Doctoral Dissertation

博士論文

Identification of a novel redox sensor that mediates delayed activation of stress-responsive MAPK pathways

（新規レドックス・センサー分子による SAPK シグナル制御機構の解析）

A Dissertation Submitted for the Degree of Doctor of Philosophy

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1. Abstract

Cells are exposed to various kinds of environmental stresses such as ultraviolet rays, ionizing radiation, genotoxins, heat shock and oxidative stress. Especially, oxidative stress causes damage to proteins, DNA and lipids, thereby leading to cell death, tumorigenesis and inflammation. Hence eukaryotic organisms have developed intracellular stress response devices that sense stresses and make cell fate decisions such as cell repair, cell cycle arrest and cell death. Such a crucial decision between survival and death is partly mediated by the stress-activated p38 and JNK MAPK (SAPK) pathways. SAPK (p38/JNK) pathways, composed of three-tiered SAPKKK, SAPKK and SAPK, are activated in response to various stresses. Reflecting the presence of a wide range of cellular stresses, mammalian cells express more than a dozen SAPKKKs. Upon stress stimuli, these SAPKKKs activate a limiting number of their cognate SAPKKs (MKK3/4/6/7), which in turn activate SAPKs, thereby regulating reparative and/or apoptotic responses. We have previously identified MAP three kinase 1 (MTK1) as a SAPKKK. We have also reported that MTK1 activation is initiated by GADD45 that is induced by various stimuli such as DNA damage and tumor growth factor β (TGF-β). Indeed, I found that MTK1 was activated in a GADD45-mediated manner by 12-O-tetradecanoylphorbol 13-acetate (TPA).

In this study, I found that MTK1 was also activated in response to oxidative stress, but the oxidative stress-induced MTK1 activation occurred in a GADD45-independet manner. I demonstrated that upon oxidative stress, MTK1 was oxidized directly at Cys residues of GADD45-binding domain (GBD) and subsequent reduction of oxidized MTK1 by thioredoxin (Trx) was required for its activation. Furthermore, this study showed that MTK1 was responsible for delayed-phase activation of SAPK (p38/JNK) under oxidative stress conditions that contributed to cell death and cytokine production such as IL-6. In addition, I also found that early-phase activation of SAPK was mediated by apoptosis signal-regulating kinase 1 (ASK1), a SAPKKK. Therefore, I concluded that MTK1 and ASK1 cooperatively make cell fate decision under oxidative stress conditions by inducing early and delayed SAPK activation, respectively.

Index

2. Introduction

2-1 SAPK pathways

Cells are exposed to various kinds of environmental stresses such as reactive oxygen species (ROS), ultraviolet (UV), chemical compounds and so on. In general, in cases of the intensity of the damage is moderate, cells seek to repair the damage. However, when the damage is too severe to be repaired, damaged cells are eliminated by apoptosis to reduce the risk of disorder such as cancer. Intracellular signal transduction is a mechanism to response to various environmental conditions. Intracellular signal transduction systems take place via activity changes of enzymes and are essential for cell fate decisions such as cell growth, death and stress responses. Previous studies have shown that there are various signaling pathways to adapt to surrounding environment of cells. One of the most pivotal ways of signal transduction is phosphorylation of proteins. Phosphorylation regulates protein enzymatic activities by altering protein charges and inducing conformational changes of proteins. Kinases are responsible enzymes for protein phosphorylation, are known to key regulators of intracellular signaling pathways.

Mitogen-activated protein kinase (MAPK) pathways are constituted by growth factor-inducible ERK pathway and stress responsible SAPK (p38 and JNK) pathways (Figure 1). The SAPK pathways, composed of three-tiered protein kinases, SAPK kinase kinase (SAPKKK), SAPK kinase (SAPKK) and SAPK, are activated by various stresses. Reflecting the presence of a wide range of cellular stresses, mammalian cells express more than a dozen SAPKKKs such as MEK Kinase 1/2/3 (MEKK1/2/3), MAP three kinase 1 (MTK1) (also known as MEKK4), TGF-β activated kinase 1 (TAK1) and Apoptosis signal-regulating kinase 1 (ASK1). Upon stress stimuli, these SAPKKKs activate a limiting number of their cognate SAPKKs (MKK3/4/6/7), which in turn activate SAPKs, thereby regulating reparative and/or apoptotic responses [1]. Although SAPK pathways are important for cell fate decisions under stress conditions, the molecular mechanism of how SAPK pathways are activated upon stress is largely unknown.

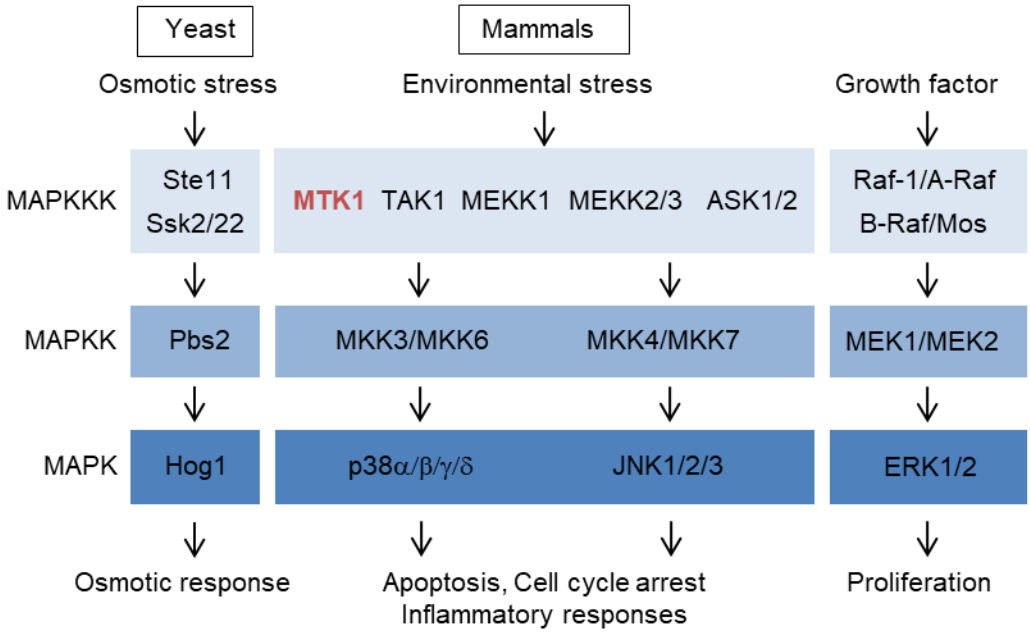

Figure 1 MAPK pathways in yeast and mammals.

Mammalian MAPK pathways are composed of stress responsive pathways (SAPK: p38/JNK pathway) and growth factor pathway (ERK pathway)

2-2 MTK1

A previous study has identified MTK1, the human homolog of the yeast Ssk2/Ssk22 MAPKKKs, as a specific mediator of stress-induced SAPK activation [2]. MTK1 is required for neural tube closure during embryogenesis and TGF-β mediated IFN-γ production from T helper type 1 (Th1) cells [3, 4]. In unstimulated cells, MTK1 is kept catalytically inactive through an autoinhibitory interaction between the amino-terminal regulatory domain and the carboxy-terminal kinase domain [5]. MTK1 activation is initiated by binding of the growth arrest and DNA damage-inducible 45 (GADD45) family proteins (GADD45α/β/γ), whose expression is induced by various stresses [6]. GADD45 binds to GADD45-binding domain (GBD) in MTK1 near its N-terminal autoinhibitory domain, thereby leading to the dissociation of the inhibitory N-C interaction of MTK1 and subsequent MTK1 homodimer formation. Dimerized MTK1 is fully activated by *trans*-autophosphorylation at T1493 residue, the essential activating phosphorylation site of MTK1 (Figure 2) [7]. However, it remains unclear if there is another mechanism and physiological role of MTK1.

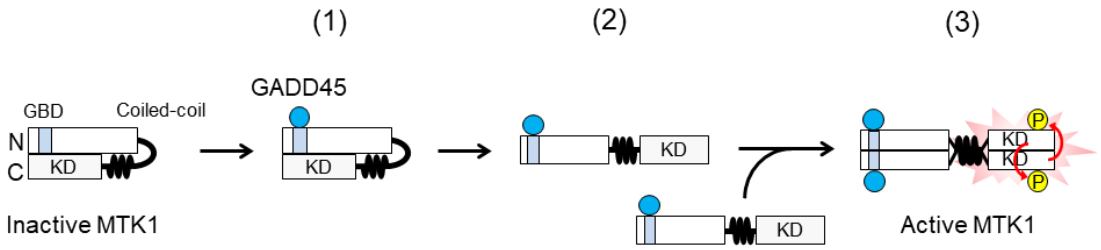

Figure 2　Schematic model of GADD45-induced activation of MTK1.

(1) Stress-inducible GADD45 binds to MTK1 GBD and (2) induces MTK1 conformational change. Then (3) MTK1 dimerizes and is activated by *trans*-autophosphorylation.

2-3 Oxidative stress

Under oxidative stress conditions, elevated intracellular reactive oxygen species (ROS) cause damage to proteins, DNA and lipids [8]. In addition, ROS also function as signaling transduction mediators [9]. ROS is synthesized by mitochondrial electron transport chain and oxidase such as NADPH oxidase (NOX) in response to respiration, inflammation and infection [10, 11]. ROS targets thiol residues of proteins under oxidative stress conditions. Protein oxidation forms intra- or inter-molecular disulfide bonds, which then induces conformational and functional changes of proteins (Figure 3) [12]. Thus, continuous oxidative stress induces severe cellular damage, for example, inflammation and apoptotic- or necrotic-cell death. Cells protect themselves from ROS-induced damages by activating reductants such as catalase, thioredoxin (Trx) and glutathione (GSH). These reductases eliminate ROS and reduce oxidized proteins. Hence, dysregulation of intracellular ROS levels increases the risk of intractable diseases such as chronic inflammatory diseases, cancer, cardiovascular diseases and neurodegenerative diseases [13-15]. However, the mechanism by which cells sense oxidative stress is largely unclear.

2-4 Thioredoxin

Trx is a reductant that converts oxidized thiol residues to reduced ones. When Trx reduces disulfide bonds of oxidized proteins, Trx binds to the disulfide bond (S-S bond) of target proteins via its catalytic Cys32 residue. Trx further attacks the S-S bond via another catalytic Cys35 residue, thereby leading to reduction of target proteins [16]. The intramolecular S-S bond between Cys32 and Cys35 of oxidized Trx is converted to reduced Trx by Trx reductase (TrxR), which allows Trx to reduce oxidized proteins repeatedly under oxidative stress conditions (Figure 4) [17, 18].

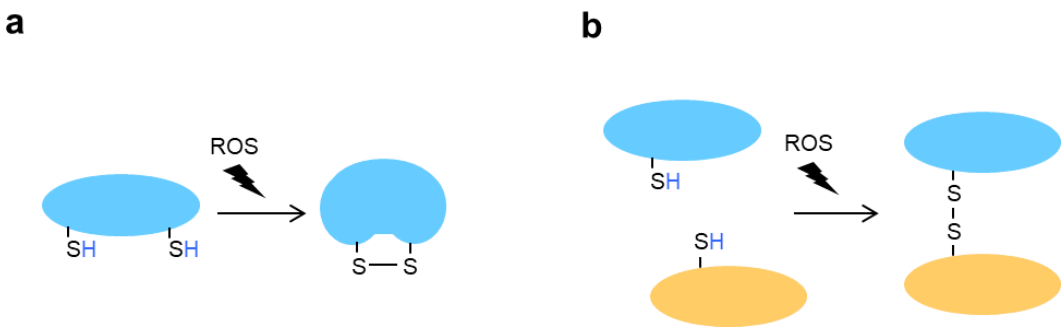

Figure 3 ROS targets thiol and changes protein functions.

(**a**) Oxidation of thiol induces formation of intra-molecular disulfide bond.

(**b**) Oxidation of thiol induces formation of inter-molecular disulfide bond.

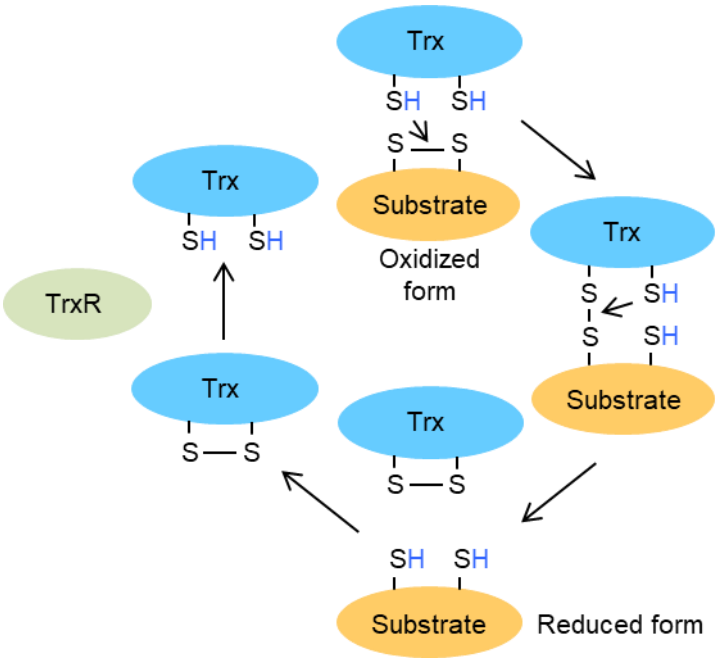

Figure 4 Trx catalyzes reduction of oxidized thiol of proteins.

Trx reduces its oxidized substrates through Trx Cys32 and Cys35. Trx attacks S-S bond of the substrate and reduces it by Trx oxidation. Oxidized Trx is reduced and recycled by TrxR.

2-5 ASK1

Upon oxidative stress, Trx regulates oxidative stress induced activation of ASK1. ASK1 has the two coiled-coil domains in the N-terminal region (NCC) and C-terminal region (CCC) [19, 20]. Under unstimulated conditions, Trx interacts with the ASK1 N-terminal region and suppresses ASK1 activation [21, 22]. When cells are exposed to oxidative stress, Trx is oxidized and released from ASK1. This dissociation allows ASK1 to form a homodimer through NCC and CCC. The dimerization of ASK1 induces its activation by *trans*-autophosphorylation at T845 (Figure 5) [20].

2-6 Macrophage and cytokine

Macrophages are phagocytotic cells, which are involved in innate immune responses. They are differentiated from monocyte cells and locate in various tissues of organisms. Upon invasion of microorganisms, macrophages engulf invaders and present antigens to T cells. In response to infection, monocyte cells are differentiated into macrophages. Macrophages promote proinflammatory responses by producing ROS and proinflammatory cytokines such as TNFα, IL-1β and IL-6. (Figure 6) [23].

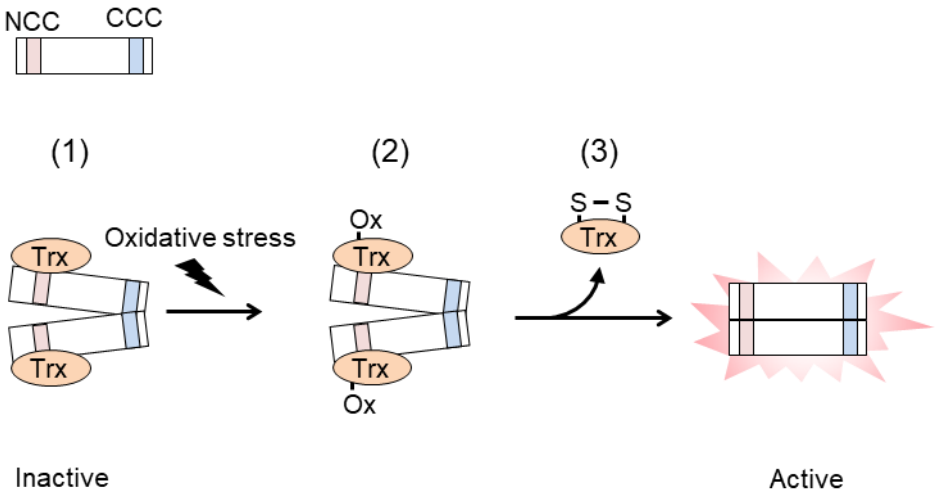

Figure 5　Schematic model of oxidative stress-induced activation of ASK1.

ASK1 has two coiled-coil domains both in N-terminal (NCC) and C-terminal (CCC) regions. In the inactive state, NCC is masked by Trx (1). When cells are exposed to oxidative stress, Trx is oxidized directly (2), released from ASK1 (3), and then ASK1 is activated by *trans*-autophosphorylation.

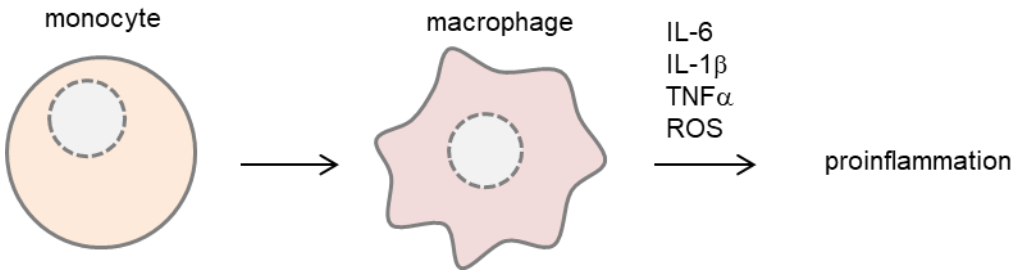

Figure 6 Macrophages are involved in inflammatory responses.

Macrophages are differentiated from monocyte cells and induce proinflammatory cytokines and ROS.

3. Aim

Previous studies have shown that MTK1 is activated by a variety of stimuli such as methyl methanesulfonate (MMS) and TGF-β that induce the GADD45 expression [6, 24]. In this study, I found that TPA also induced GADD45 expression and thereby activated MTK1. In addition, I found that oxidative stress also activated MTK1. But, interestingly, its activation occurred in a GADD45-independent manner because MTK1 activation under oxidative stress was observed even when GADD45 expression was inhibited by a protein synthesis inhibitor, cycloheximide. These data suggest that in addition to GADD45-induced MTK1 activation, MTK would be activated in response to oxidative stress by a GADD45-independent mechanism. Therefore, I have tried to elucidate the molecular mechanism of how MTK1 is activated upon oxidative stress.

4. Results

4-1 Oxidative stress stimulation activates MTK1 in a GADD45-independent manner.

Previous studies showed that the stress-inducible GADD45 family proteins were specific activators of MTK1[6, 7, 24]. To investigate if MTK1 is activated through stress-mediated production of GADD45 protein, Myc-MTK1 stably expressing cells were stimulated with TPA, a mitogen which activates not only ERK but also SAPKs [25]. TPA stimulation strongly promoted expression of GADD45β mRNA and production of GADD45β protein (Figure 7b, left panel and Figure 7c, lanes 1-3). Under TPA stimulated condition, MTK1 was markedly phosphorylated (Figure 7a, lanes 1-3). Then, Myc-MTK1 stably expressing cells were pretreated with cycloheximide (CHX), a protein synthesis inhibitor, to suppress de novo synthesis of the GADD45 proteins. Pretreatment with CHX markedly inhibited TPA-induced production of GADD45β protein and activation of MTK1 (Figure 7c, lane 3 and Figure 7a, lanes 4-6). These data suggested that TPA stimulation could activate MTK1 through GADD45 proteins. Different from TPA stimulation, H_2O_2 stimulation did not induce the expression of any of the GADD45 family mRNAs and GADD45β protein (Figure 7b, right panel and Figure 7c, lanes 4-6). Interestingly, activation of MTK1 was observed under oxidative stress conditions with H_2O_2 even when cells were pretreated with CHX (Figure 7a, lanes 7-12). H_2O_2-induced activation of MTK1 was sustained at least until 240 minutes after H_2O_2 stimulation (Figure 7a, lanes 13-16). Therefore, these data suggest that there is an unidentified, GADD45-independent mechanism of MTK1 activation in response to oxidative stress.

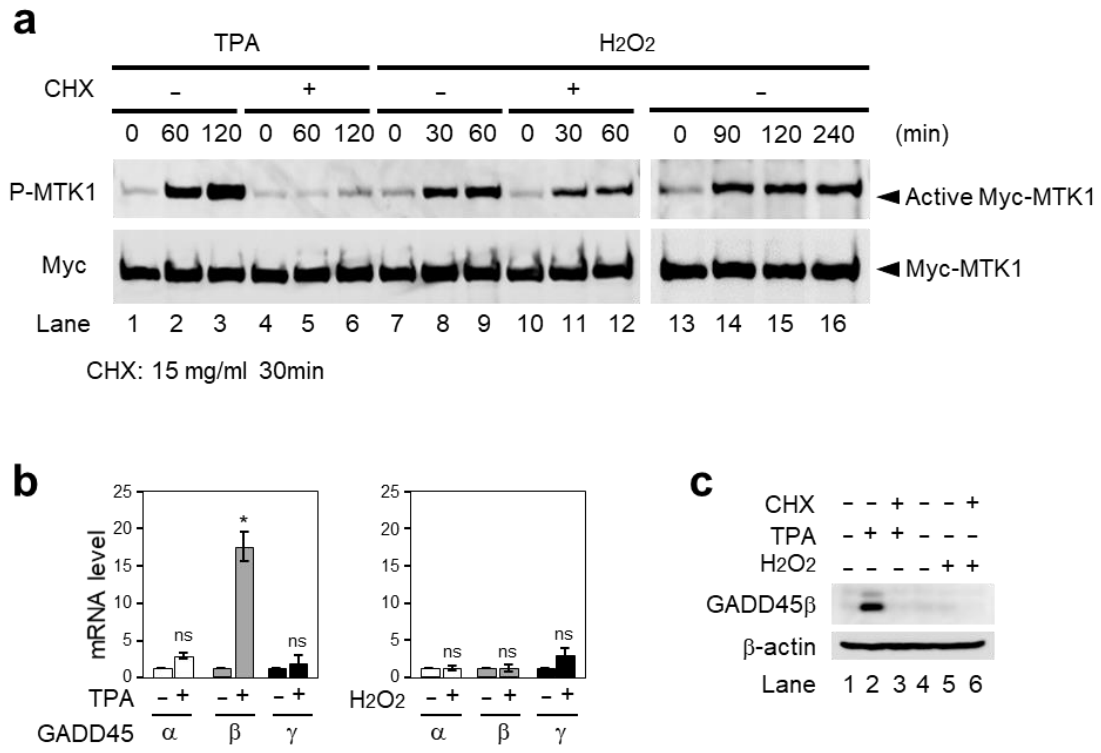

Figure 7 MTK1 is activated in GADD45-independent manner in response to oxidative stress.

(**a**) GADD45 mediates TPA-induced MTK1 activation, whereas H_2O_2-induced MTK1 activation occurs in a GADD45-independent manner. HEK293A cells stably expressing Myc-MTK1 were treated with CHX (15 μg/ml) for 30 min, followed by TPA (80 nM) or H_2O_2 (1 mM) for the indicated time. Immunoprecipitated Myc-MTK1 was probed with P-MTK1 and Myc antibodies (top and bottom panel).

(**b**) GADD45 transcription was not induced in response to oxidative stress. HEK293A cells were treated with TPA (80 nM) for 120 min or H_2O_2 (1 mM) for 60 min. Purified mRNAs were analyzed for expression level of GADD45β, α and γ by qRT-PCR.

(**c**) GADD45 protein was not induced under oxidative stress conditions. HEK293A cells were treated with CHX (15 μg/ml) for 30 min, followed by TPA (80 nM) for 120 min or H_2O_2 (1 mM) for 60 min. Lysates were probed with GADD45β and β-actin antibodies.

4-2 Oxidation and subsequent reduction of MTK1 are required for its activation under oxidative stress conditions.

Next, I examined how oxidative stress activated MTK1. To investigate whether MTK1 is oxidized directly under oxidative stress conditions, I performed non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In this method, cell lysates were separated without 2-mercaptoethanol (2ME), a reducing agent, therefore oxidation-mediated S-S bond of proteins were preserved. Myc-MTK1 stably expressing cells were treated with H_2O_2 for 5 minutes and cell lysate was separated by non-reducing SDS-PAGE, then immunoblotted to monitor Myc-MTK1. As a result, H_2O_2-treatment induced a remarkable band-shift of Myc-MTK1 to high-molecular-weight species (Figure 8a, top left lanes 1-2). This band-shift disappeared in reducing SDS-PAGE, in which cell lysate was incubated with 2ME (Figure 8a, top right). These data suggest that MTK1 is rapidly oxidized under oxidative stress conditions, resulting in forming a complex with other proteins through S-S bonds. Treatment of H_2O_2 in Myc-MTK1 stably expressing cells rapidly induced oxidation of MTK1 within 5 min, and the oxidized MTK1 was maintained up to 20 min and then gradually returned to the reduced form after 40 min (Figure 8a, top left). In contrast, H_2O_2 treatment induced the activation of MTK1 gradually. This activation was concurrent with the reduction of oxidized MTK1. (Figure 8a, middle). The same results were observed for endogenous MTK1 in HEK293 cells (Figures 8b and c). These data suggest that the reduction of oxidized MTK1 is necessary for its activation. To investigate if reduction of oxidized MTK1 is required for its activation, Myc-MTK1 stably expressing cells were pretreated with a catalase inhibitor 3-amino-1,2,4-triazole (3-AT) [26]. In consequence, activation of MTK1 was suppressed when reduction of oxidized MTK1 was inhibited by 3-AT (Figure 8d). Hence, a redox regulation (oxidation and subsequent reduction) of MTK1 is essential for its activation in response to oxidative stress.

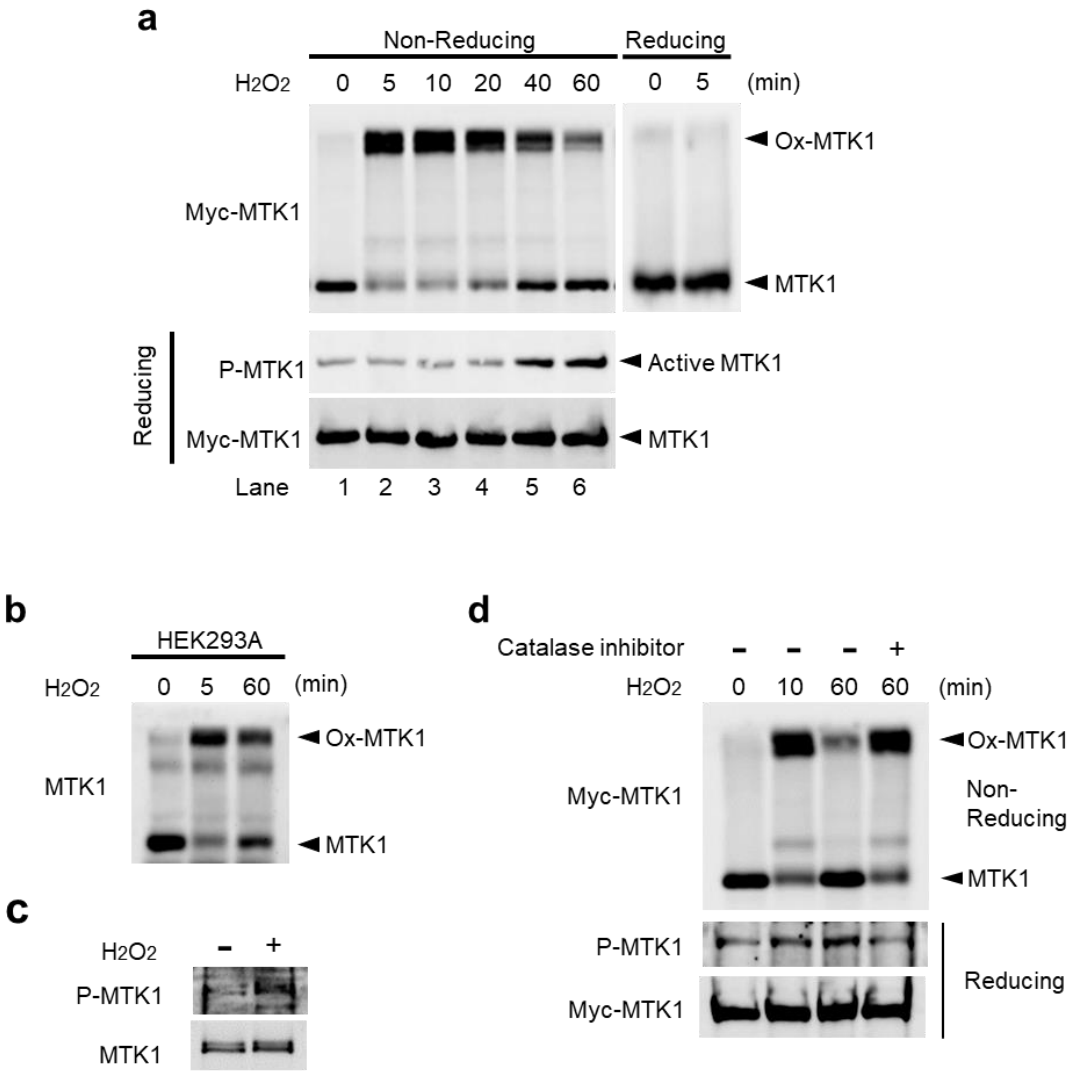
a
Non-Reducing
Reducing
H2O2
0 5 10 20 40 60 0 5 (min)
Ox-MTK1
Myc-MTK1
MTK1
Reducing
P-MTK1
Active MTK1
Myc-MTK1
MTK1
Lane 1 2 3 4 5 6
b
HEK293A
H2O2
0 5 60 (min)
Ox-MTK1
MTK1
MTK1
c
H2O2
- +
P-MTK1
MTK1
d
Catalase inhibitor
- - - +
H2O2
0 10 60 60 (min)
Ox-MTK1
Myc-MTK1
Non-Reducing
MTK1
P-MTK1
Myc-MTK1
Reducing

Figure 8 Reduction of oxidized MTK1 is required for oxidative stress-induced MTK1 activation.

(**a**) MTK1 is oxidized quickly and its subsequent activation occurs after reduction of oxidized MTK1. HEK293A stably expressing Myc-MTK1 cells were treated with 1 mM H_2O_2. The lysates were probed with Myc antibody by non-reducing (top left panel) or reducing (top right panel) SDS-PAGE. Immunoprecipitated Myc-MTK1 was probed for its activation (middle and bottom panels).

(**b**) Endogenous MTK1 are oxidized by H_2O_2 treatment. Myc-MTK1 stably expressing HEK293A cells were treated with H_2O_2 (1 mM) for the indicated time. The lysates were probed with MTK1 antibody by non-reducing SDS-PAGE (2ME (-)) SDS-PAGE.

(**c**) Endogenous MTK1 is activated in response to oxidative stress. HEK 293A cells were treated with H_2O_2 (1 mM) for 60 minutes . Immunoprecipitated MTK1 was analyzed for its activation.

(**d**) The reduction of oxidized MTK1 is required for MTK1 activation in response to oxidative stress. Myc-MTK1 stably expressing HEK293A cells were treated with a catalase inhibitor 3-AT (0.5 mM) for 60 minutes , followed by H_2O_2 (0.5 mM) for the indicated time. The lysates were probed with Myc antibody by non-reducing SDS-PAGE (top panel). Immunoprecipitated Myc-MTK1 was probed with P-MTK1 and Myc antibody by reducing SDS-PAGE (middle and bottom panel).

4-3 Oxidation and reduction of C218 in GBD is required for oxidative stress-induced MTK1 activation.

Next, to determine the oxidized Cys residues of MTK1, I generated various deletion mutants of MTK1, and their oxidation states were monitored by Western blot analysis under non-reducing conditions. In consequence, the oxidative stress-induced band-shift of MTK1 was not observed in the MTK1 mutant lacking GADD45-binding domain (MTK1ΔGDB) (Figures 9a and Figure 9b, lanes 1-4). In general, Cys residues are targets of protein oxidation and form S-S bond between their thiols (Figure 10). Thus, I generated triple Cys-to-Ser (3C/S) mutant, which has mutations of all three Cys residues within GBD (C163, C193, and C218) (Figure 9a). Oxidative stress-induced band shift was also inhibited in MTK1 3C/S expressing cells (Figure 9b, lanes 5 and 6). However, MTK1 single C/S mutants, which were mutated each of three Cys residues to Ser, were still strongly oxidized (Figure 11a). Taken together, these results indicate that multiple Cys residues in GBD are the targets of oxidation.

To investigate the association between oxidation of MTK1 and oxidative stress-induced activation of MTK1, activation states of these MTK1 mutants were monitored by immunoblotting. As a result, both MTK1-ΔGBD and MTK1-3C/S mutants were not activated by H_2O_2 treatment (Figure 9c). However, MTK1-3C/S mutants were robustly activated by coexpressing with GADD45β (Figure 12). These results indicate that the cysteine residues in GBD are essential for oxidative stress-induced activation of MTK1 but not for GADD45-induced activation. Furthermore, whereas H_2O_2-induced activation of MTK1 was observed in C163S or C193S mutant expressing cells, MTK1 C218S mutant was not activated by H_2O_2 (Figure 11b). Therefore, the redox regulation of Cys218 in GBD is essential for MTK1 activation under oxidative stress conditions. Interestingly, Cys218 is highly conserved among MTK1 orthologues in various species of vertebrates (Figure 13). It suggests that this oxidation-mediated regulatory system of MTK1 might be conserved in diverse organisms.

Previous studies have revealed a molecular mechanism of GADD45-mediated MTK1 activation [5-7]. When GADD45 binds to GBD, the inhibitory interaction of N-terminal autoinhibitory domain and C-terminal kinase domain is dissociated. Then MTK1 dimerizes via coiled-coil domain and is activated by *trans*-autophosphorylation (Figure 2). Thus, N-C dissociation and homodimerization of MTK1 are the two essential

steps of the activation process. To investigate if the oxidation and reduction of MTK1 regulate these steps for activation, I tested whether oxidation of MTK1 induces intermolecular MTK1 interaction. Myc-MTK1 and Flag-MTK1 were transfected to HEK293A cells and stimulated with H_2O_2. As a result, Flag-MTK1 interacted with Myc-MTK1 (WT) when cells were stimulated with H_2O_2, but not with Myc-MTK1 (3C/S) (Figure 14a). These results show that at least two molecules of MTK1 interact with each other by S-S bond through their Cys residues in GBD. Next, I tested whether the inhibitory N-C interaction would be dissociated by oxidative stress. The MTK1 N-terminal (MTK1-N) and C-terminal (MTK-C) fragments were expressed as two separated molecules (Figure 14b), which can interact with each other. This interaction mimics the N-C interaction, and it can be disrupted by coexpressing of GADD45β [7] . These fragments were transfected to cells and treated with H_2O_2. As a result, the N-C interaction was not significantly changed at 10 minutes , at the timing that MTK1 is highly oxidized and remains inactive (Figure 14c, lane 4), but it was markedly decreased at 90 minutes when oxidized MTK1 is reduced and activated (Figure 14c, lane 5). These data indicate that the disruption of the inhibitory N-C interaction, which is needed for MTK1 activation, occurs when oxidized MTK1 is reduced. To investigate whether the dimerization of MTK1 via the coiled-coil domain is also required for activation of MTK1 under oxidative stress conditions, I used a dimerization-defective MTK1 mutant in which three hydrophobic residues essential for the coiled-coil-mediated helix bundle formation (L997, I1001, and V1008) were substituted by a helix-disrupting proline (hereafter 3P mutant) [7] (Figure 14b). Although MTK1-3P mutant was oxidized (Figure 14d) by H_2O_2 treatment, its activation was not observed (Figure 14e). Therefore, these results indicate that these two activation steps (i.e., the disruption of the inhibitory N-C interaction and the subsequent dimerization via the coiled-coil domain) are also required for oxidative stress-induced MTK1 activation.

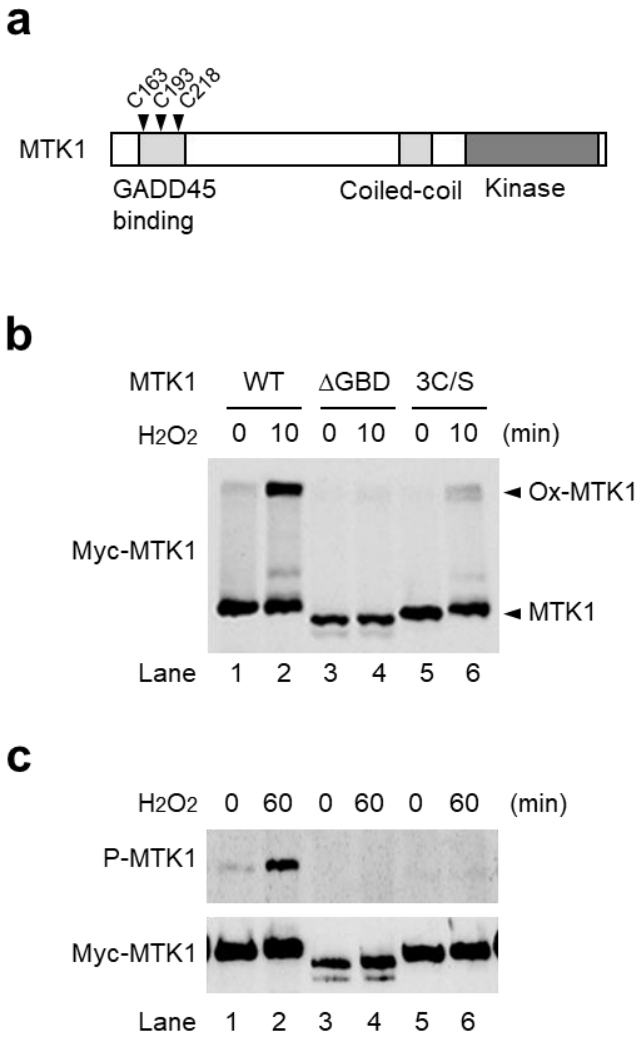

Figure 9 Cys residues in the GADD45-binding domain (GBD) are essential for oxidative stress-induced activation of MTK1.

(**a**) The summary of MTK1 structure. GADD45-bindig domain; Coiled-coil domain; Kinase domain.

(**b, c**) Oxidation of Cys residues in GBD was required for MTK1 activation. HEK293A cells stably expressing Myc-MTK1-WT, ΔGBD or 3C/S mutants, were treated with H_2O_2 (1 mM) for the indicated time. Oxidation levels of MTK1 were analyzed by immunoblotting using Myc antibody with non-reducing SDS-PAGE (**b**). Phosphorylation state of MTK1 (T1493) was assessed by immunoblotting using P-MTK1 antibody with reducing SDS-PAGE (**c**).

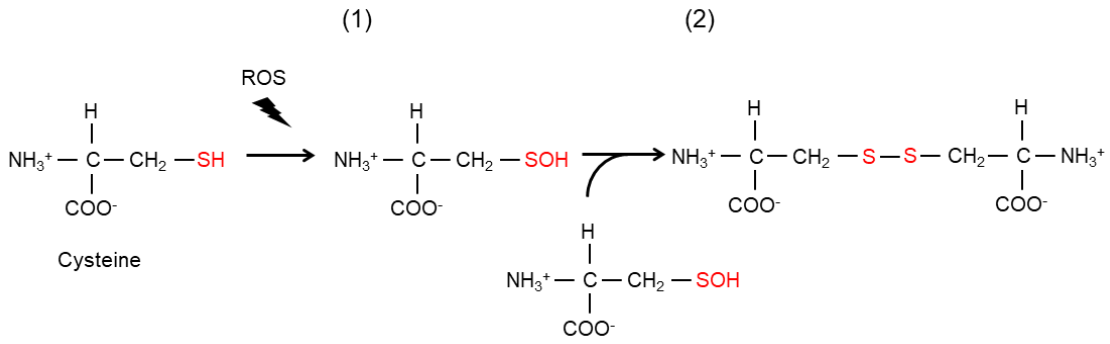

Figure 10　Thiols of Cys residues are targets of protein oxidation.

ROS oxidizes thiol of Cys residue (1), then two Cys residues form S-S bond through their oxidized thiol (2).

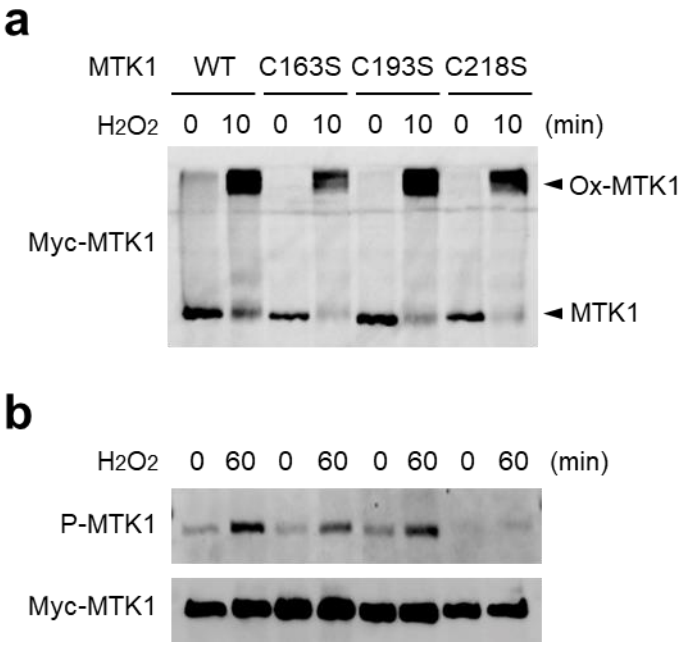

Figure 11 Cys218 is required for oxidative stress-induced activation of MTK1.

(**a, b**) The oxidation and reduction of Cys218 residue were required for MTK1 activation under oxidative stress. HEK293A cells stably expressing Myc-MTK1-WT, C163S, C193S or C218S were treated with H_2O_2 (1 mM) for the indicated time. Cell lysates were detected with Myc antibody by non-reducing SDS-PAGE (**a**). Immunoprecipitated Myc-MTK1 was probed with P-MTK1 and Myc antibody by reducing SDS-PAGE (**b**).

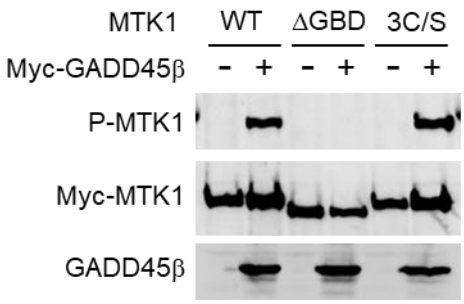

Figure 12　Cys residues in GBD are not essential for GADD45-induced activation of MTK1.

The GADD45-induced MTK1 activation was observed in MTK1-3C/S mutant, but not in ΔGBD mutant. COS7 cells were transfected with Myc-MTK1-WT, ΔGBD or 3C/S mutants together with GADD45β. Immunoprecipitated Myc-MTK1 was probed with P-MTK1 and Myc antibodies (top and middle panels). Myc-GADD45β expression levels in lysates were analyzed by immunoblotting with Myc antibody (bottom panel).

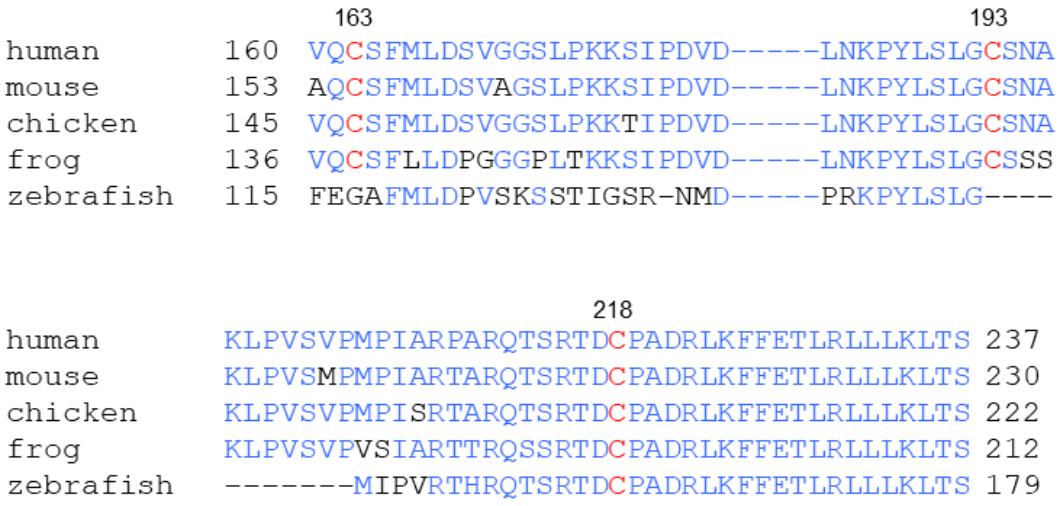

Figure 13 Cys218 in GBD is conserved in a wide range of species.

C163, C193 and C218 residues (red) in GBD of human MTK1 are conserved in various animal species such as mouse, chicken and frog. Cys218 is conserved in from human to zebrafish. Multiple alignment of MTK1 Cys residues was constructed by ClustalW.

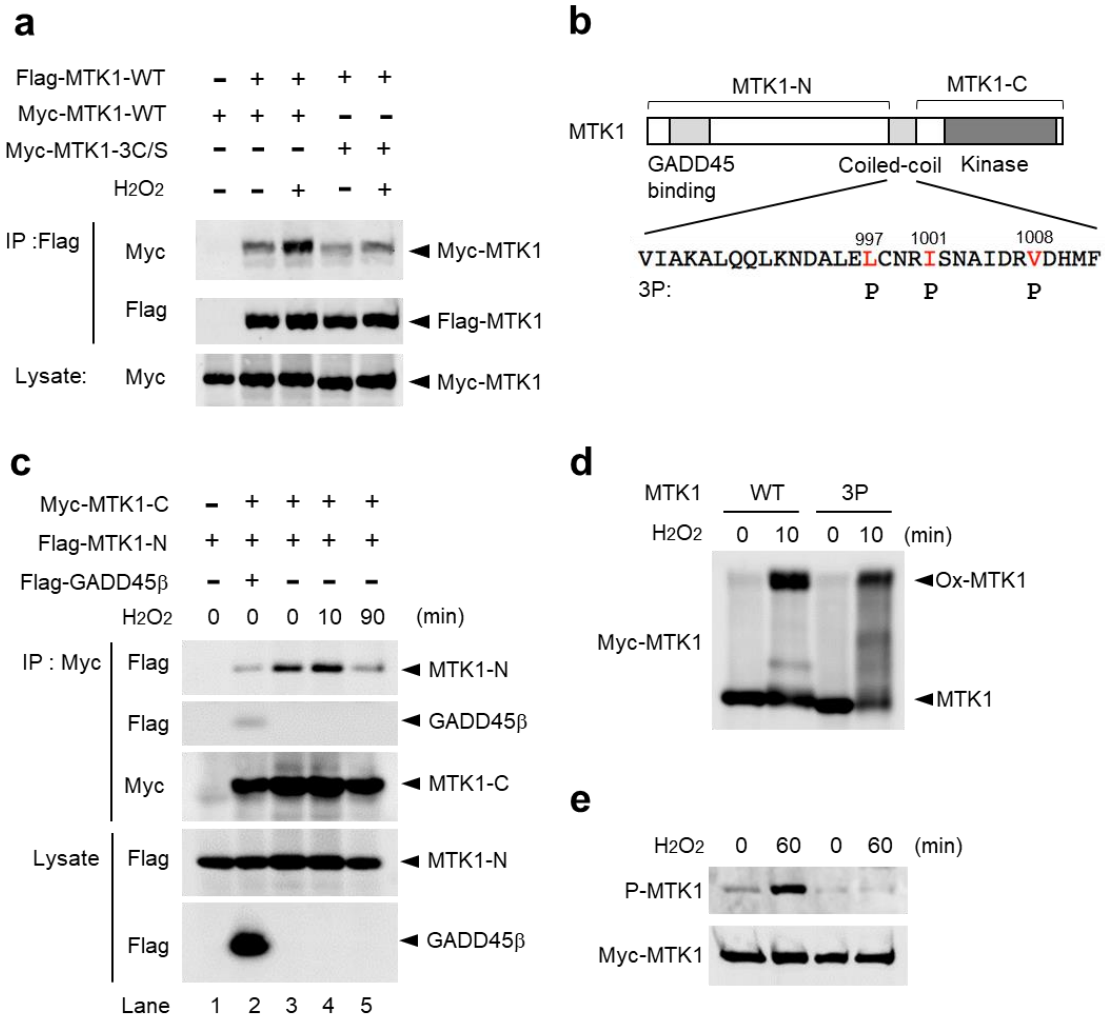
a
Flag-MTK1-WT
Myc-MTK1-WT
Myc-MTK1-3C/S
H2O2
IP :Flag
Myc
Flag
Lysate:
Myc
Myc-MTK1
Flag-MTK1
Myc-MTK1
b
MTK1-N
MTK1-C
MTK1
GADD45 binding
Coiled-coil
Kinase
997
1001
1008
VIAKALQQLKNDALELCNRISNAIDRVDHMF
3P:
P
P
P
c
Myc-MTK1-C
Flag-MTK1-N
Flag-GADD45β
H2O2
0 0 0 10 90 (min)
IP : Myc
Flag
Flag
Myc
Lysate
Flag
Flag
MTK1-N
GADD45β
MTK1-C
MTK1-N
GADD45β
Lane 1 2 3 4 5
d
MTK1
WT
3P
H2O2
0 10 0 10 (min)
Myc-MTK1
Ox-MTK1
MTK1
e
H2O2
0 60 0 60 (min)
P-MTK1
Myc-MTK1

Figure 14 MTK1 conformational change is induced when oxidized MTK1 is reduced.

(**a**) At least two molecules of MTK1 interacted with each other through their Cys residues in GBD. HEK293A cells transiently expressing Myc-MTK1-WT or 3C/S and Flag-MTK1-WT were treated with H_2O_2 (1 mM) for 10 minutes .

Immunoprecipitated Flag-MTK1 was probed for coprecipitating Myc-MTK1. (**b**) The summary of MTK1 N-terminal fragment, C-terminal fragment and 3P mutant.

(**c**) The inhibitory interaction of MTK1 N-terminal regulatory domain and C-terminal kinase domain was dissociated when oxidized MTK1 was reduced. Cos7 cells transiently expressing Myc-MTK1-C and Flag-MTK1-N, were treated with H_2O_2 (1 mM) for the indicated time. Myc-MTK1-C was immunoprecipitated, and coprecipitating Flag-MTK1-N.

(**d, e**) The dimerization of MTK1 via coiled-coil domain was required for MTK1 activation under oxidative stress. HEK293A cells stably expressing Myc-MTK1-WT or 3P mutant, were treated with H_2O_2 (1 mM) for the indicated time. Cell lysates were probed with Myc antibody by non-reducing antibody (**d**). Immunoprecipitated Myc-MTK1 was probed with P-MTK1 and Myc antibodies by reducing SDS-PAGE (**e**).

4-4 Thioredoxin (Trx) activates MTK1 under oxidative stress conditions by reducing oxidized MTK1.

Since the oxidation and subsequent reduction of MTK1 were required for activation of MTK1 under oxidative stress conditions, I tried to identify the reductant which is responsible for reduction of oxidized MTK1. Generally, S-S bonds between oxidized cysteine residues are reduced by reductants such as thioredoxin (Trx) family proteins. Therefore, I investigated whether Trx family proteins such as Trx, Nucleoredoxin (NRX) and 32 kDa thioredoxin-related protein (TRP32) could reduce the oxidized MTK1. Trx family proteins have two redox-active Cys residues in their conserved catalytic site sequence (CxxC), and the mutation of second Cys in this motif to Ser makes them to trap their specific substrates (Figure 15) [16, 27]. I performed coimmunoprecipitation assay by using these substrate-trapping mutants. As a result, MTK1 slightly binds to wild-type Flag-Trx and robustly interacted with a substrate-trapping Trx (C35S) mutant but did not bind to redox-inactive Trx (C32S/C35S) mutant when cells were treated with H_2O_2 (Figure 16a). The redox-inactive mutants neither interact with nor reduce their substrate because they have mutation at both two catalytic Cys residues. Furthermore, the oxidation-defective MTK1(3C/S) mutant did not bind to Trx even when cells were treated with H_2O_2 (Figure 16b). On the other hand, MTK1 did not interact with substrate-trapping mutant of NRX, TRP32 and wild-type (WT) Glutathione S-transferase P (GSTP1), a reductase, even when cells were treated with H_2O_2 (Figures 17a-c). Thus, these results indicate that Trx specifically interacts with oxidized form of MTK1 through the its catalytic Cys residues. Endogenous Trx is also confirmed to interact with oxidized MTK1 (Figure 18). Moreover, cells were treated with a Trx inhibitor, 2,4-dinitro-1-chlorobenzene (DNCB), which prevents recycling of oxidized Trx by inhibiting TrxR, to inhibit endogenous Trx activity. As a result, pretreatment of cells with DNCB strongly suppressed the reduction of MTK1 and enhanced the oxidation (Figure 19). These data suggest that MTK1 is a physiological substrate of Trx.

To investigate if Trx-mediated reduction of oxidized MTK1 would directly induce MTK1 activation, I performed *in vitro* kinase activation assay by using purified Trx and MTK1 proteins. For this purpose, immunopurified oxidized Myc-MTK1 from H_2O_2-treated Myc−MTK1 stably expressing cells were incubated with recombinant Trx (WT, C35S or C32S/C35S). Then, the kinase activity of MTK1 was monitored by its

ability to undergo autophosphorylation at T1493 in an *in vitro* kinase assay. Incubation with recombinant Trx induced the reduction of oxidized MTK1 (Figure 20) and promoted its kinase activity (Figures 21a and b). In contrast, the catalytically inactive Trx(C32S/C35S) and Trx(C35S) mutants failed to reduce oxidized MTK1 and did not induce MTK1 activation (Figure 20 and Figures 21a-d). Therefore, these data indicate that the reduction of oxidized MTK1 by Trx contributes to MTK1 activation.

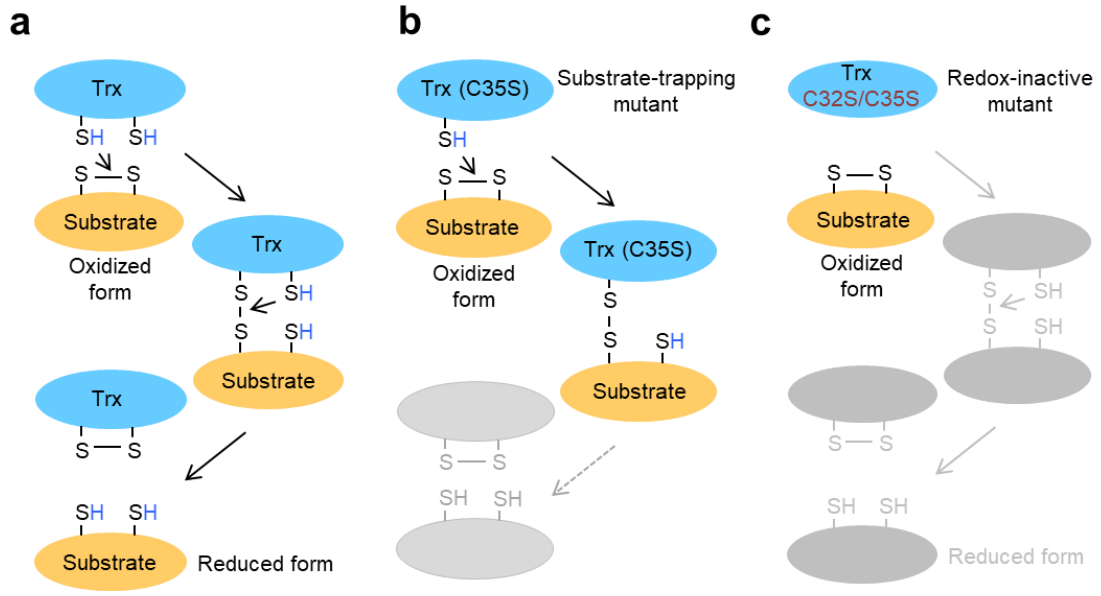

Figure 15 Summary of Trx mutants.
Trx (WT) can reduce substrate proteins through two Cys residues in its catalytic center (**a**). C35S substrate-trapping mutant can interact with substrates but cannot be released from it (**b**). C32S/C35S redox-inactive mutant cannot interact with substrates (**c**).

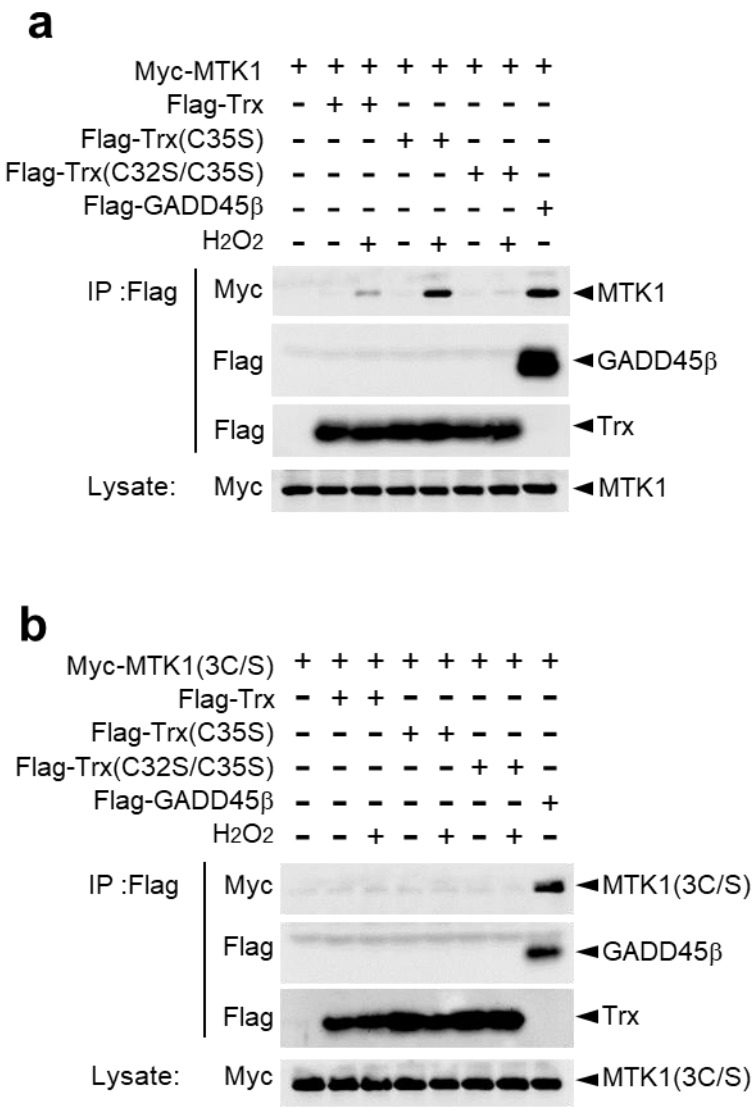

Figure 16　Trx interacts with oxidized MTK1 through Trx catalytic center cysteine.

(**a, b**) Trx preferentially bound to oxidized MTK1 under oxidative stress conditions. HEK293A cells stably expressing Myc-MTK1-WT (**a**) or 3C/S (**b**), were transfected as indicated. After cells were treated with H_2O_2 (1 mM) for 20 minutes, then Flag-Trx or Flag-GADD45β was immunoprecipitated, and coprecipitating Myc-MTK1 was probed.

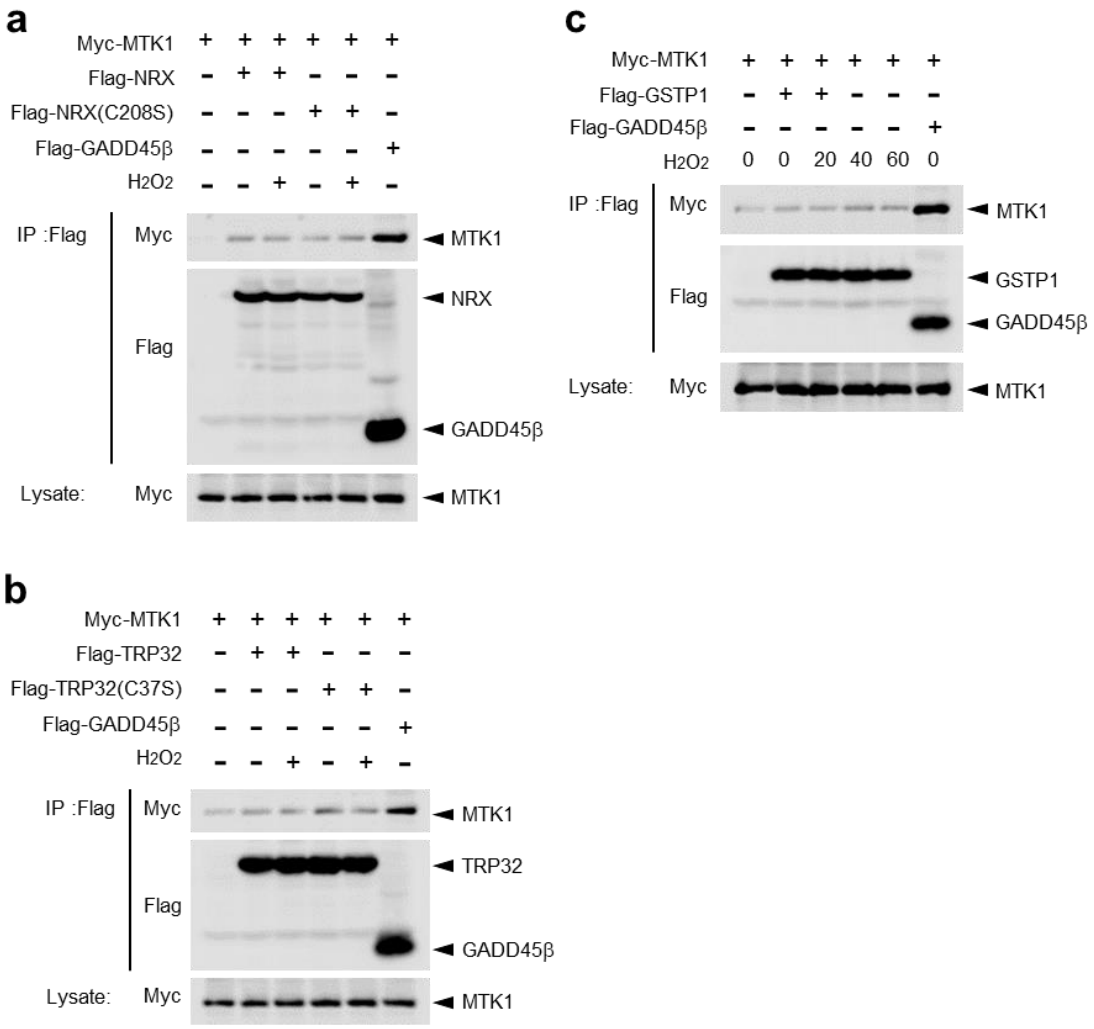

Figure 17　NRX, TRP32 and GSTP1 do not bind with oxidized MTK1.

(**a-c**) Reductants such as NRX, TRP32 and GSTP1, did not bind to oxidized MTK1. Flag-NRX (**a**), Flag-TRP32 (**b**) and Flag-GSTP1 (**c**) were transfected into Myc-MTK1-WT stably expressing HEK293A cells. After these cells were treated with H_2O_2 (1 mM) for 20 minutes, then Flag-NRX (**a**), Flag-TRP32 (**b**), Flag-GSTP1 (**c**) or Flag-GADD45β was immunoprecipitated, and coprecipitating Myc-MTK1 was probed.

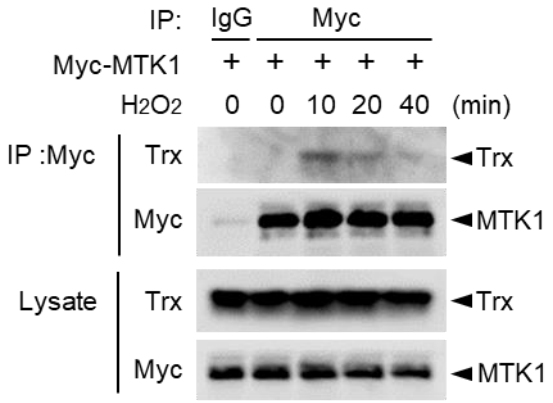

Figure 18　Endogenous Trx interacts with Myc-MTK1 in response to oxidative stress.

HEK293A cells stably expressing Myc-MTK1 were treated with H_2O_2 (1 mM) for the indicated time. Myc-MTK1 was immunoprecipitated, and coprecipitating endogenous Trx was probed.

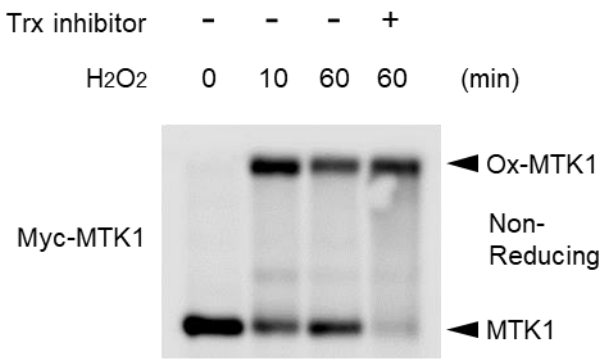

Figure 19 Trx inhibitor treatment prevents reduction of oxidized MTK1.

Trx inhibitor, 1-chloro-2,4-dinitrobenzene (DNCB), blocked the Trx-mediated reduction of oxidized MTK1. HEK293A cells stably expressing Myc-MTK1 were treated with DNCB (100 μM), followed by H_2O_2 (1 mM) for the indicated time. Cell lysates were probed with Myc antibody.

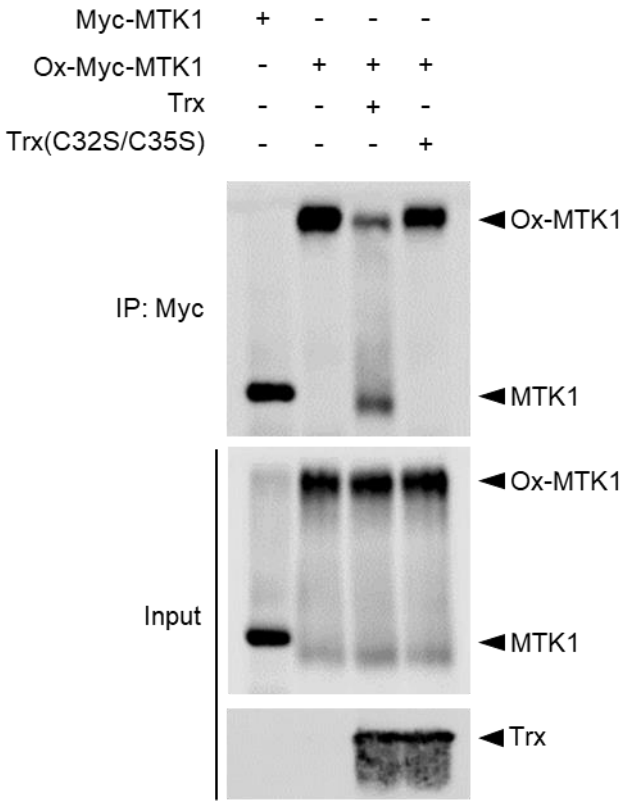

Figure 20 Trx reduces oxidized MTK1 *in vitro*.

HEK293A cells stably expressing Myc-MTK1-WT was treated with H_2O_2 (1 mM) for 10 minutes. Immunoprecipitated Myc-MTK1 was incubated with recombinant Trx at 37 ˚C for 30 minutes. Oxidation states of MTK1 were analyzed by immunoblotting with anti Myc antibody (top and middle panels). Trx expression level was analyzed by immunoblotting with Trx antibody (bottom panel).

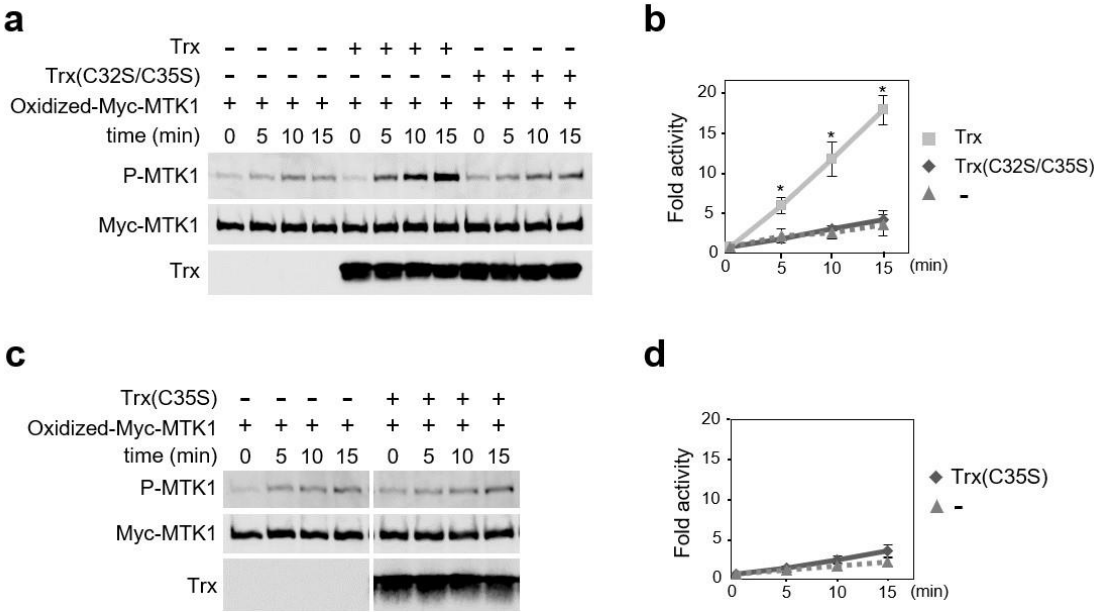

Figure 21　Trx-mediated reduction of oxidized MTK1 induces MTK1 activation *in vitro*.

(**a-d**) HEK293A cells stably expressing Myc-MTK1-WT were treated with H_2O_2 (1 mM) for 10 minutes. Immunoprecipitated Myc-MTK1 was firstly incubated with recombinant Trx-WT, C32S/C35S (**a, b**) or C35S (**c, d**) at 16 ˚C for 30 min, then incubated with ATP (200 μM) at 16 ˚C for the indicated time. Phosphorylation states of MTK1 and Trx loading levels were analyzed by immunoblotting (**a, c**). (**b, d**) All data are the means of three independent experiments. Error bars indicate s.e.m. *<p=0.05.

4-5 MTK1 contributes to delayed activation of p38/JNK under oxidative stress conditions.

Previous studies have shown that MTK1 induces p38/JNK activation in response to various stimuli which promote the expression of GADD45 family genes [6]. To investigate whether MTK1 is responsible for the activation of p38/JNK under oxidative stress conditions, I generated MTK1 knockout (KO) HEK293A cells. These cells were treated with H_2O_2 (1 mM), then SAPKs and SAPKKs activities were monitored by immunoblotting. In consequence, activation of SAPKs and SAPKKs at 120 minutes after H_2O_2 stimulation were declined in MTK1 KO cells, however their activation at 30 minutes following stimulation were not altered (Figure 22). These data suggest that MTK1 is involved in delayed activation of SAPK pathways but not early activation of SAPK pathways. Therefore, next, I attempted to identify the SAPKKK which is responsible for early-phase activation of SAPK pathways (~30 minutes) under oxidative stress conditions. Since ASK1, a SAPKKK, is well known to be activated in response to oxidative stress [21, 28], I generated ASK1 KO cells. As a consequence, ASK1 KO cells showed decreased p38/JNK signals at 30 minutes after H_2O_2 stimulation but not at 120 minutes (Figure 23a). Furthermore, I established MTK1/ ASK1 double KO cells and monitored p38/ JNK activation. As a result, activation of p38 and JNK were declined in MTK1/ASK1 KO cells both 30 minutes and at 120 minutes (Figure 24). These results indicate that ASK1 is responsible for early-phase activation of p38/ JNK, and MTK1 contributes to delayed-phase activation of p38/JNK. Interestingly, a lower concentration of H_2O_2 (0.1 mM) induced only short term-activation of p38/JNK (~30 minutes) after stimulation. In this condition, phosphorylation states of p38/JNK (~30 minutes) were declined in ASK1 KO cells (Figure 23b). Therefore, a brief period of SAPK activation which is caused by a low concentration of H_2O_2 is induced mainly by ASK1.

Previous studies have shown that activation of p38/JNK is involved in apoptosis or inflammatory responses [1]. To investigate if MTK1 regulates oxidative stress-induced cell death, I performed 3-(4, 5-Dimethylthial-2-yl)-2, 5-Diphenyltetrazalium Bromide (MTT) cell viability assay. HEK293A WT cells, MTK1 KO cells and Myc-MTK1 re-expressing MTK1 KO cells were treated with H_2O_2 for 6 hours. As a result, I found that the ratio of viable cells was higher in MTK1 KO cells compared with HEK 293A WT cells and Myc-MTK1 re-expressing MTK1 KO cells

(Figure 25). This result shows that MTK1 is involved in oxidative stress-induced cell death.

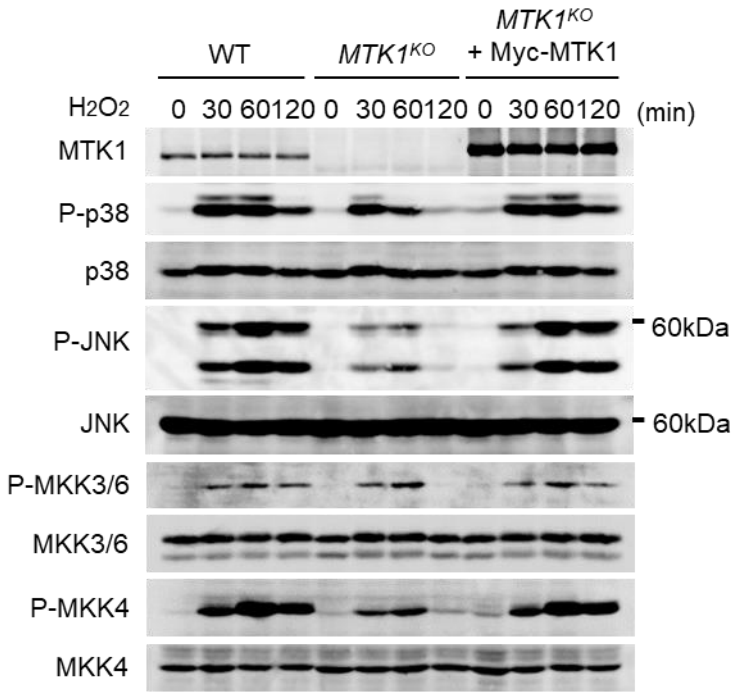

Figure 22 MTK1 contributes to delayed p38/JNK activation in response to oxidative stress.

Parental HEK293A cells (WT), MTK1 knock-out (KO) HEK293A cells and Myc-MTK-WT re-expressing MTK1 KO HEK293A cells were treated with H_2O_2 (1 mM) for the indicated time. Phosphorylation levels of p38 and JNK were analyzed by immunoblotting.

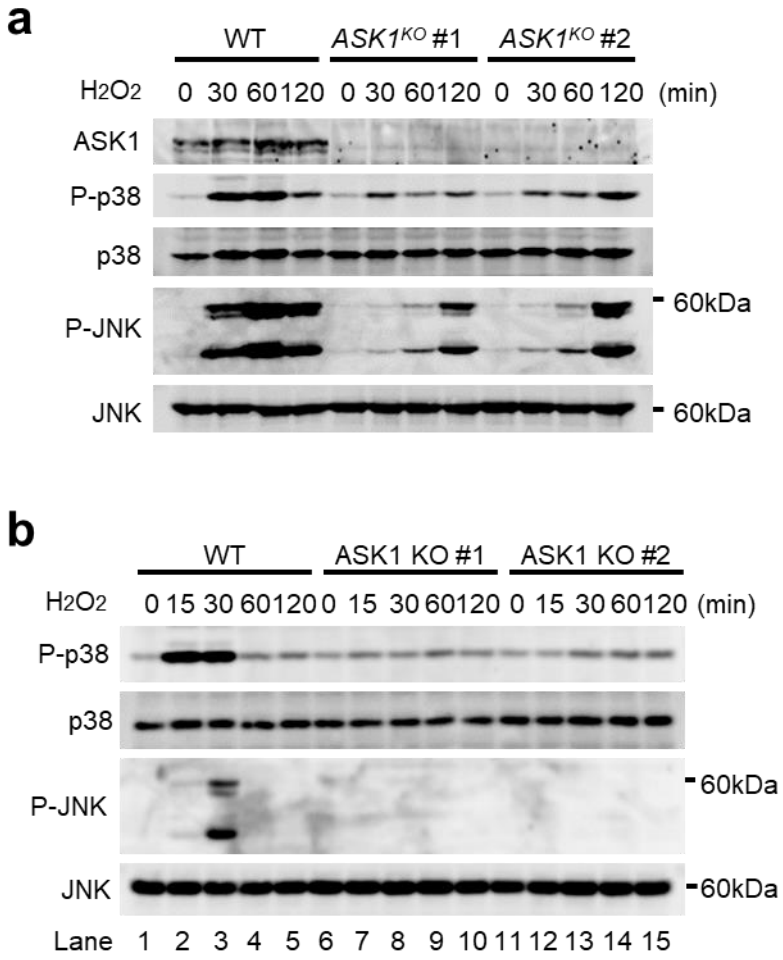

Figure 23 ASK1 induces p38/JNK activation in early-phase in response to both high and low concentrations of H_2O_2.

(**a**) ASK1 induced early SAPK activation in response to oxidative stress. WT HEK293A cells, ASK1 KO #1 and #2 HEK293A cells were treated with H_2O_2 (1 mM) for the indicated time. Phosphorylation levels of p38 and JNK were analyzed by immunoblotting.

(**b**) A low concentration of H_2O_2-induced p38/JNK activation was mainly induced by ASK1. WT HEK293A cells, ASK1 KO #1 and #2 HEK293A cells were treated with H_2O_2 (0.1 mM) for the indicated time. Phosphorylation levels of p38 and JNK were analyzed by immunoblotting.

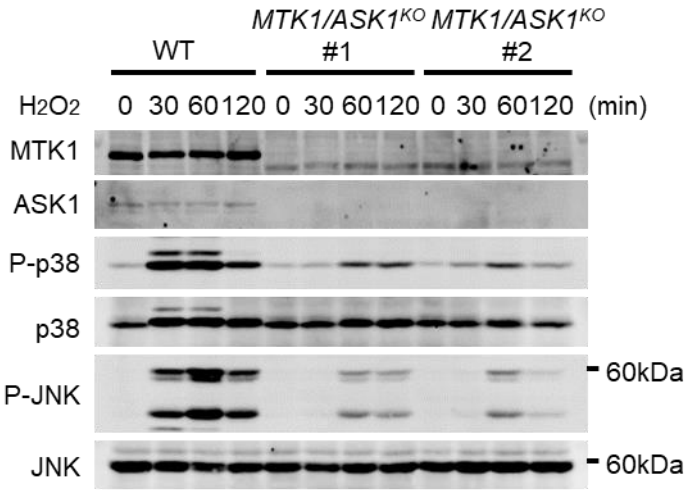

Figure 24　The oxidative stress-induced SAPK activation is blocked in MTK1/ASK1 double KO HEK293A cells.

HEK293A WT, MTK1/ASK1 double KO #1 and #2 cells were treated with H_2O_2 (1 mM) for the indicated time. Phosphorylation levels of p38 and JNK were analyzed by immunoblotting.

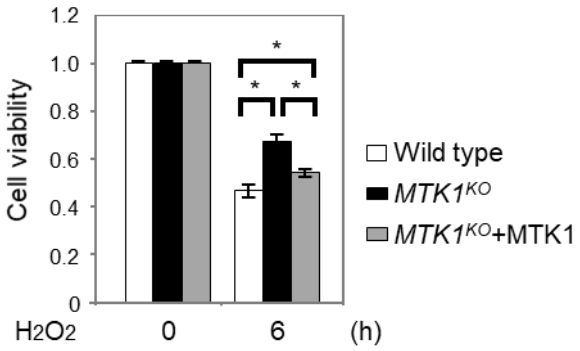

Figure 25 MTK1 is involved in the H_2O_2-induced cell death.

HEK293A WT, MTK1 KO cells and MTK1 KO cells re-expressing Myc-MTK1 were treated with H_2O_2 (0.5 mM) for the indicated time. Cell numbers were counted by MTT assay.

Macrophages are known to generate ROS during phagocytosis [29]. Therefore, to investigate if oxidative stress-induced activation of MTK1 contributes to inflammatory responses in macrophage, THP-1 human monocyte cells were differentiated to macrophages. The macrophages were treated with H_2O_2 and MTK1 oxidation was monitored by immunoblotting in non-reducing conditions. As a result, MTK1 was oxidized in response to H_2O_2 stimulation in THP-1 macrophage (Figure 26).

These data suggest that MTK1 also functions as a redox sensor in THP-1 macrophage. We next generated MTK1 knock down (KD) THP-1 cells by stably expressing shRNA against human MTK1 (Figure 27a). These cells also showed a decrease in p38 activity at 120 min after H_2O_2 stimulation (Figure 27b). These data indicate that MTK1 induces delayed activation of SAPK in THP-1 macrophage in response to oxidative stress.

Furthermore, to investigate the physiological role of oxidative stress-induced MTK1 activation in THP-1 macrophages, the control and MTK1-KD macrophages were stimulated for 2-6 h with zymosan, which contains cell wall components of *Saccharomyces cerevisiae* and mimics fungal infection. mRNAs were then purified from the macrophages and analyzed for cytokine expression by quantitative real-time PCR. In control macrophages, zymosan treatment rapidly induced TNFα mRNA expression with a peak at 2-hours post-stimulation (Figure 28b), whereas IL-6 mRNA expression increased more slowly (Figure 28a). These findings suggest that these two cytokines have different responsiveness to zymosan. Furthermore, compared with control macrophages, MTK1 KD macrophages showed markedly declined IL-6 gene expression at 6-hours after zymosan stimulation. In contrast, there was no significant difference in the level of TNFα mRNA expression between control and MTK1-KD macrophages (Figures 28a and b). These data show that MTK1 is important for zymosan-induced, delayed IL-6 expression, but not for early TNFα expression. I confirmed that zymosan stimulation induced a comparable level of ROS production both in control (Figure 29a) and MTK1 KD macrophages (Figure 29b). Zymosan-induced expression of IL-6 mRNA was profoundly inhibited by pretreatment with N-acetyl cysteine (NAC), an anti-oxidant regent (Figure 30), confirming that zymosan-induced ROS was involved in IL-6 gene expression. Zymosan-induced IL-6 gene expression was also suppressed by pretreatment with SAPK inhibitors (SB239063 and SP600125) (Figure 31). These data indicate that MTK1 is involved in IL-6 production in response to phagocytosis and phagocytosis-induced oxidative stress.

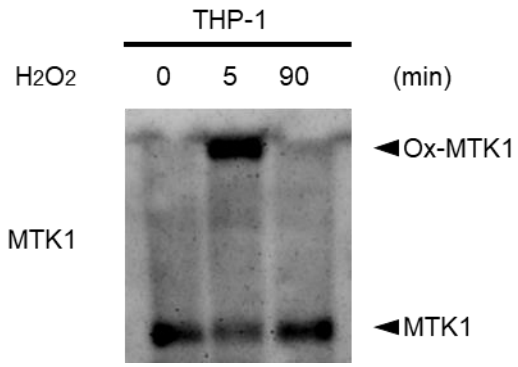

Figure 26　MTK1 is oxidized in THP-1 derived macrophages.

MTK1 was oxidized in THP-1 cells. THP-1 cells were differentiated into macrophages by treatment with TPA (80 nM) for 24 hours. Macrophages were treated with H_2O_2 (1 mM) for the indicated time. Oxidation of MTK1 was monitored by using non-reducing SDS-PAGE with anti-MTK1 antibody.

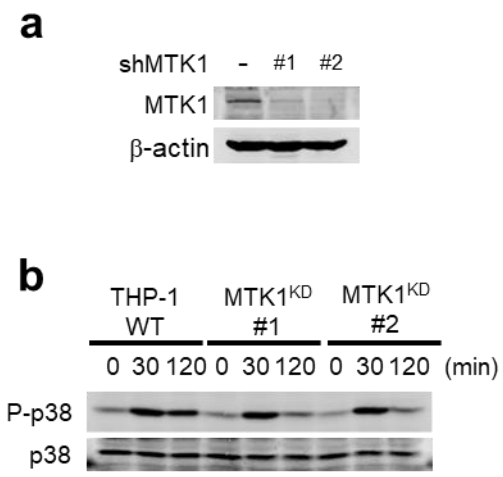

Figure 27　MTK1 contributes delayed phase-activation of p38 in THP-1 derived macrophage.

(**a**) MTK1 KD THP1 cells were generated by stably expressing shRNAs against human MTK1. Expression levels of MTK1 were detected by immunoblotting with MTK1 antibody. β-actin was loading control.

(**b**) H_2O_2 stimulation induced p38 activation. THP-1 cells were differentiated into macrophage by treatment with TPA (80 nM) for 24 hours. Macrophages were treated with H_2O_2 (1 mM) for the indicated time. Phosphorylation levels of p38 were analyzed by immunoblotting.

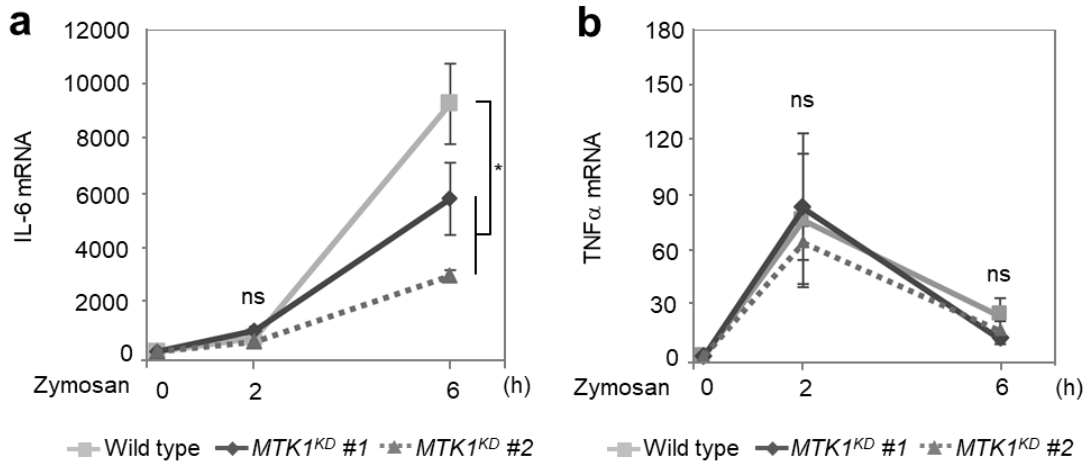

Figure 28 MTK1 contributes zymosan-induced IL-6 mRNA expression.

(**a, b**) mRNA expression levels of IL-6 (**a**) and TNFα (**b**) was declined in MTK1 KD THP-1 cells after zymosan stimulation. THP-1 parental cells and MTK1 KD THP-1 cells were derived into macrophage by pre-treatment of TPA (80 nM) for 24 hours. These cells were stimulated with zymosan (0.2 mg/ml) for the indicated time. Expression levels of IL-6 mRNA and TNFα were monitored by qRT-PCR. All data are the means of three independent experiments. Error bars indicate s.e.m. *<p=0.05.

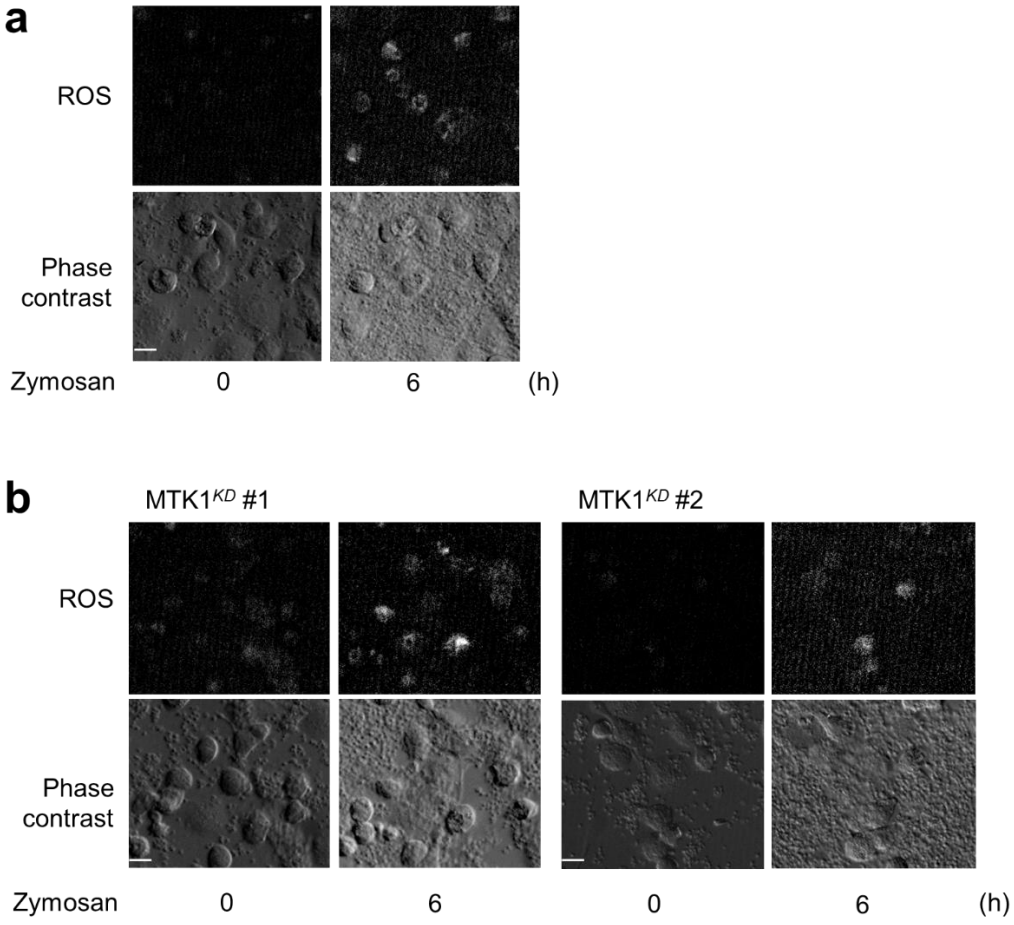

Figure 29 Zymosan induced ROS production in THP-1 macrophages.

Zymosan induced ROS production both in THP-1 parental (**a**) and MTK1 KD cells (**b**). THP-1 parental cells and MTK1 KD cells were treated with zymosan (0.2 mg/ml) for 6 hours. ROS levels were monitored by fluorescent ROS probe, CellROX Green. Scale bar, 30 μm.

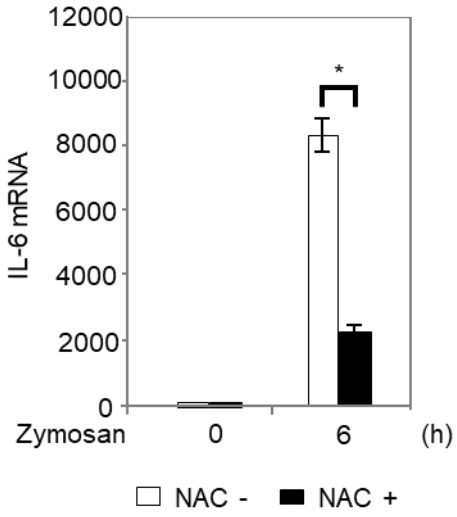

Figure 30　Zymosan-induced oxidative stress contributes to IL-6 mRNA production in THP-1 macrophages.

Zymosan-induced expression of IL-6 mRNA was suppressed by NAC treatment. THP-1 parental cells were derived into macrophage by pretreatment of TPA (80 nM) for 24 hours. These cells were pretreated with NAC (5 mM) for 20 minutes then stimulated with zymosan (0.2 mg/ml) for the indicated time. Expression levels of IL-6 mRNA was monitored by qRT-PCR. All data are the means of three independent experiments. Error bars indicate s.e.m. *<p=0.05.

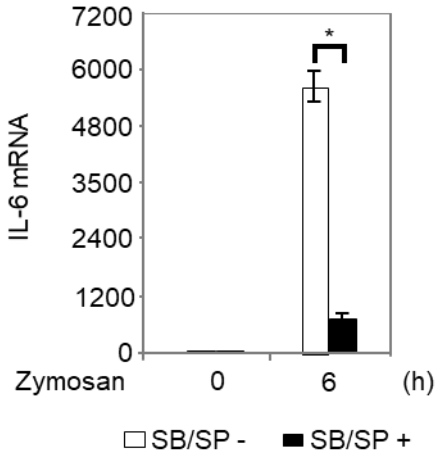

Figure 31 SAPKs contribute zymosan-induced IL-6 mRNA expression.

mRNA expression levels of IL-6 were suppressed by SB/SP treatment. THP-1 parental cells were differentiated into macrophages by pre-treatment of TPA (80 nM) for 24 hours. These cells were treated with SB239063 (20 μM) and SP600125 (60 μM) for 1 hour, then treated with zymosan (0.2 mg/ml) for the indicated time. Expression levels of IL-6 mRNA were monitored by qRT-PCR. All data are the means of three independent experiments. Error bars indicate s.e.m. *<p=0.05.

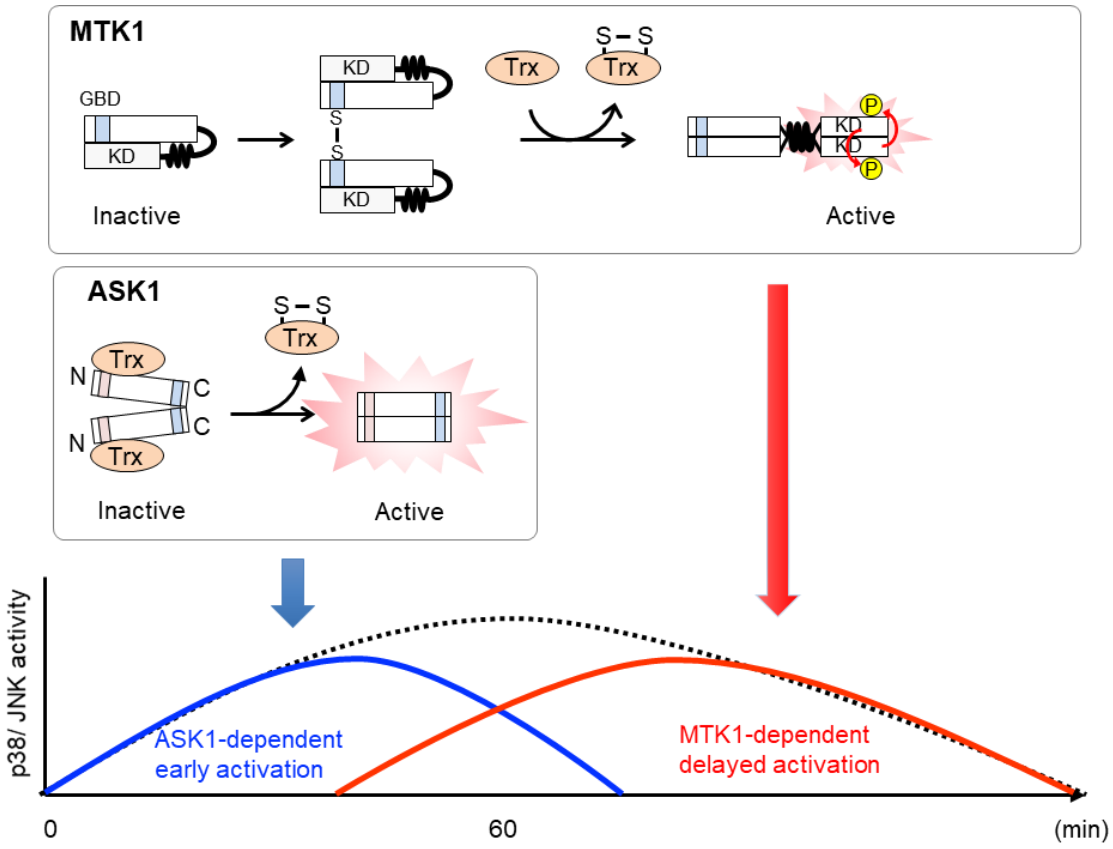

Figure 32　Schematic model of oxidative stress-induced activation of MTK1, ASK1, p38 and JNK.

The oxidative stress-induced activation of MTK1 needs not only oxidation but also subsequent reduction of oxidized MTK1 (top panel). In contrast, ASK1 is activated by dissociation of Trx in response to oxidative stress (middle panel). ASK1 induces early phase-activation of p38/JNK and MTK1 contributes to delayed phase-activation of p38/JNK under oxidative stress conditions (bottom panel).

5. Discussion

In this study, I found that MTK1 functioned as a redox sensor and was responsible for SAPK activation in the delayed phase after H_2O_2 stimulation. Under oxidative stress conditions, MTK1 is rapidly oxidized at Cys residues in GBD, then Trx binds to this oxidized MTK1 through its catalytic Cys residues and reduces MTK1. Reduction of oxidized MTK1 induces dissociation of the N-terminal autoinhibitory domain from the C-terminal kinase domain of MTK1. This conformational change of MTK1 enables dimerization of MTK1 kinase domain through the coiled-coil region and induces *trans*-autoactivation of MTK1 (Figure 32, top). From these results, I concluded that GBD of MTK1 not only interacts with its activator GADD45 but also functions as a redox-sensor domain to induce its kinase activity. Furthermore, oxidative stress-induced MTK1 activation is responsible for the delayed activation of SAPK pathways following H_2O_2 stimulation. On the other hand, ASK1 KO cells showed decreases in SAPK activation in the early-phase response against both high and low level of oxidative stress. Since the activation of SAPK in response to a low concentration of H_2O_2 scarcely continued after 30 minutes post-stimulation, ASK1 mainly regulates SAPK pathways at a low concentration of oxidative stress stimulation. Previous data also showed that the oxidative stress-induced activation of SAPKs was inhibited until 60 minutes after H_2O_2 stimulation in TRAF6, which is an activator of ASK1, depleted cells but the activation of SAPKs was observed after 90 minutes following H_2O_2 stimulation even in TRAF6 KO cells [30]. These data suggest that (an)other SAPKKK(s) is/are possibly related with oxidative stress-induced delayed phase activation of SAPKs.

Previous studies indicated the molecular mechanism of ASK1 activation in response to oxidative stress [21]. ASK1 has two coiled-coil domains at the N-terminal region (NCC) and the C-terminal region (CCC). The kinase domain of ASK1 is located between these two coiled-coil regions [19, 20]. But the homodimerization of ASK1 kinase domain is inhibited by interaction with Trx [21, 22]. Under oxidative stress conditions, Trx is oxidized and dissociates from ASK1. This dissociation enables the dimerization of ASK1 kinase domain and induces ASK1 activation (Figure 32, middle) [20]. Therefore, ASK1 activation is occurred via one step (Trx oxidation) but MTK1 needs two steps (MTK1 oxidation and reduction). These distinctions of activating mechanisms between

MTK1 and ASK1 are considered to cause different responsiveness to oxidative stress (Figure 32, bottom).

Previous studies have shown that some signaling molecules function as redox sensors, such as MAPK phosphatases (MKPs) [31], protein tyrosine phosphatases (PTPs) [32] and phosphatase and tensin homolog (PTEN) [33]. These molecules are directly oxidized at catalytic Cys residues, thereby leading to decrease in their phosphatase activities. When the oxidized and inactive phosphatases are reduced by Trx, the reduced phosphatases gain them phosphatase activities again. For instance, a Cys residue in PTPs signature motif in MKPs is oxidized directly, resulting in inhibiting MKP phosphatase activity. MKPs are negative regulators of p38/JNK, thus, the inhibition of MKP by oxidative stress induces sustained activation of these SAPKs, thereby resulting in both apoptotic and necrotic cell death [31]. Since weak phosphorylation of p38 and JNK are still observed in MTK1/ASK1 double KO cells (Figure 24), MKPs may contribute to SAPK activation in response to oxidative stress. Moreover, other SAPKKKs are also possibly involved in the SAPK activation.

Oxidative stress is known to induce both apoptotic and necrotic cell death. Previous studies showed that sustained activation of p38/JNK contributed to oxidative stress-induced cell death [31]. In this study, MTT cell viability assay showed that the oxidative stress-induced cell death was suppressed in MTK1 KO cells. Therefore, MTK1 activation under oxidative stress conditions might be involved in both apoptotic and necrotic cell death.

Zymosan is one of the components of pathogen-associated molecular patterns (PAMPs). PAMPs are recognized through Toll-like receptor, which induces ROS by mitochondrial NADPH oxidase. These ROS are essential for immune responses by innate and adaptive immune cells. Hence macrophages induce productions of ROS and pro-inflammatory cytokines (IL-1β, TNFα and IL-6 *etc.*) to eliminate pathogens and to activate other immune cells. IL-6 production is known to be induced by infections and tissue injuries. IL-6 transduces its signal via IL-6 receptor and induces inflammatory responses or monocyte recruitment to inflammation sites [34]. In this study, I found that mRNA expression levels of IL-6 from macrophages in response to zymosan stimulation were declined in MTK1 KD cells (Figure 28). I also observed that zymosan-induced ROS and SAPK activation were involved in the IL-6 mRNA expression (Figures 30 and

31). Interestingly, previous studies reported that ASK1 is required for lipopolysaccharide (LPS)-induced cytokine production [35]. LPS is a component of PAMPs and induces ROS production. Therefore, MTK1 and ASK1 may also function coordinately in innate immune responses. IL-6 is also known to be involved in various inflammatory diseases, for example, Crohn's disease, rheumatoid arthritis [36] and atherosclerosis [37]. Especially, foam cell macrophage-secreted IL-6 in atherosclerotic plaques is involved in pro-atherosclerotic responses [37]. MTK1 contributes IL-6 production from macrophages in response to zymosan stimulation. Therefore, MTK1 might be a new therapeutic target for immune diseases.

6. Conclusion

In this study, MTK1 was identified as a novel redox sensor. Under oxidative stress conditions, MTK1 activity was regulated by direct oxidation of Cys residues in GBD and subsequent Trx-mediated reduction. The reduction of oxidized MTK1 induces conformational change and dimerization of MTK1, then MTK1 is activated by *trans*-autophosphorylation at T1493. Oxidative stress-induced activation of MTK1 contributes to delayed activation of p38/ JNK SAPK pathways. In contrast, another stress responsive MAPKKK, ASK1 is involved in the activation of p38/ JNK in early-phase following oxidative stress stimulation. Thus, MTK1 activation upon oxidative stress requires multi-steps, which is the reason why MTK1 activation is slower than that of ASK1. From these results, I propose the following model. Under oxidative stress conditions, ASK1 is firstly activated and induces the earlier activation of p38/ JNK. Then MTK1 is activated and leads to delayed activation of p38/ JNK. These findings show that MTK1 and ASK1 coordinately function in response to oxidative stress and regulate cell death and IL-6 gene expression.

7. Materials and Methods

7-1 Media and buffers

Lysis buffer A:

20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM dithiothreitol and 0.5% deoxycholate.

Lysis buffer B:

20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 25 mM N-ethylmaleimide, 1.5 mM indole-3-acetic acid and 0.5% deoxycholate.

Lysis buffer C:

20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 25 mM N-ethylmaleimide, 1.5 mM indole-3-acetic acid

Lysis buffer D:

20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 0.5% Nonidet P-40, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 25 mM N-ethylmaleimide, 1.5 mM indole-3-acetic acid

Lysis buffer E:

20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin.

IP wash buffer A:

20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol.

IP wash buffer B:
20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF and 1 mM phenylmethylsulfonyl fluoride.

IP wash buffer C:
20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 0.5% Nonidet P-40, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF and 1 mM phenylmethylsulfonyl fluoride.

IP wash buffer D:
20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 2 mM EDTA, 10% (vol/vol) glycerol, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium vanadate, 10 mM NaF and 1 mM phenylmethylsulfonyl fluoride.

Kinase wash buffer:
25 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.25 mM sodium vanadate and 4 mM EGTA.

Kinase reaction buffer:
25 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 10 mM β-glycerophosphate and 12.5 mM sodium vanadate.

Sodium dodecyl sulfate (SDS) loading buffer A:
65 mM Tris-HCl (pH6.8), 2% SDS, 0.1% bromophenol blue, 5% (vol/vol) 2-mercaptoethanol and 10% glycerol.

Sodium dodecyl sulfate (SDS) loading buffer B:
65 mM Tris-HCl (pH6.8), 2% SDS, 0.1% bromophenol blue and 10% glycerol.

GST buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM, phenylmethylsulfonyl fluoride and 5 μg/mL aprotinin.

PreScission buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1 mM EDTA.

7-2 Plasmids

The mammalian expression vector pDON5Myc was used to generate Myc-MTK1-WT and Myc-MTK1-ΔGBD. MTK1 3C/S, C163S, C193S, C218S mutants, MTK1-N terminal fragment, C terminal fragment, Trx C32S/C35S, C35S mutants, NRX C208S or TRP32 C37S were generated by PCR mutagenesis. pcDNA4Myc and pQCXIP4Myc vectors were used to generate Myc-tagged MTK1-3C/S, C163S, C193S, C218S mutants, MTK1-C and GADD45β. pcDNA3Flag vector was used to generate Flag-tagged MTK1-N, GADD45β, Trx WT, C32S/C35S, C35S mutants, NRX WT, C208S mutant, TRP32 WT and C37S mutant. pSpCas9(BB)-2A-Puro vector was used to generate MTK1 knockout, ASK1 knockout construct. pQCXIH4Myc vector was used for generating Myc-MTK1 re-expressing construct. pGEX-GST vector was used for generating recombinant Trx-WT, C32S/C35S and C35S. pSUPER.retro.puro vector was used to generate shMTK1 constructs.

7-3 Cells

HEK293A parental cell, HEK293A cells stably expressing Myc-MTK1-WT, ΔGBD, 3C/S, C163S, C193S and C218S mutants were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, L-glutamate, penicillin and streptomycin. THP-1 and cells stably expressing shMTK1 were maintained in RPMI 1640 medium with 20% fetal bovine serum and L-glutamate. THP-1 was provided by RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan.

Cells stably expressing MTK1 (wild-type and its mutant derivatives) were generated by retroviral infection. GP2 293 packaging cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and L-glutamate. GP2 293 cells cultured in 60-mm dishes were transfected with the appropriate expression plasmids and pVSV-G envelope vector by using X-tremeGENE 9 (Roche). HEK293A cells were infected with the packaged retrovirus and selected with neomycin for Myc-MTK1-WT and Myc-MTK1-ΔGBD, puromycin for Myc-MTK1-3C/S, Myc-MTK1-C163S, Myc-MTK1-C193S and Myc-MTK1-C218S, respectively.

The expression levels of the infected proteins in stably expressing cells were confirmed by western blotting analysis.

7-4 Macrophage derived from THP-1

THP-1 cells were treated with TPA (80 nM) for 24 hours in RPMI 1640 medium with L-glutamate and without fetal bovine serum, then incubated in RPMI 1640 medium with 10% fetal bovine serum and L-glutamate for 24 hours.

7-5 Transient transfection

Cells cultured in 60-mm dishes were transfected with the appropriate expression plasmids using X-tremeGENE 9 (Roche). The total amount of plasmid DNA was adjusted to 1 μg per plate with vector (pcDNA3).

7-6 Western blotting analyses

The following antibodies were used for western blotting analysis: anti-cMyc monoclonal antibody (mAb) 9E10 (Santa Cruz), anti-Flag mAb M2 (Sigma), anti-phospho-p38, anti-phospho-JNK, anti-JNK, anti-phospho-MKK3/6, anti-phospho-MKK4 (CST), anti-p38, anti-MKK3/6, anti-MKK4, anti-ASK1 (Santa Cruz) and anti-MTK1 (Santa Cruz or made in house [4]). An anti-phospho-T1493 rabbit polyclonal antibody has been made in house [7].

7-7 Knockout cells

MTK1 knockout, ASK1 knockout and MTK1/ASK1 knockout cells were generated by CRISPR/Cas9 system by using pSpCas9(BB)-2A-Puro. Single guide RNAs (sgRNAs) were designed by using CRISPRdirect (http://crispr.dbcls.jp/).
Following sgRNA primer were used:

MTK1 KO	Forward	5' CACCGTCGGGTTCTGACTCGGTCTC 3'
MTK1 KO	Reverse	5' AAACGAGACCGAGTCAGAACCCGAC 3'
ASK1 KO #1	Forward	5' CACCGTGCCCCTGGCATCGGTTGTC 3'
ASK1 KO #1	Reverse	5' AAACGACAACCGATGCCAGGGGCAC 3'
ASK1 KO #2	Forward	5' CACCGCGGGGGCAGCCGACGGACCA 3'
ASK1 KO #2	Reverse	5' AAACTGGTCCGTCGGCTGCCCCCGC 3'

HEK293A cells cultured in 35-mm dishes were transfected with the appropriate expression plasmids using X-tremeGENE 9 (Roche). The total amount of plasmid DNA was adjusted to 1 μg per plate with vector. Knockout cells were subcloned by limiting dilution. MTK1/ASK1 knockout cells were generated by transfection of ASK1 knockout sgRNA to subcloned MTK1 knockout cells.

7-8 Expression and purification of recombinant Trx

pGEX-Trx-WT, C32S/C35S and C35S were transformed into *E. coli* DH5α. *E. coli* cells containing pGEX-Trx plasmids were pre-cultured in 25 mL LB medium containing 100 μg/mL Ampicillin at 37 ˚C for overnight. Overnight cultures were transferred 225 mL fresh LB medium and cultured at 37 ˚C for 3 hours. After that, isopropyl-β-D(-)-thiogalactopyranoside (IPTG) was added to a final concentration 0.1 M and cultured at 30 ˚C for 2 hours. Cells were harvested by centrifuge and re-suspended with GST buffer. Cells were disrupted by sonication and added Triton-X100 to a final concentration 1%. Cell lysates were incubated with Glutathione Sepharose 4B (GSH beads) (GE Healthcare) at 4 ˚C for overnight. Recombinant protein conjugated GSH beads were washed with ice-cold TBST once then washed with ice-cold TBS twice. PreScission protease (16 U) was added to recombinant protein conjugated GST beads in PreScission buffer and incubated at 4 ˚C for overnight. The supernatant was reduced with DTT (2 mM) for 30 minutes on ice, then dialyzed with Micro Float-A-Lyzer (Spectrum) at 4 ˚C for overnight. The dialyzed liquid was concentrated by using Vivaspin 500 (Sartorius). The concentrate was used as reduced Trx recombinant protein for experiments.

7-9 Detection of MTK1 Thr-1493 phosphorylation

Cell lysates were prepared in lysis buffer A. The lysates were incubated with anti-cMyc 9E10 for 2 hours or longer at 4 ˚C to precipitate Myc-MTK1. Immune complexes were recovered with the aid of protein G-Sepharose beads and washed three times with lysis buffer A without aprotinin and leupeptin. Then resuspended in SDS loading buffer A and separated by SDS-polyacrylamide gel electrophoresis (PAGE) for western blotting analyses with P-T1493. Endogenous MTK1 was precipitated with anti-MTK1 for overnight at 4 ˚C and immune complex were recovered with the aid of protein A Sepharose beads.

7-10 Detection of oxidized MTK1 band-shift

Cell lysates were prepared in lysis buffer B, mixed with SDS loading buffer B, and separated by SDS-PAGE for western blotting analyses with anti-Myc or anti-MTK1.

7-11 RNA extraction and quantitative real-time PCR

Total RNA was extracted by using TRIzol and 0.5 μg total RNA was reverse transcribed using Prime Script-RT Master Mix (TaKaRa). Quantitative real-time PCR was performed in Dice Real Time TP800 by using Thunderbird SYBR qPCR Mix (TOYOBO). The following primer sequences were used:

GADD45α	Forward	5' AGAGCAGAAGACCGAAAGGATGGA 3'
GADD45α	Reverse	5' GCAGGATGTTGATGTCGTTCTCGC 3'
GADD45β	Forward	5' ATTGCAACATGACGCTGGAAGAGC 3'
GADD45β	Reverse	5' GGATGAGCGTGAAGTGGATT 3'
GADD45γ	Forward	5' GACACAGTTCCGGAAAGCAC 3'
GADD45γ	Reverse	5' TCAAGACTTTGGCTGACTCG 3'
IL-6	Forward	5' AAAGAGGCACTGGCAGAAAA 3'
IL-6	Reverse	5' TTTCACCAGGCAAGTCTCCT 3'
TNF-α	Forward	5' CGAGTGACAAGCCTGTAGC 3'
TNF-α	Reverse	5' GGTGTGGGTGAGGAGCACAT 3'
GAPDH	Forward	5' ACCCACTCCTCCACCTTTGA 3'
GAPDH	Reverse	5' CTGTTGCTGTAGCCAAATTCGT 3'
ACTB	Forward	5' TCCCTGGAGAAGAGCTACGA 3'
ACTB	Reverse	5' TGAAGGTAGTTTCGTGGATGC 3'

7-12 Coimmunoprecipitation assay for protein binding

Cell lysates were prepared in lysis buffer C or D, immunoprecipitated with antibody-conjugated protein A or G Sepharose for 4 hours at 4 ˚C and washed three times with 5 minutes intervals with wash buffer B or C. Then resuspended in SDS loading buffer A and separated by SDS-PAGE for western blotting analyses with each antibody.

7-13 IP kinase assay

Cells lysates from Myc-MTK1 stably expressed cells were prepared in lysis buffer E, immunoprecipitated with anti-Myc antibody at 4 ˚C for overnight, washed with IP wash buffer D, and washed with IP wash buffer B twice. Then washed kinase wash buffer twice, reacted with recombinant Trx at 16˚C for 30 minutes and kinase reaction with 200 μM ATP in kinase reaction buffer at 16˚C for 0-15 minutes.

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