Positive Regulation of Apoptosis Signal-regulating Kinase 1 by hD53L1*

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Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase family member that plays a central role in cytokine- and stress-induced apoptosis by activating c-Jun N-terminal kinase and p38 signaling cascades. ASK1-induced apoptotic activity is up-regulated by two cellular factors, Daxx and TRAF2, through direct protein-protein interactions. Daxx and TRAF2 are death receptor-associated proteins in Fas and tumor necrosis factor-α pathways, respectively. Recent studies suggest that calcium signaling may regulate ASK1 pathway. Here we report that human D53L1, a member of the tumor protein D52 family involved in cell proliferation and calcium signaling, up-regulates the ASK1-induced apoptosis. The human D53L1 physically interacts with the C-terminal regulatory domain of ASK1 and promotes ASK1-induced apoptotic activity by activating autophosphorylation and kinase activity in mammalian cells. In luciferase reporter assays, hD53L1 activates c-Jun N-terminal kinase-mediated transactivation in the presence of ASK1. Expression of hD53L1 enhances autophosphorylation and kinase activity of ASK1 but has no effect on ASK1 oligomerization that is necessary for kinase activity and on binding of ASK1 to M KK6, a downstream factor of ASK1. Taken together, these results suggest that activation of ASK1 by hD53L1 may provide a novel mechanism for ASK1 regulation.

Apoptosis is a highly regulated process that controls normal development and homeostasis of multicellular organisms (1, 2). The inability to control the tightly regulated apoptosis causes many human diseases such as cancer, autoimmune diseases, and various neurodegenerative disorders (3). Extensive studies in recent years have revealed that apoptotic cell death occurs through an orchestrated sequence of intracellular signaling cascades. In particular, the mitogen-activated protein kinase (MAPK) signaling pathways have been known as highly conserved cascades for regulation of cell death and survival from yeast to humans (4, 5). There are at least six independent MAPK signaling units in mammalian systems (6, 7). Three of them, the extracellular signal-regulated kinase pathway, the c-Jun amino-terminal kinase (JNK; also known as stress-activated protein kinase (SAPK)) pathway, and the p38 pathway, have been extensively characterized. The extracellular signal-regulated kinase signaling pathway is often stimulated by mitogens, whereas the JNK/SAPK and the p38 signaling pathways are responsive to proinflammatory cytokines (e.g. TNF-α and interleukin-1) and environmental stress-related stimuli, including UV irradiation, H2O2, ischemia and reperfusion, and removal of growth factors. Several lines of evidence suggest that the JNK/SAPK signaling cascade plays a role in apoptotic cell death induced by stress-related stimuli (4, 5, 8, 9).

Apoptosis signal-regulating kinase 1 (ASK1) is a key MAP kinase kinase kinase (MAPKKK) that activates both the JNK/SAPK and the p38 signaling cascades by directly phosphorylating and, therefore, activating MKK4 and -7 (SAPK pathway) and MKK3 and -6 (p38 pathway) (10). Overexpression of ASK1 induced apoptotic cell death, and a dominant negative mutant of ASK1 reduced TNF-α- and Fas-induced apoptosis, indicating the involvement of ASK1 in TNF-α and Fas-induced apoptotic pathways (10, 11). Functional activations of ASK1 by TNF-α and Fas signals are mediated by bindings of ASK1 to TNF receptor-associated factor 2 (TRAF2) and the Fas-associated protein Daxx, respectively (11, 12). The Daxx- or TRAF2-induced ASK1 activation leads to apoptotic cell death through the activation of the JNK/SAPK signaling pathway (11, 13).

There are several antagonistic cellular partners for ASK1 including thioredoxin (Trx), glutaredoxin (Grx), 14-3-3 proteins, and protein serine/threonine phosphatase 5. Thioredoxin (Trx) was identified as a negative regulator of the ASK1-JNK/p38 pathway through yeast two-hybrid screening for ASK1-binding proteins (14). In resting cells, ASK1 constantly forms an inactive complex with Trx, but upon treatment of cells with TNF-α or reactive oxygen species such as H2O2, ASK1 is dissociated from Trx and activated by subsequent modifications, including oligomerization and auto- and/or cross-phosphorylation (10, 14, 15). It was recently reported that 14-3-3 proteins and Grx directly bind to ASK1, and that overexpression of 14-3-3 and Grx proteins blocked ASK1-induced apoptosis and JNK1 activity, respectively (16, 17). In addition, protein serine/threonine phosphatase 5 was identified as a negative regulator of the activated ASK1. Protein serine/threonine phosphatase 5 binds to and dephosphorylates ASK1 in response to H2O2, enabling inactivation of ASK1 by negative feedback (18). Moreover, intramolecular interaction, probably between the N-ter-

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* The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; ASK1, apoptosis signal-regulating kinase 1; GST, glutathione S-transferase; HA, hemagglutinin; SAPK, stress-activated protein kinase; Trx, thioredoxin; Grx, glutaredoxin; PARP, poly(ADP-ribose) polymerase.

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minal and C-terminal domains of ASK1, may be required to maintain the inactive form of ASK1 (11). Thus, ASK1 is associated with the mechanism for apoptotic cell death. As a result, execution of apoptosis induced by ASK1 must be strictly regulated by intracellular partners. However, the molecular mechanism by which ASK1 activity is regulated in cells is not understood completely.

To better understand the mechanism for the regulation of the ASK1 activity, we searched for its binding partners using the yeast two-hybrid screening method. Among several positive clones, we have identified one of them as hD53L1, a novel hD53 splicing variant encoding 131 amino acids. The hD52 gene was originally identified through its elevated expression in human breast carcinoma. The hD52 gene encodes a 184-amino acid polypeptide including a potential glycosylation site and several potential phosphorylation sites (19). Studies of hD52 homologues from other species have indicated that hD52 may play roles in calcium-mediated signal transduction and cell proliferation (20). Two homologues of hD52, hD53 and hD54, have also been identified, demonstrating the existence of a novel protein family (21). The hD52-like protein sequences are all predicted to contain a coiled-coil domain, which is responsible for homo- and heteromeric interactions. Yeast two-hybrid screening showed that hD52 could specifically interact with hD52 as well as its family members (22). Thus, D52-like proteins appear to exert and regulate their activities through specific interactions with other hD52-like proteins (23).

In the present study, we show that hD53L1 physically interacts with ASK1 and acts as a positive regulator of ASK1. Activation of ASK1 by hD53L1 leads to the activation of the caspase-3-dependent apoptosis pathway. Expression of hD53L1 induces ASK1 autophosphorylation and JNK/p38 activation but has no effect on ASK1 self-oligomerization and binding to MKK6. The ASK1-activating action of hD53L1 appears to be a novel function of this tumor protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney (HEK) 293, 293T, and BOSC 23 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 0.238% HEPES (Invitrogen), 0.375% sodium bicarbonate (Sigma), 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 10% fetal bovine serum (Gibco) in a 5% CO2 incubator at 37°C. After binding, the beads were collected by centrifugation at 30°C at 13,000 rpm for 15 min (24). The soluble fractions were incubated with 1.4 s medium without serum. For transient transfection, cells were washed once with the lysis buffer, twice with a solution containing 20 mM Tris-HCl, pH 7.5, 0.5% deoxycholate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. Cell extracts were cleared by centrifugation, and the supernatants were immunoprecipitated with anti-FLAG M2 antibody or hD53L1-specific antiserum. For phosphorylation assays, BOSC 23 cells were transfected as described above. After 48 h of transfection, cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 12 mM β-glycerophosphate, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 3 mM dithiothreitol, 1 mM sodium orthovanadate, and 1 μg/ml aprotinin.

As an example, we can observe how the text is structured and what information is included. The text discusses the identification and properties of hD53L1, a novel splicing variant of hD53 that interacts with ASK1. It describes the methods used for identifying binding partners and the experimental procedures involved in these studies. The text also outlines the results obtained from these experiments, highlighting the role of hD53L1 in regulating ASK1 activity.

For instance, the text states:

“...the hD52 gene was originally identified through its elevated expression in human breast carcinoma. The hD52 gene encodes a 184-amino acid polypeptide including a potential glycosylation site and several potential phosphorylation sites (19). Studies of hD52 homologues from other species have indicated that hD52 may play roles in calcium-mediated signal transduction and cell proliferation (20). Two homologues of hD52, hD53 and hD54, have also been identified, demonstrating the existence of a novel protein family (21). The hD52-like protein sequences are all predicted to contain a coiled-coil domain, which is responsible for homo- and heteromeric interactions. Yeast two-hybrid screening showed that hD52 could specifically interact with hD52 as well as its family members (22). Thus, D52-like proteins appear to exert and regulate their activities through specific interactions with other hD52-like proteins (23).”

This example illustrates how the text is organized and what information is conveyed. It emphasizes the role of hD53L1 in the regulation of ASK1, pointing to the methods used to identify binding partners and the experimental procedures involved. It also highlights the results obtained from these experiments, which show the role of hD53L1 in regulating ASK1 activity.

The text continues to describe the experimental procedures used to study the interaction between ASK1 and hD53L1. It mentions the use of yeast two-hybrid screening to identify binding partners and the methods used for immunoprecipitation and phosphorylation assays. The text also discusses the results obtained from these experiments, which show that hD53L1 activates ASK1 by promoting its autophosphorylation and activating the caspase-3-dependent apoptosis pathway.

In summary, the text provides a detailed description of the experimental procedures used to study the interaction between ASK1 and hD53L1. It emphasizes the role of hD53L1 in regulating ASK1 activity and highlights the methods used to identify binding partners and the experimental procedures involved. It also discusses the results obtained from these experiments, which show the role of hD53L1 in regulating ASK1 activity.
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say was performed with the protocol described by Duke (25) with the following modifications. Approximately 8 × 10^6 cells were lysed with 500 μl of lysis buffer containing 5 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 0.5% Triton X-100 on ice for 20 min. Cell extracts were clarified by centrifugation at 13,000 rpm at room temperature for 5 min. The lysate was incubated with 0.1 mg/ml RNase A at 37 °C for 20 min. DNA was purified by standard phenol-chloroform extraction and ethanol precipitation. Dry DNA pellets were then resuspended in 20 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) containing 0.02 mg/ml RNase A. Samples were loaded on a 1.5% agarose gel and visualized by ethidium bromide staining.

Luciferase Reporter Assay—293T cells were grown to 50–80% confluence in 60-mm plates and transfected with 1 μg each of pFR-Luc and 0.05 μg of pCMV-β-gal. 24 h after transfection, medium containing 1% fetal bovine serum was added, and the cells were incubated for an additional 17 h. Medium was then changed with medium containing 0.5% fetal bovine serum and incubated for another 24 h before harvesting. The luciferase activity was determined using an assay system (Promega) with a luminometer. The relative -fold induction of luciferase activity was determined and normalized to β-galactosidase activity. All transfections were repeated at least three times.

RESULTS

ASK1 Physically Interacts with hD53L1—In order to identify a new binding partner of ASK1, we carried out yeast-two-hybrid screening with a HeLa cDNA library using the C-terminal domain (residues 649–1375) of ASK1 as a bait (26). The C-terminal part contains a kinase domain (residues 649–940) and a regulatory domain (residues 941–1375). We have obtained and identified positive clones, some of which (e.g. MAPKK6 and 14-3-3 proteins) have already been reported to interact with ASK1 (17, 27). One of the de novo positive clones was identified as a full-length protein that is a splicing variant of tumor protein D53 and termed as human D53-like 1 (hD53L1) (GenBank™ accession number AF208012). Sequence alignment of hD53L1 to hD53 shows that the C-terminal 76 amino acids of hD53 protein were deleted due to alternative splicing.

To confirm the interaction between ASK1 and hD53L1, we carried out interaction assays with transfected mammalian cell extract. HEK 293 cells were transiently co-transfected with FLAG-ASK1 and GST-hD53L1 expression plasmids. GST-hD53L1 protein was pulled down by glutathione beads, followed by immunoblotting with appropriate antibodies to detect ectopically expressed ASK1 in the pulled-down hD53L1 complexes (Fig. 1A). ASK1 was detected in the pulled-down hD53L1 complexes, whereas GST alone did not form a complex with ASK1. We also performed co-immunoprecipitation assays with expressed HA-ASK1 and FLAG-hD53L1 proteins and found HA-ASK1 in the immunoprecipitated FLAG-hD53L1 complex (data not shown), suggesting that ASK1 and hD53L1 interact with each other, regardless of tags used. To test whether ASK1 could bring down hD53L1 in a reciprocal way, HA-hD53L1 and FLAG-ASK1 were expressed and ASK1 was immunoprecipitated by anti-FLAG beads, followed by immunoblotting with anti-HA antibody. HA-hD53L1 protein was detected in the pulled-down ASK1 complexes (Fig. 1B). The results showed that these two proteins interact with each other. To examine the interaction between the two endogenous proteins, we produced antisera specific to hD53L1 protein was pulled down by glutathione beads, followed by immunoblotting with anti-ASK1 antibody also confirmed the interaction of the two endogenous proteins (Fig. 1D). In a subsequent experiment, we performed co-immunoprecipitation assays to determine which domain of ASK1 binds to hD53L1 in vitro. The C-terminal regulatory domain (residues 941–1375) of ASK1 was found to be responsible for the binding to hD53L1, whereas the kinase (residues 649–940) domain showed no affinity to hD53L1 (Fig. 1E). Collectively, these data suggest that ASK1 specifically interacts with hD53L1 through its C terminus in mammalian cells.

Expression of hD53L1 Protein Potentiates ASK1-induced Apoptosis—Since hD53L1 interacts with ASK1, we next examined whether it could regulate ASK1-induced cell death. HEK 293 cells were co-transfected with ASK1 and hD53L1 expression plasmids. When cell death was determined by a trypan blue exclusion assay, ASK1-induced cell death was increased in the presence of hD53L1 in a dose-dependent manner (Fig. 2A), suggesting that hD53L1 is involved in the ASK1-induced cell death. Whereas overexpression of hD53L1 alone induced cell death only slightly, co-expression of hD53L1 and ASK1 significantly enhanced ASK1-mediated cell death.

Based on the above observation, we further examined whether the hD53L1-ASK1 signal is accompanied by apoptotic features such as DNA fragmentation and induction of caspase-3 activity. DNA fragmentation is a process that appears in the later stage of apoptosis and results from the activation of endonucleases during the apoptotic program. These endonucleases degrade chromatin into 200-bp laddering of DNA fragments (28). Chromosomal DNA samples were prepared from HEK 293 cells co-transfected with appropriate expression plasmids and were analyzed by electrophoresis. As shown in Fig. 2B, DNA fragmentation was not detected in samples from mock-transfected, hD53L1-transfected, and catalytically inactive ASK1K709R mutant-transfected cells, whereas ASK1-transfected and H2O2-treated cell samples displayed the apoptotic DNA laddering feature. Co-expression of ASK1 and hD53L1 gave strong laddering intensity in preference to ASK1 expression alone. Also, increasing concentration of hD53L1 elevated DNA fragmentation. On the other hand, expression of the catalytically inactive ASK1K709R mutant failed to induce DNA fragmentation in the presence of expressed hD53L1, suggesting that a phosphorylation event is necessary for the apoptosis. These findings indicate that overexpression of ASK1 effectively induces apoptosis in HEK 293 cells through MAPK pathways and that ASK1-mediated apoptosis can be enhanced by the addition of hD53L1. These results, therefore, suggest that hD53L1 may contribute to activation of the proapoptotic activity of ASK1 by partly, if not completely, enhancing kinase activity of ASK1.

Overexpression of hD53L1 Activates Caspase-3 in an ASK1-dependent Manner—To directly test whether hD53L1 can regulate the apoptotic activity of ASK1, we measured the ASK1-induced caspase-3 activity in the presence of transiently expressed hD53L1. Caspase-3 has been implicated as a key protease activated during the early stage of apoptosis (29). Active caspase-3, found in cells undergoing apoptosis, consists of a heterodimer of 17- and 12-kDa subunits that are derived from the inactive 32-kDa proenzyme. Active caspase-3 proteolytically cleaves and activates other caspases. Since members of the caspase family are crucial mediators of apoptosis (30, 31), we examined the requirement of caspase-3 activities for ASK1-induced apoptosis using DEVD-7-amino-4-methylcoumarin as
a substrate. Previous studies showed that caspase signaling is responsible for ASK1-induced apoptosis (32). HEK 293 cells were transfected with HA-ASK1 and FLAG-hD53L1 expression plasmids, followed by serum deprivation after 24 h of transfection. Following 16 h of serum starvation, ASK1-induced caspase-3 activity was measured in vitro (Fig. 3A).

Whereas hD53L1-transfected cells gave no significant increase of caspase-3 activity, ASK1-expressing cells showed an apparent increase of caspase-3 activity. Cells co-transfected with ASK1 and hD53L1 expression plasmids show higher caspase-3 activity than cells transfected with the ASK1 expression plasmid alone, suggesting that hD53L1 up-regulates the ASK1-induced caspase-3 activity.

**Fig. 1. ASK1 binds to hD53L1 in vivo.** A, HEK 293 cells were transfected with 1 μg of pEBG-hD53L1 and either FLAG-ASK1 or empty pFLAG-CMV2 plasmid. After 48 h of transfection, lysates were prepared and were incubated with glutathione-Sepharose 4B beads. Bound proteins were eluted and subjected to SDS-PAGE. Immunoblotting was performed with anti-FLAG M2 antibody (top panel). Whole cell extracts were immunoblotted with anti-GST antibody (middle panel) and an anti-FLAG M2 antibody (bottom panel) to show expression levels of proteins. B, HEK 293 cells were co-transfected with plasmids as indicated. Co-immunoprecipitation assays were performed with anti-FLAG M2 affinity gel, followed by immunoblotting with an anti-HA antibody. Cell lysates were subjected to immunoblotting using anti-FLAG M2 (middle) and anti-HA (bottom) antibodies to show protein expression levels of transfected genes. IP, immunoprecipitation; IB, immunoblot. C, cell lysates from the untransfected HEK 293 or HeLa cells were immunoprecipitated with mouse preimmune IgG agarose or mouse anti-ASK1 antibody agarose. The immunopellets were subjected to SDS-PAGE and immunoblotted with hD53L1-specific antiserum. D, HEK 293 cell lysates were immunoprecipitated with mouse preimmune serum or mouse ASK1 antibody serum. The immunopellets were subjected to SDS-PAGE and immunoblotted with hD53L1-specific antiserum, followed by immunoblot analysis with anti-ASK1 antibody. E, domain localization of ASK1 for binding to hD53L1. Top panel, HA-ASKK (amino acids 649–840) or HA-ASKC (amino acids 941–1375) expression plasmid was co-transfected into BOSC 23 cells along with either pEBG-hD53L1 or empty plasmid. Binding assays were performed with glutathione-Sepharose 4B beads, followed by immunoblotting with an anti-HA antibody. Bottom panel, immunoblot of expressed ASK1 mutants in cell extracts.

**Fig. 2. Effect of hD53L1 on ASK1-dependent cell death.** A, HEK 293 cells were transfected with 1 μg of HA-ASK1 and 1 μg (+) or 2 μg (++) of FLAG-hD53L1 plasmids as indicated using the calcium phosphate precipitation method. After 24 h of transfection, cells were washed with phosphate-buffered saline and then incubated for 40 h in serum-free medium. To minimize the influence of transfection reagents, mock-transfected cells were used as 100% viability. Cells exposed to 1 mM H2O2 for 9 h were used as positive control. Cell viability was determined by trypan blue exclusion assay. Results shown are mean S.E. (n = 3). B, transfections were done as described in A. Positive control cells were stimulated with 1 mM H2O2 for 9 h. Laddering assay was performed as described under “Experimental Procedures.” Soluble cleavage chromatin were detected by ethidium bromide staining. The first lane shows a 100-bp size marker.
To confirm that the increase of caspase-3 activity is directly related to the promotion of the in vivo caspase-3 activity, immunoblot analysis was performed to detect the protein level of intact PARP (Fig. 3B). Because caspase-3 is responsible for the proteolytic cleavage of PARP, PARP was chosen as an in vivo marker of caspase-3 activity. Intact PARP signal was decreased in an hD53L1 dose-dependent manner, suggesting that PARP was cleaved by activated caspase-3. This caspase-3 activity was further confirmed by Western blotting of a 17-kDa active caspase-3 fragment (Fig. 3C). With co-expression of hD53L1 and ASK1, detection of the active caspase-3 was more apparent than that of cells expressing ASK1 only.

The hD53L1 Protein Activates ASK1-mediated MAPK Pathway but Does Not Increase ASK1 Oligomerization and Substrate Binding for Activity—Since hD53L1 induces ASK1-mediated apoptosis through MAPK signaling cascades as described above, we performed reporter assays to determine the effect of hD53L1 on ASK1-mediated transactivation activities of c-Jun, which is a downstream target of JNK (Fig. 4). For these assays, the N-terminal region (amino acids 1–223) of c-Jun fused with the DNA binding domain of yeast GAL4 and a reporter plasmid (pFR-Luc) carrying 5× GAL4 binding sequences in the promoter region that controls expression of the luciferase gene were used. ASK1 expression plasmid together with the GAL4-c-Jun expression plasmid in the presence or in the absence of hD53L1 expression plasmid was co-transfected into 293T cells. Whereas overexpression of ASK1 was sufficient for stimulations of c-Jun activity, co-expression of hD53L1 further enhanced its activity.

There have been several reports that oligomerization of ASK1 is necessary for its kinase activity (15, 33). Since auto-phosphorylation of ASK1 by oligomerization is important for ASK1 kinase activity, we tested whether hD53L1 could enhance ASK1 autophosphorylation and its kinase activity. ASK1 was immunoprecipitated from the same cell extracts co-expressing hD53L1 and ASK1 and effects of hD53L1 on ASK1 activity in the immunocomplex were determined by autophosphorylation assays and in vitro kinase assays using GST-MKK6 as a substrate. Results show that ASK1 is more auto-phosphorylated and more active in the presence of hD53L1 than in the absence of hD53L1 (Fig. 5A). The effects of phosphorylation on ASK1 and MKK6 induced by the expression of hD53L1 are lower than those induced by the addition of 1 mM H$_2$O$_2$ but similar to those induced by 100 μM H$_2$O$_2$. Since
hD53L1 has no apparent kinase activity on ASK1, we conclude that ASK1 is autophosphorylated at a higher level in the presence of hD53L1 than in the absence of hD53L1. Since activation of ASK by hD53L1 may result from the enhanced ASK1 oligomerization, we then tested whether hD53L1 induces ASK1 dimerization to increase ASK1 activity. HEK 293 cells expressing HA-ASK1 and FLAG-ASK1 in the presence or absence of hD53L1 were lysed, and then cell extracts were analyzed by immunoprecipitation, followed by immunoblotting. As shown in Fig. 5B, there is no apparent increase of ASK1 dimerization regardless of the hD53L1 expression level, suggesting that hD53L1-dependent activation of ASK1 is not induced by the modulated oligomerization of ASK1.

Activation of ASK1 by hD53L1 could result from the increased interaction between ASK1 and its substrate. This was tested in in vitro binding experiments using the immunoprecipitated FLAG-ASK1 and GST-MKK6 protein. After incubation of the immunoprecipitated ASK1 with GST-MKK6, the complexes were washed and subjected to SDS-PAGE, followed by immunoblot with anti-GST antibody. No evidence of increased interaction in the presence of hD53L1 was observed (data not shown), indicating that activation of ASK1 by hD53L1 is not due to change of ASK1 affinity to its substrate.

**DISCUSSION**

Recent extensive studies on ASK1-mediated signaling pathway have identified three important signaling routes that could activate ASK1 kinase activity. TNF-α, Fas, and endoplasmic reticulum stress signals activate ASK1 kinase activity through direct interactions between ASK1 and receptor-associated proteins (TRAF2 for TNF and endoplasmic reticulum stress signals and Daxx for Fas signal) (11, 12, 34).

However, there are several recent reports that other regulatory mechanisms such as Ca²⁺ signaling may be involved in the regulation of ASK1. In neuronal cells of *C. elegans*, NSY-1, which is the homolog of the human ASK1, is activated by calcium/calmodulin-dependent protein kinase II, UNC-43, suggesting that ASK1 is controlled by calcium influx to regulate cell fate (35). In addition, the report that the ASK1-MKK4-JNK pathway is activated by Ca²⁺-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors in the hippocampal CA1 region after ischemia provides further evidence that ASK1 may be regulated by calcium influx (36). It also has been reported that D52 tumor proteins are involved in Ca²⁺ signaling to regulate proliferation and Ca²⁺-stimulated secretory activity (37, 38). Recently, it has been reported that calcium/calmodulin-dependent protein kinase II phosphorylates a Ca²⁺-regulated heat-stable protein of 28 kDa (CRHSP-28; a member of the tumor protein D5 family) in cultured mucosal T84 cells (39). Thus, our finding that hD53L1, a member of the tumor protein D5 family, activates the ASK1 pathway suggests that it may play a role in the Ca²⁺-dependent ASK1-regulating pathway. The functional and biochemical basis of D52 family proteins, however, remains unknown. D52-like proteins have been found to interact with MAL2, a novel member of the MAL phospholipid family, as well as annexin VI (40, 41). Both D52-interacting proteins are found in membrane fractions, whereas D52 proteins are present as peripheral membrane proteins that are recovered in the soluble fraction after alkaline treatment. In addition, binding of D52 to annexin VI is Ca²⁺-dependent, whereas binding to MAL2 is not. Interestingly, the D52 orthologue R10 in Japanese quail was identified as a chimeric cellular cDNA in proliferation-stimulated quail neuroretina cultures infected with RAV-1 retrovirus (42). This report and our results suggest that D52 proteins may participate in an unidentified signaling pathway that is involved in cell proliferation, despite the lack of evidence for what is located upstream of D52-ASK1 pathway.

It is worth noting that both hD53L1 and ASK1 contain coiled-coil motifs for protein-protein interactions. The C-terminal coiled-coil motif (residues 1236–1293) of ASK1 is conserved among ASK1s from different species and involved in the ASK1 oligomerization and activation. The coiled-coil domain of hD53L1 may participate in binding to that of ASK1. However, we found that oligomerization of ASK1 was not altered by expression of hD53L1, suggesting that hD53L1 activates ASK1 without inducing oligomerization. TRAF2, an activator of ASK1, enhances ASK1 oligomerization probably by mediating the reactive oxygen species-mediated dissociation of Trx from ASK1 (43). Therefore, we could conclude that the activation mechanism of ASK1 by hD53L1 is different from the TRAF2-mediated ASK1 activation mechanism. One possible explanation for this observation is that hD53L1 acts on the preformed oligomer of ASK1 to induce conformational change to lead to
the activation of ASK1-mediated apoptosis. In fact, it has been reported that ASK1 forms a silent homo-oligomer in non-stressed cells and then undergoes conformational change upon exposure to H₂O₂ (33). In addition, binding of hD53L1 to ASK1 did not alter the binding affinity of ASK1 to its substrate, MKK6, which suggests that hD53L1 regulates ASK1 through a mechanism independent of physical interaction between ASK1 and MKK6.

Interestingly, hD53L1 did not enhance the kinase activity of ASK1 as strong as other adapter activators such as Daxx of Fas signaling and TRAF2 of TNF signaling (11, 12). It is possible that hD53L1 may act as a competitive inhibitor of 14-3-3 or Grx proteins that bind to the C-terminal portion of ASK1 and suppress its activation (16, 17, 44), resulting in prevention of 14-3-3 proteins or Grx from binding to ASK1 in a concentration-dependent manner. This possible role of hD53L1 as an inhibitor of ASK1-inhibiting proteins may explain why the kinase activity of ASK1 is not induced significantly. Whereas little is known of hD52 family protein function, a recent report that hD53 (TPD 52L1) and hD54 (TPD52L2) contain 14-3-3 binding motifs suggests possible roles of hD52 family members in 14-3-3 signaling and TRAF2 of TNF signaling (11, 12). It is possible that hD53L1 on ASK1 and apoptosis.

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