TAK1 Mediates the Ceramide Signaling to Stress-activated Protein Kinase/c-Jun N-terminal Kinase*

(Received for publication, December 19, 1996, and in revised form, January 22, 1997)

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Ceramide has been proposed as a second messenger molecule implicated in a variety of biological processes. It has recently been reported that ceramide activates stress-activated protein kinase (SAPK, also known as c-Jun NH2-terminal kinase (JNK), c-Jun amino-terminal kinase; SAPK, MAPK). Recent studies have demonstrated that the MAPK superfamily is independently regulated by particular sets of extracellular stimuli and therefore may have distinct functions in various biological processes. We have recently identified a novel member of mammalian MAPKKK, TAK1. Several lines of evidence suggested that TAK1 functions in the signal transduction pathways triggered by members of the transforming growth factor-β (TGF-β) superfamily (10). During the course of further biochemical characterization of TAK1, we have found that TAK1 can be activated in response to several stimuli that have been shown to activate SAPK/JNK. Moreover, we noticed that these stimuli are known to induce an increase in ceramide. Ceramide has recently emerged as a second messenger molecule that induces multiple cellular responses such as cell cycle arrest, differentiation, and apoptosis (11, 12). Most recently, it has been reported that ceramide activates SAPK/JNK and the ceramide-induced activation of SAPK/JNK is required for stress- and ceramide-initiated apoptosis (13). This may highlight the role of SAPK/JNK as a mediator of cellular responses induced by ceramide. But the molecular mechanism by which ceramide induces the activation of SAPK/JNK is unknown.

In this report, we first show that TAK1 is activated in cells treated with agents and stresses that induce the generation of ceramide. We then show that ceramide itself can activate TAK1. Furthermore, expression of an active form of TAK1 induced activation of SAPK/JNK and its activator, SEK1/MKK4. In addition, expression of a kinase-deficient form of TAK1 interfered with the activation of SAPK/JNK induced by ceramide. These results indicate that TAK1 acts as a mediator for the ceramide-induced activation of SAPK/JNK.

MATERIALS AND METHODS

Cell Culture—A673 human rhabdomyosarcoma cells, COS7 cells, and rat 3Y1 cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

Preparation of Recombinant Proteins—Both kinase-negative MPK2 (KN-MPK2) and wild-type SAPKs were expressed as His-tagged proteins and purified using a Ni2+-affinity column (14). Wild-type XMEK2/SEK1 and kinase-negative MAPKs were expressed as glutathione S-transferase fusion proteins and purified as described (14).

Plasmids and Transfection—Wild-type and kinase-negative forms of TAK1 cDNAs were subcloned into pSR-HA vector (15) to generate pSR-HA-TAK1 and pSR-HA-KNTAK1, respectively. Rat SAPKα mouse SEK1, Xenopus SAPK, and Xenopus SAPKK cDNAs were also subcloned into pSR-HA vector to generate pSR-HA-SAPK, pSR-HA-SEK1, pSR-HA-MAPK, and pSR-HA-MAPKK, respectively. TAB1 (1–418) and a kinase-negative form of TAK1 were previously expressed in the pEF vector (16). These plasmids were transfected into COS7 cells by the LipofectAMINE method according to the manufacturer’s instructions. The cells were lysed in an extraction buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EDTA, 10 mM NaF, 2 mM dithiothreitol, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). The cell lysates were diluted and precleared with protein A beads. The sera were precipitated with protein A beads and treated with 5% SDS and boiled before use.

Immunoprecipitation of Endogenous TAK1—The cells were lysed in an extraction buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EDTA, 10 mM NaF, 2 mM dithiothreitol, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). The cell lysates were diluted and precleared with protein A beads. The sera were precipitated with protein A beads and treated with 5% SDS and boiled before use.
The activity of immunoprecipitated TAK1 was assayed in a coupled kinase assay. TAK1 activity increased in cells exposed to these agents, peaked after 5–10 min, and declined gradually with time (Fig. 1B). We next examined whether TAK1 could be activated by treatment of cells with stresses. An expression construct encoding epitope-tagged TAK1 (HA-TAK1) was subjected to an in vitro kinase assay. The immunoprecipitate was assayed by its ability to activate recombinant XMEK2/SEK1, whose activity was assayed by its ability to activate recombinant KN-MPK2. The activity of immunoprecipitated recombinant XMEK2/SEK1 (0.5 mg/ml) in a solution containing 20 mM Tris-Cl, pH 7.5, 10 mM MgCl$_2$, and 100 µM ATP for 20 min at 30 °C. Then, KN-MPK2 (5 mg/ml, 3 µl) and [$\gamma^\text{32P}$]ATP (5 µCi) was added, and the mixtures were incubated further for 10 min at 30 °C. The activity of immunoprecipitated recombinant c-Jun, recombinant SAPK, SEK1, MAPK, and MAPKK was assayed by their ability to phosphorylate recombinant c-Jun, recombinant SAPK, myelin basic protein, and recombinant kinase-negative form of MAPK (KN-MAPK), respectively.

**RESULTS AND DISCUSSION**

A673 human rhabdomyosarcoma cells were stimulated with TNF-α, IL-1, or anti-Fas antibody, and the immunoprecipitate with anti-TAK1 antibody was subjected to an in vitro coupled kinase assay. This antibody specifically immunoprecipitated endogenous TAK1 (Fig. 1A). TAK1 activity increased in cells exposed to these agents, peaked after 5–10 min, and declined gradually with time (Fig. 1B). We next examined whether TAK1 could be activated by treatment of cells with stresses. An expression construct encoding epitope-tagged TAK1 (HA-TAK1) was transiently transfected into COS7 cells. After treatment of the cells with UV irradiation or sorbitol, TAK1 activity was determined in a coupled kinase assay. TAK1 activity increased within 5 min of exposure of cells to UV irradiation and peaked at 10 min (Fig. 1C). Exposure of the cells to hyperosmolality with 0.5 M sorbitol also caused an increase in TAK1 activity (Fig. 1C). TAK1 activity was maximal after 30 min, and the high level of activity was sustained for 60 min (Fig. 1C).

Because TNF-α (17, 18), IL-1 (19, 20), anti-Fas antibody (21, 22), and several stresses (13) were all known to induce an increase in ceramide, we tested whether TAK1 could be activated by treatment of cells with ceramide itself. COS7 cells were transfected with HA-TAK1 and treated with the mem-
brane-permeable ceramide analogue, C₂₇-ceramide. HA-TAK1 was isolated by immunoprecipitation, and its activity was determined. TAK1 activity increased within 5 min of exposure of cells to C₂₇-ceramide, was maximal after 10–30 min, and declined with time (Fig. 2B). C₂₇-ceramide stimulated TAK1 activity in a dose-dependent manner (Fig. 2C). This activation was not observed in immune complexes from cells transfected with an HA epitope-tagged, kinase-negative form of TAK1 (Fig. 2A). The endogenous TAK1 was also activated by treatment with C₂₇-ceramide (Fig. 2D). Taken together, these results have demonstrated that ceramide is capable of inducing TAK1 activation.

We tested the effect of other lipids on the TAK1 activity. C₂₇-dihydroceramide, which lacks the trans double bond at C₄–C₅ of the sphingoid base backbone, and 1,2-dioctanoyl-sn-glycerol, which is an activator of protein kinase C, failed to activate TAK1 activity efficiently (Fig. 2E). Arachidonic acid also failed to activate TAK1 (Fig. 2E). Thus, C₂₇-ceramide specifically activates TAK1.

TAK1 was originally identified as a mediator of the signal transductions triggered by the TGF-β superfamily (10). Most recently, one of TAK1-binding proteins, TAB1, has been identified as an activator for TAK1 (16). A truncated form of TAB1 lacking the TAK1-binding domain (TAB1 (1–418)) was shown to act as a dominant-negative inhibitor of TGF-β-induced gene expression (16). Overexpression of TAB1 (1–418) almost completely suppressed the activation of TAK1 induced by TGF-β stimulation but had little effect on the ceramide-induced activation of TAK1 (data not shown). These results may indicate that ceramide and TGF-β activate TAK1 through TAB1-independent and -dependent pathways, respectively.

A recent report of Verheij et al. (13) suggested the central role of SAPK/JNK in the ceramide-induced signal transduction. We therefore examined whether TAK1 participates in SAPK/JNK activation induced by ceramide. We have previously shown that a truncated form of TAK1 lacking NH₂-terminal 20 amino acids (TAK1ΔN) is constitutively active (10). The expression vector encoding TAK1ΔN was co-transfected into COS7 cells with an expression vector encoding HA-tagged SAPK/JNK or HA-tagged MAPK, and the activity of SAPK/JNK or MAPK was determined in the immunoprecipitate obtained with anti-HA antibody. Co-expression of TAK1ΔN induced activation of SAPK/JNK (Fig. 3A) but not that of MAPK (Fig. 3B). Co-expression of TAK1ΔN with SEK1/MKK4 or MAPKK, direct activators of SAPK/JNK and MAPK, respectively, resulted in activation of SEK1/MKK4 (Fig. 3C) but not that of MAPKK (Fig. 3D). These results demonstrated that TAK1 is able to activate the SEK1/MKK4 → SAPK/JNK cascade but unable to activate the MAPKK → MAPK cascade. Furthermore, when a kinase-negative form of TAK1 was co-expressed, the C₂₇-ceramide-induced activation of SAPK/JNK was suppressed markedly (Fig. 3E), indicating that TAK1 is required for SAPK/JNK activation induced by ceramide.

In this study we have shown that TAK1, a recently identified MAPKK family molecule, is activated in cells treated with cytokines and stresses that are known to induce the generation of ceramide (13, 17–22). We have here further shown that ceramide itself is able to activate TAK1 (Fig. 2). TAK1 activation was not observed in cells treated with other types of lipids, including diacylglycerol and arachidonic acid (Fig. 2). These results thus suggest that TAK1 may participate in the signal transduction pathway initiated by ceramide.

In the present study, expression of a constitutively active mutant of TAK1 resulted in the activation of SAPK/JNK and its direct activator SEK1/MKK4 (Fig. 3, A and C). Expression of a kinase-negative mutant of TAK1 suppressed the activation of TAK1 Mediates the Ceramide Signaling to SAPK/JNK.

Fig. 2. Ceramide induces TAK1 activation. A, COS7 cells were transiently transfected with HA tagged wild-type (WT) or kinase-negative (KN) form of TAK1 expression plasmids. The cells were treated with (+) or without (−) C₂₇-ceramide (100 μM) for 10 min, and HA-TAK1 (WT) or HA-KNTAK1 (KN) was immunoprecipitated and assayed. The radioactivity of the KN-MPK2 bands was shown (upper panel). Immunoprecipitated TAK1 (HA-TAK1 or HA-KNTAK1) was immunoblotted with anti-TAK1 antibody (lower panel). B, COS7 cells were transfected with an HA-TAK1 expression plasmid and treated with the C₂₇-ceramide (100 μM) for the indicated times. HA-TAK1 was immunoprecipitated and assayed. The phosphorylation state of KN-MPK2 was shown (upper and middle panels). Immunoprecipitated HA-TAK1 was immunoblotted with anti-TAK1 antibody (lower panel). C, transfected COS7 cells were treated with the indicated concentrations of C₂₇-ceramide for 5 min, and HA-TAK1 was immunoprecipitated and assayed. The phosphorylation state of KN-MPK2 was shown (upper and middle panels). Immunoprecipitated HA-TAK1 was immunoblotted with anti-TAK1 antibody (lower panel). D, rat 3Y1 cells were treated with Cer or without Control the C₂₇-ceramide (100 μM) for 30 min, and endogenous TAK1 was immunoprecipitated and assayed. The phosphorylation state of KN-MPK2 was shown (upper panel). Immunoprecipitated HA-TAK1 was immunoblotted with anti-TAK1 antibody (lower panel).
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Fig. 3. Requirement of TAK1 in the activation of SAPK/JNK induced by ceramide. COS7 cells were transfected with pSRα-HA-SAPK (A), pSRα-HA-MAPK (B), pSRα-HA-SEK1 (C), or pSRα-HA-MAPKK (D) in the presence of an expression vector encoding an active form of TAK1 (TAK1) or an empty vector (v). HA-tagged proteins were immunoprecipitated and assayed for kinase activity using recombinant c-Jun (A), myelin basic protein (B), recombinant SAPKα (C), or recombinant kinase-negative form of MAPK (D) for substrates. Phosphorylated proteins were subjected to SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. In each panel, the arrowhead indicates the position of the substrate. E, COS7 cells were transfected with a pSRα-HA-SAPK plasmid in combination with an expression vector encoding kinase-negative form of TAK1 (ΔTAK1), myelin basic protein (ΔM), for indicated times, and HA-SAPK was immunoprecipitated and visualized by autoradiography. In each panel, the arrowhead indicates the position of the substrate.

SAPK/JNK induced by ceramide (Fig. 3E). Therefore, TAK1 is necessary and sufficient for the activation of SAPK/JNK induced by ceramide. Although Raf-1, another member of MAPKK family, has been shown to be a mediator of ceramide signaling to classical MAPK (23–25), a molecular link between ceramide and SAPK/JNK has been missing. Our present results have identified TAK1 as a crucial mediator of ceramide signaling to SAPK/JNK.

The mechanism of TAK1 activation in response to ceramide should be elucidated. Several molecules have previously been reported as direct targets for ceramide, including ceramide-activated protein kinase (26, 27), ceramide-activated protein phosphatase (28), protein kinase C-ζ (29), and Raf-1 (30). These molecules might lie upstream of TAK1.

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