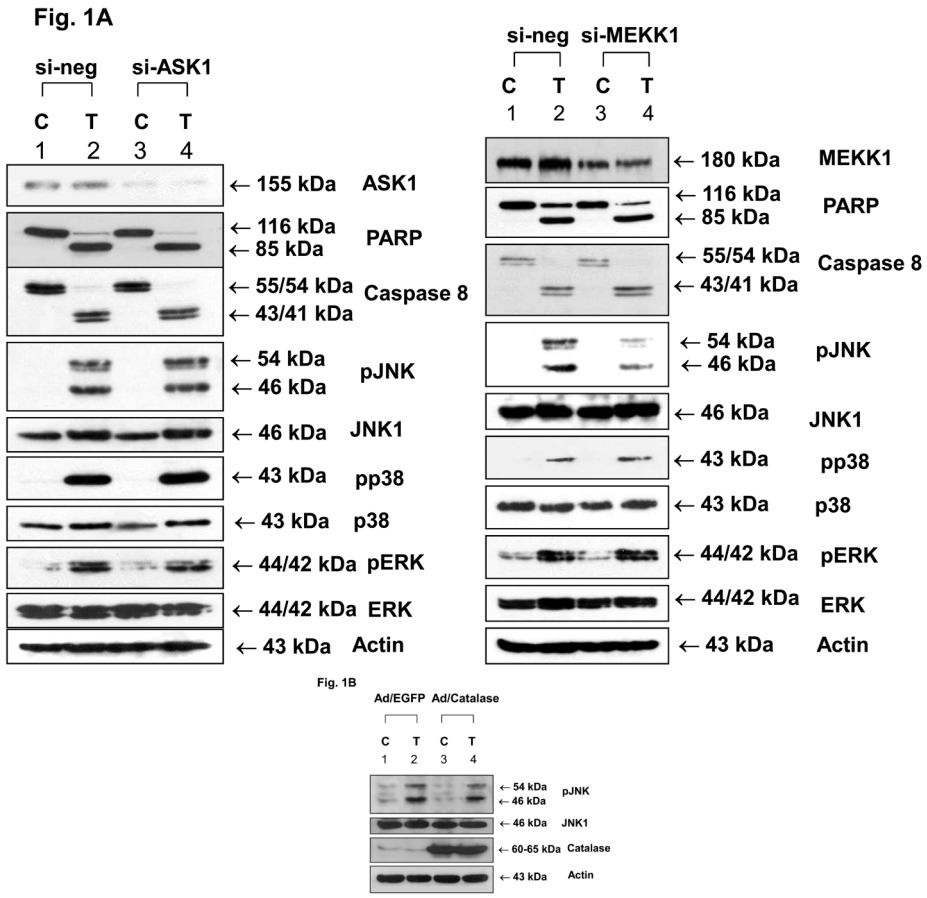

Figure 1.

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

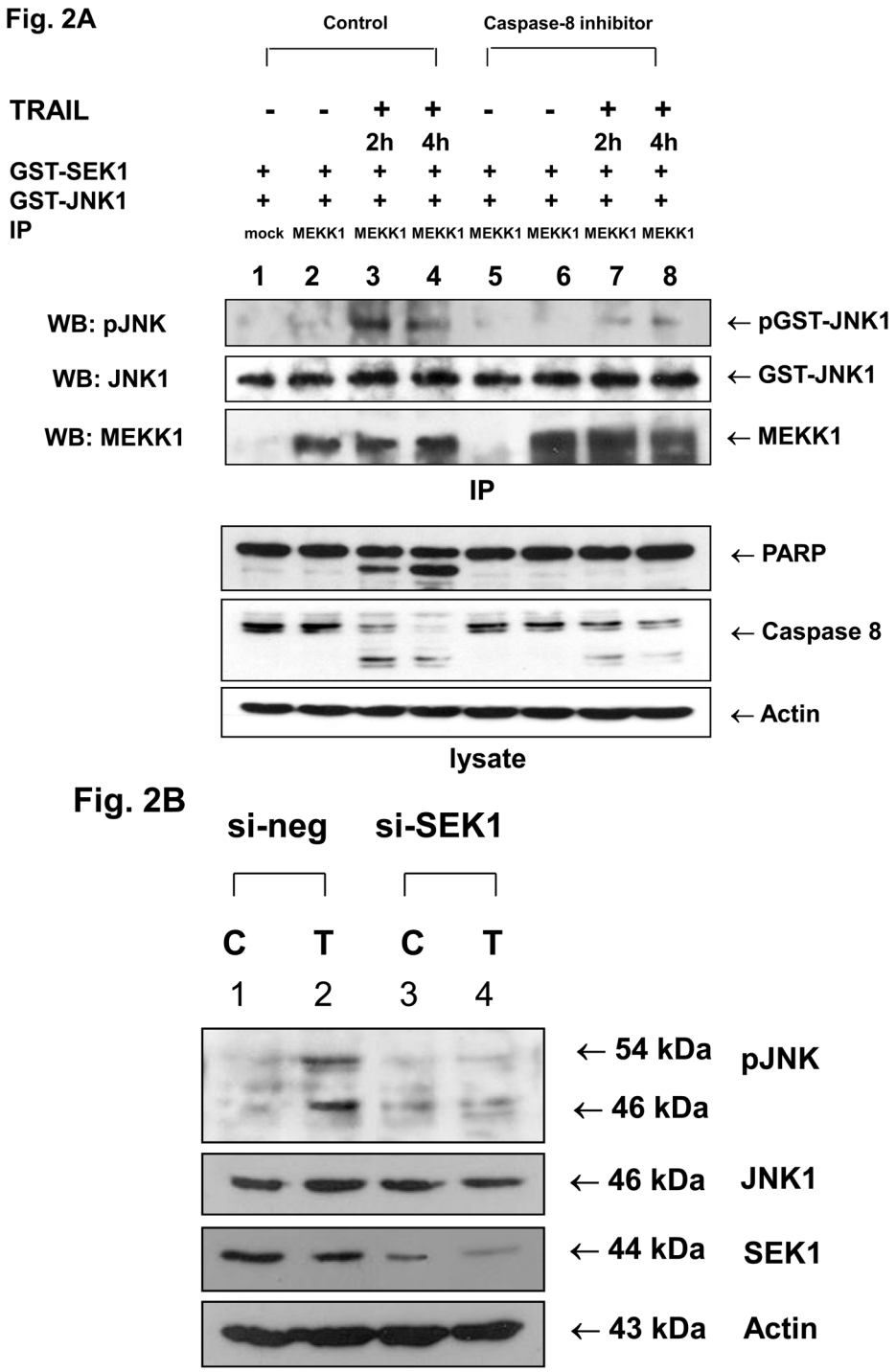

Figure 2.

Caspase 8-dependent MEKK1 activation during TRAIL treatment. (A) DU-145 cells were pretreated with 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 antibody, respectively. (B) Phosphorylated JNK in control vector–transfected (si-neg) or pSilencersi SEK1 stably transfected (si-SEK1) single cell clones was determined during TRAIL treatment from 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 and anti-actin antibody, respectively. C: control; T: TRAIL

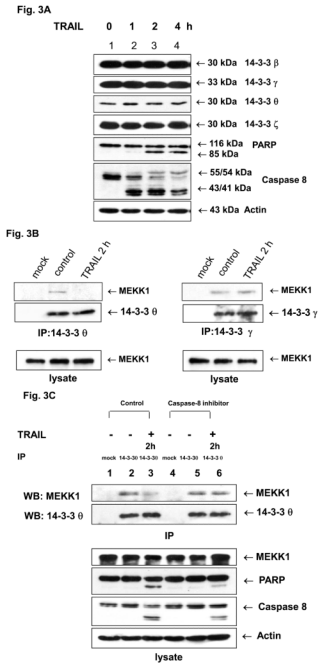

Figure 3.

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 antibody or anti-MEKK1 antibody (upper panels). Cell lysates (lower panel) were immunoblotted with MEKK1 antibody. (C) DU-145 cells were pretreated 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 panels). Cell lysates (lower panel) were immunoblotted with MEKK1 antibody, anti-PARP antibody, caspase-8 antibody, or anti-actin antibody, respectively.

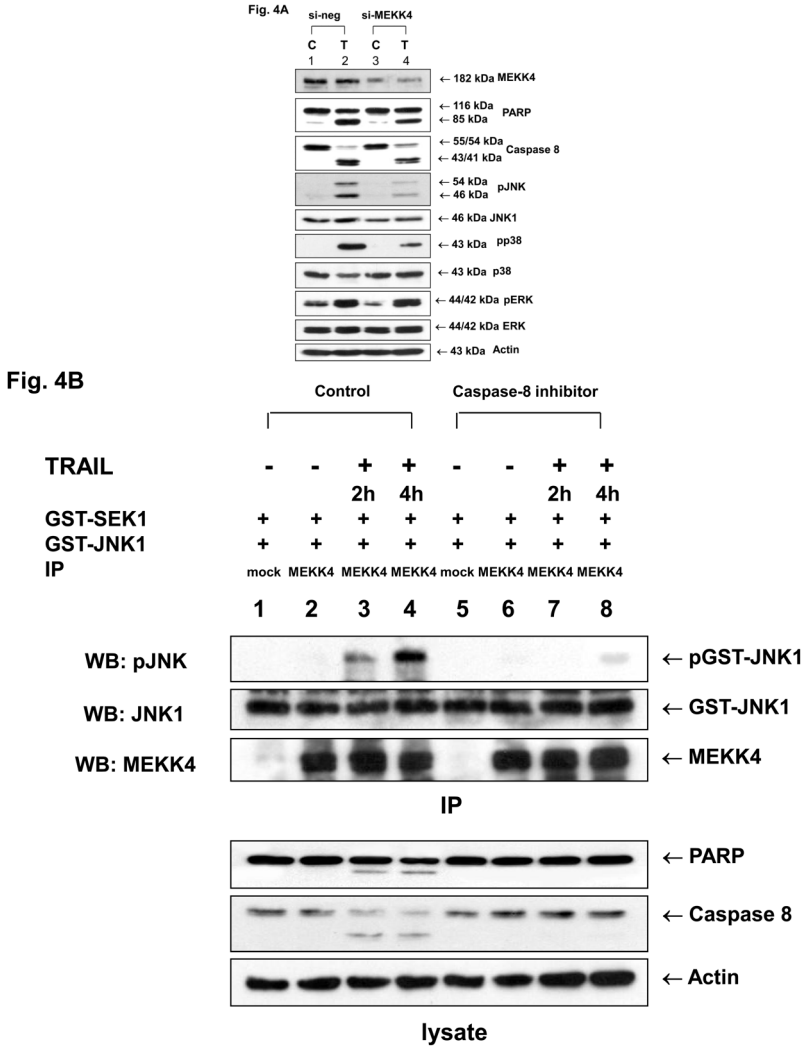

Figure 4.

Involvement of MEKK4 in MAPKs phosphorylation during TRAIL treatment. (A) MAPKs phosphorylation induced by TRAIL treatment (200 ng/ml, 4 h) was examined after transfection of control siRNA or MEKK1 siRNA (left) or MEKK4 siRNA (right) into DU-145 cells. (B) DU-145 cells were pretreated with 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 antibody, respectively. C: control; T: TRAIL