The apoptosis signal-regulating kinase 1 (ASK1)-JNK/p38 signaling pathway is pivotal component in cell apoptosis and can be activated by a variety of death stimuli including tumor necrosis factor (TNF) α and oxidative stress (reactive oxygen species). However, the mechanism for ASK1 activation is not fully understood. We have recently identified ASK1-interacting protein (AIP1) as novel signal transducer in TNFα-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. In the present study, we employed yeast two-hybrid system using the N-terminal domain of AIP1 as bait and identified homeodomain-interacting protein kinase 1 (HIPK1) as an AIP1-associated protein. Interestingly, we showed that TNFα induced HIPK1 desumoylation concomitant with a translocation from nucleus to cytoplasm at 15 min followed by a return to nucleus by 60 min. The kinetics of HIPK1 translocation correlates with those of stress-induced ASK1-JNK/p38 activation. A specific JNK inhibitor blocked the reverse but not the initial translocation of HIPK1, suggesting that the initial translocation is an upstream event of ASK1-JNK/p38 signaling and JNK activation regulates the reverse translocation as a feedback mechanism. Consistently, expression of HIPK1 increased, whereas expression of a kinase-inactive form (HIPK1-D315N) or small interference RNA of HIPK1 decreased stress-induced ASK1-JNK/p38 activation without effects on IKK-NF-κB signaling. Moreover, a sumoylation-defective mutant of HIPK1 (KR5) localizes to the cytoplasm and is constitutively active in ASK1-JNK/p38 activation. Furthermore, HIPK1-KR5 induces dissociation of ASK1 from its inhibitors 14-3-3 and thioredoxin and synergizes with AIP1 to induce ASK1 activation. Our study suggests that TNFα-induced desumoylation and cytoplasmic translocation of HIPK1 are critical in TNFα-induced ASK1-JNK/p38 activation.

Apopotosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase family, is an upstream activator of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase signaling cascades (1). ASK1 can be activated in response to diverse stresses including proinflammatory cytokine tumor necrosis factor (TNF) α, reactive oxygen species (ROS), death receptor Fas, disruption of microtubule structures, protein aggregation in endoplasmic reticulum, and genotoxic stress from nucleus (2, 3). In vitro data suggest that activation of ASK1 triggers various biological responses such as apoptosis, inflammation, differentiation, and survival in different cell types (4–7). Studies from ASK1-deficient mice indicate that ASK1 is critical for TNFα and ROS-induced apoptosis signaling (8).

ASK1 is a 170-kDa protein that functionally is composed of an inhibitory N-terminal domain, an internal kinase domain, and a C-terminal regulatory domain. The mechanism by which stress stimuli activate ASK1 is not fully understood. Several cellular factors including thioredoxin (Trx), glutaredoxin, and 14-3-3 have been reported to interact with different ASK1 domains and inhibit ASK1 activity (6, 7, 9–12). The C-terminal domain of ASK1 binds to TNFα receptor-associated factor 2 (TRAF2), and this association is required for ASK1 activation by TNFα (3, 13). Dissociation from Trx is required for ASK1 binding to TRAF2, which in turn induces ASK1 oligomerization. ASK1 oligomerization leads to autophosphorylation of ASK1 at Thr-845 within the activation loop in the kinase domain (11, 14). Activated ASK1 subsequently recruits and activates its downstream targets mitogen-activated protein kinase kinase (MEKK3/7 and MEKK4/7) and mitogen-activated protein kinase (JNK and p38). Thus, ASK1 activation appears to involve several sequential steps including release of cellular inhibitors such as Trx, glutaredoxin, glutathione S-transferase Mu, heat shock proteins, and 14-3-3; ASK1 oligomerization/autophosphorylation at Thr-845; and scaffold protein-mediated association of ASK1 with downstream MEKK and JNK.
The release of Trx and 14-3-3 from ASK1 appears to be a critical step in ASK1 activation. Trx (cytosolic Trx1 and mitochondrial Trx2) in a reduced form binds to the N-terminal domain of ASK1, whereas 14-3-3 binds to the C-terminal domain of ASK1 via phosphoserine 967 to maintain ASK1 in an inactive state. Because the oxidized form (intramolecular disulfide between Cys-32 and Cys-35) or the redox-inactive form (the double mutation at catalytic sites Cys-32 and Cys-35) of Trx binds to ASK1 (7, 9, 11, 15), we have proposed that oxidation of Trx1 is likely attributed to the dissociation of Trx from ASK1 in response to TNFα/ROS (7, 15). The mechanism for 14-3-3 release has been recently characterized. Phosphoserin-binding protein 14-3-3 associates with ASK1 via its Ser(P)-967, which is basally phosphorylated. In response to TNFα/ROS, ASK1 is dephosphorylated at Ser-967 leading to release of 14-3-3. The phosphate(s) responsible for dephosphorylation of ASK1 at Ser(P)-967 has not been identified, although a protein phosphatase 2A-like enzyme has been proposed (16). We have recently identified a novel ASK1-interacting protein (AIP1) that plays a critical role in 14-3-3 release and ASK1-JNK activation by TNFα/ROS (17). AIP1, a new member of Ras-GAP protein family, via its protein kinase C-conserved (C2) domain binds to a sequence surrounding the 14-3-3-binding site (Ser(P)-967) on ASK1 but preferentially to a dephosphorylated active form of ASK1. More importantly, AIP1 facilitates disassociation of 14-3-3 from ASK1 leading to an enhanced ASK1 activity (17). However, how AIP1 facilitates release of 14-3-3 from ASK1 is not known.

SUMO is a ubiquitin-like protein that can be covalently attached to a large number of proteins through the formation of isopeptide bonds with specific lysine residues of target proteins (18, 19). A large number of sumoylated proteins, including Ran-GAP1, PML, IKB, p53, p53, C-Jun, Sp3, Elk-1, p300, and many nuclear receptors, have been identified (18, 19). Sumoylation is a dynamic process that is mediated by activating, conjugating, and ligating enzymes and that is readily reversed by a family of SUMO-specific proteases (20). Several members of SUMO-specific proteases have been reported in the mammalian system (21–26). Different members of these SUMO-specific proteases appear to localize in different cellular compartments where they regulate protein function by modifying the protein stability, cellular localization, and protein-protein interactions (21–27).

Homeodomain-interacting protein kinase 1 (HIPK1) is one of three closely related serine/threonine protein kinases that regulate the activity of a broad range of transcription factors (28). The HIPKs were originally identified as nuclear protein kinases that function as co-repressors for various homeodomain-containing transcription factors (28). Recently the growth inhibitory and tumor suppressor functions for HIPK1 and HIPK2 have been extensively studied. HIPK1 and HIPK2 appear to act cooperatively with the tumor suppressor protein p53. Further studies indicate that HIPK2 phosphorylates p53 on Ser-46, resulting in activation of p53-dependent transcription, cell growth regulation, and apoptosis initiation (29, 30). However, the study from HIPK1$^{−/−}$ mice appears to support that HIPK2 functions as an oncogene because the mice develop few tumors than HIPK1$^{−/−}$ mice (31). HIPKs have been shown to interact with other proteins involved in apoptosis and signal transduction in a cellular localization-dependent manner. In the nucleus, HIPK2 can promote apoptosis by down-regulating the transcriptional co-repressor CtBP (32). In cytoplasm, HIPK1, HIPK1, and HIPK3 appear to transduce signals by death receptors through interaction with TRADD and FADD (33, 34). However, HIPKs are primarily localized in the nucleus where it is sumoylated (35, 36). This raises the possibility that HIPK translocation from nucleus to cytoplasm is involved in TNFα/Fas signaling.

In the present study, we identified HIPK1 as an AIP1/ASK1-associated protein. In resting endothelial cells (EC), HIPK1, AIP1, and ASK1 are primarily localized in the nucleus, plasma membrane, and cytoplasm, respectively. TNFα induces de-sumoylation of HIPK1 concomitant translocation to cytoplasm where it associates with the AIP1-ASK1 complex to induce the release of Trx and 14-3-3 from ASK1. Our study suggests that HIPK1 is novel signal transducer in TNFα-induced ASK1-JNK/p38 activation.

MATERIALS AND METHODS

**Plasmid Construction**—Expression plasmids for Daxx was generously provided by Dr. Jacques Landry, Université Laval, Quebec, Canada, and SUMO was from Dr. Ronald T. Hay (University of St. Andrews, St. Andrews, Scotland, UK). Expression plasmids for ASK1 and AIP were described previously (6, 17, 37). For yeast expression plasmid, AIP1-N was amplified by PCR using a 5′ primer with an NdeI site and a 3′ primer with SalI. The PCR product was inserted into the NdeI and SalI sites of the expression vector pGBK7 (Clontech) to generate a new AIP1 clone in which AIP1 was fused in-frame with the DNA-binding domain of yeast transcriptional activator GAL4. The expression plasmids for GFP-tagged and Myc-tagged HIPK1 were generated by inserting mouse HIPK1 cDNA into pEGFP-C2 and pCS3+ MBTX, respectively, as described previously for HIPK2 (28, 35). The mutant HIPK1 (KR and D315N) were constructed by site-directed mutagenesis using a QuikChange™ site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer.

**Antibodies**—A rabbit polyclonal antibody against phospho-specific antibodies against phospho-ASK1 (Ser(P)-967 and Thr(P)-845) and phospho-p38 from Cell Signaling, phospho-JNK from Bioresources. We obtained anti-ASK1 (H300), anti-p38, anti-Myc, anti-SUMO, and anti-IκBα from Santa Cruz Biotechnology. Anti-hemagglutinin was from Roche Applied Science. Anti-hemagglutinin and anti-FLAG was from Sigma. Rabbit polyclonal antibodies against AIP1 and HIPK1 were generated in our laboratories.

**Cells, Cytokines, and Inhibitors**—Human umbilical vein endothelial cells (HUVEC) and bovine aortic EC (BAEC) were purchased from Clonetics (San Diego, CA). HUVEC were cultured in modified M199 culture medium containing 20% (v/v) heat-inactivated bovine fetal calf serum, 100 μg/ml heparin sodium salt, 30 μg/ml endothelial cell growth supplement, 2 μg/ml L-glutamine, 60 units/ml penicillin, and 0.5 μg/ml streptomycin at 37°C, in 5% CO2 on gelatin-coated tissue culture plastic. The cells were used at passages 2–4. Human recombinant TNFα was from R & D Systems Inc. (Minneapolis, MN) and used at 10 ng/ml. Chemical inhibitors were purchased from Calbiochem.

**Yeast Two-hybrid Screening**—AIP1-N bait was used to screen a pre-transformed human heart cDNA library (Clontech). The yeast two-hybrid screening was performed according to the instructions of the manufacturer (Clontech). In brief, the yeast strain AH109 harboring pGBK7-AIP1 was mated with Y190 harboring a human heart cDNA library. Mating zygotes were selected on synthetic dropout agar plates lacking Trp, Leu, His, and Ade (QDO). Yeast colonies were transferred onto a nylon membrane and processed by the β-galactosidase filter assay. Plasmids from positive colonies were isolated and retransformed into the yeast strain Y190 with either pGBKT7 or pGBK7-AIP1-N to confirm that growth on QDO and β-galactosidase was AIP1-N-dependent. The cDNA inserts from true positive clones were subjected to DNA sequencing with a dye terminator cycle sequencing kit (Keck Facility at Yale).

**JNK and ASK1 Kinase Assays**—A JNK assay was performed as described previously (6, 17, 37) using glutathione S-transferase-c-Jun (1–50) fusion protein as a substrate. ASK1 assay was performed using glutathione S-transferase-MKK4 as a substrate.

**Transfection and Reporter Assay**—Transfection of HUVEC was performed by the DEAE-dextran method as described (6, 17, 37). BAEC were transfected by Lipofectamine 2000 (Invitrogen). Luciferase activity followed by Renilla activity was measured twice in duplicate using a Berthold luminometer. All of the data were normalized as relative luciferase light units/Renilla unit.

**Immunoprecipitation and Immunoblotting**—HUVEC or BAEC after various treatments were washed twice with cold phosphate-buffered saline and lysed in 1.5 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.75% Brij 96, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoro-
with TNF. BAEC were transfected with GFP-HIPK1 and treated with TNFα (10 ng/ml) for 0 (Ctrl), 15, and 60 min. Localization of GFP-HIPK1 was visualized by fluorescence microscopy. The nuclei were counterstained with DAPI. b, Daxx is not translocated in response to TNFα. Myc-HIPK1 and Daxx were co-transfected into BAEC followed by treatment with TNFα (10 ng/ml). HIPK1 and Daxx were detected by indirect immunofluorescence microscopy with anti-Myc mouse monoclonal antibody (for HIPK1) and anti-Daxx rabbit polyclonal antibody followed by Alexa 488-conjugated (green) anti-mouse and Alexa 594-conjugated (red) anti-rabbit secondary antibodies.

**RESULTS**

**HIPK1 Is Translocated from Nucleus to Cytoplasm in Response to TNFα**—We have recently identified a novel Ras-GAP as an ASK1-interacting protein (AIP1, also called DAB2-interacting protein). In response to TNFα, AIP1 binds to ASK1 and facilitates 14-3-3 dissociation from ASK1 leading to ASK1-JNK activation (17). To identify additional proteins involved in this process, we used AIP1 as bait in yeast two-hybrid and identified HIPK1 as a potential AIP1-interacting protein. The HIPK1-AIP1 interaction was also seen in EC overexpressing these molecules (see Fig. 5). However, HIPK1 is localized in the nucleus, whereas AIP1-ASK1 is in the cytoplasm in resting EC. We reasoned that HIPK1 might be translocated from nucleus to cytoplasm in response to TNFα to associate with cytoplasmic protein AIP1-ASK1 complex. To test this hypothesis, BAEC were transfected with GFP-HIPK1, and the cells were treated with TNFα (10 ng/ml) for 15–60 min. Localization of GFP-HIPK1 was visualized by fluorescence microscopy, and the nuclei were counterstained with DAPI. HIPK1 is localized in nucleus of resting cells. However, HIPK1 is translocated to cytoplasm upon TNFα treatment for 15 min. Interestingly, HIPK1 shuttled back to nucleus by 60 min (Fig. 1a). To determine whether HIPK1 translocation is specific, we compared HIPK1 with Daxx, a nuclear protein implicated in Fas-induced ASK1-JNK signaling. BAEC were co-transfected with HIPK1 (Myc-tagged) and Daxx followed by treatment with TNFα (10 ng/ml for 15 min). Localization of HIPK1 and Daxx were visualized by indirect immunofluorescence microscopy with anti-Myc antibody (for HIPK1) and anti-Daxx followed by Alexa 488-conjugated anti-mouse and Alexa 594-conjugated anti-rabbit secondary antibodies. In resting cells, both HIPK1 and Daxx were localized in the nucleus. However, TNFα induced a cytoplasmic translocation of HIPK1 but not of Daxx (Fig. 1b). In contrast, Daxx is not translocated in response to TNFα (Fig. 1b).

**HIPK1 Is Desumoylated in Response to TNFα**—We have previously shown that HIPK2, another member of HIPK family protein, is modified by sumoylation, which is known to modulate the cellular trafficking of several proteins (35). To determine whether HIPK1 is desumoylated in response to stress stimuli, BAEC were untreated or treated with TNFα (10 ng/ml). TNFα-induced activation of p38 and JNK were determined by Western blot with phospho-specific antibodies. As shown previously, activation of JNK/p38 peaked at 15 min and declined to the basal level by 60 min (Fig. 2a). TNFα treatment caused significant reduction in a modified HIPK1 band above the HIPK1 protein. This band was shown to be sumoylated HIPK1 as determined by immunoprecipitation with anti-SUMO followed by Western blot with anti-HIPK1 (Fig. 2a). These data suggest that TNFα induced HIPK1 desumoylation.

A sequencing search indicated that HIPK1 contains multiple consensus motifs for sumoylation (aKXE, where X stands for an aliphatic amino acid, K indicates the lysine residues that serves as the SUMO attachment site, and X stands for any amino acid). The lysine residues at the consensus sumoylation sites of HIPK1 (Lys-1202, Lys-556, Lys-440, Lys-317, and Lys-25) were mutated to arginine sequentially from the C terminus to the N terminus. The resulted HIPK1-KR5 contains mutations at all five consensus sites. Expression...
overexpression of HIPK1-KR5 reduced the density of the shifted band compared with HIPK1-WT and HIPK1-KR4, suggesting that HIPK1-KR5 might be defective in sumoylation (Fig. 2b).

Sumoylation of HIPK1 was determined by immunoprecipitation with anti-SUMO antibody followed by Western blot with anti-Myc antibody. Sumoylation of HIPK1 was markedly diminished in cells expressing HIPK1-KR5 compared with HIPK1-WT or HIPK1-KR1–4 (Fig. 2c), suggesting that Lys-25 is critical for HIPK1 sumoylation. This was further confirmed by a single mutation at Lys-25 (HIPK1-K25R) (not shown).

To determine whether desumoylation regulates HIPK1 cytoplasmic translocation, Myc-tagged HIPK1-WT, HIPK1-K25R, or HIPK1-KR1–5 was transfected into BAEC. Localization of the HIPK1 proteins was then detected by indirect immunofluorescence microscopy with anti-Myc. HIPK1-KR5 and HIPK1-K25R showed cytoplasmic distribution in the absence of stimuli (Fig. 2d). In contrast, HIPK1-KR4 as well HIPK1-KR1–3 were detected in the nucleus (Fig. 2d) in resting cells and were translocated to cytoplasm in response to stress stimuli (not shown). Taken together, these data suggest that desumoylation of HIPK1 is critical for HIPK1 cytoplasmic translocation from the nucleus.

**Cytoplasmic Translocation of HIPK1 Is an Upstream Event of JNK Activation That Is Critical for Subsequent Return of HIPK1 to Nucleus—** Because TNFα-induced HIPK1 translocation and JNK/p38 showed a similar kinetics, we determined whether these two events are dependent. To this end, we first examined effects of specific inhibitors on TNFα-induced HIPK1 translocation. BAEC were transfected with GFP-HIPK1, and the cells were treated with JNK inhibitor SP600125 (20 μM) or p38 inhibitor SB203580 (20 μM) for 30 min followed by TNFα treatment (10 ng/ml for 15 or 60 min). As a control, localization of phospho-JNK was determined by indirect immunofluorescence microscopy with anti-phospho-JNK. Consistent with results from Western blot, JNK phosphorylation induced by TNFα peaked at 15 min and declined by 60 min. SP600125, but not SB203580, specifically blunted TNFα-induced JNK activa-
HIPK1 Associates with AIP1-ASK1 Complex in Response to TNFα—Cytoplasmic translocation of HIPK1 prompted us to examine whether HIPK1 is associated with AIP1-ASK1 in the cytoplasm. Association of HIPK1 with AIP1-ASK1 in EC was first determined by co-localization analyses in response to TNFα. GFP-HIPK1 was co-transfected with FLAG-tagged AIP1 into BAEC, and the cells were treated with TNFα (10 ng/ml for 15 or 60 min). GFP-HIPK1 was visualized by fluorescence microscopy. FLAG-tagged AIP1 was detected by indirect immunofluorescence microscopy with anti-FLAG followed by Alex 594-conjugated anti-mouse secondary antibody. The merged pictures are shown on the right. Association of endogenous HIPK1 with AIP1 and ASK1 in response to TNFα, BAEC were treated with TNFα (10 ng/ml for 15 min). Association of HIPK1 with AIP1 and ASK1 was determined by immunoprecipitation with anti-HIPK1 followed by Western blot with anti-AIP1 and anti-ASK1, respectively. HIPK1 protein in the immunoprecipitates was determined by Western blot with anti-HIPK1. Expression of AIP1, ASK1, and phospho-p38 was determined by Western blot with respective antibodies. Association of HIPK1 with ASK1 was determined as in b (right panel).

Critical Domains in HIPK1, AIP1, and ASK1 for Their Interactions—We then mapped the interacting domains in HIPK1, AIP1, and ASK1 proteins, which are schematically depicted in Fig. 5a. To this end, Myc-tagged HIPK1-KR5 (which is constitutively expressed in cytoplasm) was co-transfected with FLAG-tagged AIP1 or ASK1 truncates containing the N-terminal domain (N), the kinase domain (K), and the N-terminal deletion (ΔN) into BAEC, and association of HIPK1 with AIP1 or ASK1 was determined by immunoprecipitation with anti-HIPK1 followed by Western blot with anti-AIP1 or anti-ASK1, respectively. Association of HIPK1 with AIP1 or ASK1 was not significantly altered by SiRNA of AIP1, suggesting that binding of HIPK1 to ASK1 is independent on AIP1 (Fig. 5a, right panel). These data suggest that HIPK1, ASK1, and AIP1 form a ternary complex in the cytoplasm in response to TNFα.
HIPK1 binds to AIP1-N, but not AIP1-PHC2 or PH, suggesting that HIPK1 binds to the GAP domain of AIP1 (Fig. 5c). We next defined the critical domain in HIPK1 for interactions with ASK1 and AIP1. HIPK1 contains the N-terminal kinase domain (K, amino acids 1–578), internal homeodomain-interacting domain (ID), the PEST sequence and the C-terminal Tyr/His-rich motif (YH). The lysine residues at the sumoylation site critical for its activation. BAEC were transfected with HIPK1-KR5 and FLAG-tagged ASK1 truncates containing the YH domain (Fig. 5d). Association of HIPK1 with ASK1 was determined by immunoprecipitation with anti-Myc followed by Western blot with anti-FLAG. HIPK1 protein in the immunoprecipitate was determined by Western blot with anti-Myc and anti-FLAG (for AIP1). HIPK1 protein in the immunoprecipitate was determined by Western blot with anti-Myc.

Critical Roles of HIPK1 in TNFα-induced Activation of ASK1-JNK/p38 and EC Apoptosis—Next we performed series of experiments to determine whether TNFα-induced translocation of HIPK1 plays a role in ASK1-JNK/p38 activation by TNFα. We first examined effects of HIPK1 on the TNFα-induced JNK/p38 reporter gene in which a c-Jun/ATF2-binding site is critical for its activation. BAEC were transfected with various forms of HIPK1 (WT, DN, and KR5) in the presence of the JNK/p38 reporter gene. BAEC were treated with TNFα (10 ng/ml for 6 h), and the reporter gene activity was measured by luciferase assay. HIPK1-KR5, but not HIPK1-DN, increased the basal activity of the JNK/p38 reporter gene (Fig. 6a), consistent with the idea that HIPK1-KR5 is a constitutively active form because of its presence in the cytoplasm prior to TNFα treatment. HIPK1-WT and HIPK1-KR5 enhanced, whereas HIPK1-DN blunted, TNFα-induced activation of the JNK/p38 reporter gene (Fig. 6a). To determine the effects of HIPK1 on endogenous ASK1-JNK/p38 signaling, BAEC were transfected with HIPK1-DN or vector control (VC), and cells were treated with TNFα (10 ng/ml) for various times (0–60 min). Activation of ASK1 was determined by Western blot with a phospho-specific antibody (pT845). Activation of p38 and IκB degradation were determined by Western blot with anti-p-p38 and IκB, respectively. HIPK1-DN blocked TNFα-induced activation of ASK1, JNK, and p38. As expected, TNFα induced IκB degradation, indicating NF-κB activation. However, HIPK1-DN had no effect on TNFα-induced IκB degradation (Fig. 6b), suggesting that HIPK1 is specifically involved in TNFα-induced ASK1-JNK/p38 signaling.

We then performed RNA interference to determine the physiological role of HIPK1 in TNFα-induced ASK1-JNK/p38 sig-
HIPK1 Activates ASK1

FIG. 6. Critical roles of HIPK1 in TNFα-induced activation of ASK1-JNK/p38 signaling. a, HIPK1-WT and KR5 increased, whereas HIPK1-DN blocked TNFα-induced activation of JNK/p38-dependent reporter gene. BAEC were transfected with HIPK1-WT, -KR5, or -DN in the presence of a JNK/p38 reporter gene. A Renilla construct was co-transfected as an internal control. The cells were treated with TNFα for 6 h, and both luciferase and Renilla units were measured. Relative luciferase activities are presented from mean of duplicate samples by taking untreated vector control as 1. Similar results were obtained from two additional experiments. The data are presented as the means of duplicates from two independent experiments. b, HIPK1-DN blocks TNFα-induced activation of ASK1-p38. BAEC were transfected with HIPK1-DN or VC. The cells were treated with TNFα (10 ng/ml) for various times (0, 2, 5, 15, 30, or 60 min). Activation of ASK1 (Thr(P)-845) and p38 was determined by Western blot with phospho-specific antibodies. IκBα degradation was determined by Western blot with anti-IκBα. Total ASK1 and HIPK1-DN were determined by Western blot with respective antibodies. Phosphorylation and total levels of ASK1 and p38 were determined. c, Critical roles of HIPK1 in ASK1-mediated apoptosis. BAEC were transfected with VC, HIPK1-WT, -DN, control, or HIPK1 short hairpin RNA. The cells were treated with TNFα (10 ng/ml) plus cycloheximide (CHX, 10 µg/ml) for 6 h. EC apoptosis was determined by DAPI staining for nuclei fragmentation. The apoptosis rate is shown. The data are presented as the means of duplicates from two independent experiments. *, p < 0.05. IB, immunoblots.

naling. BAEC were transfected with a control or HIPK1 RNAi, and the cells were left untreated or treated with TNFα for the indicated times (0, 15, and 60 min). HIPK1 expression was knocked down by RNAi of HIPK1 by 80% without effects on expression of ASK1 or AIP1 (Fig. 6c). Phosphorylation of ASK1 (pT845) and p38 were determined as described. The results showed that TNFα-induced activation of ASK1 and p38 was significantly blunted by RNAi of HIPK1 (Fig. 6c), highlighting the role of HIPK1 in the ASK1-JNK/p38 signaling pathway.

Finally, we determined the roles of HIPK1 in TNFα-induced EC apoptosis in HIPK1 overexpression and knockdown systems. BAEC were transfected with VC, HIPK1-WT, DN, a control, or HIPK1 SiRNA. The cells were treated with TNFα (10 ng/ml) plus cycloheximide (10 µg/ml) for 6 h. EC apoptosis was determined by DAPI staining for nuclei fragmentation. Similar to effects of HIPK1 on ASK1-JNK/p38 activation, HIPK1-WT and KR5 enhanced, whereas HIPK1-DN or HIPK1 RNAi blocked, TNFα (plus cycloheximide)-induced EC apoptosis (Fig. 6d).

HIPK1 Enhances Releases of Trx and 14-3-3 from ASK1 and EC Apoptosis—To understand the mechanism by which HIPK1 enhances ASK1 activation, we next determined the role of HIPK1 in the dissociation of ASK1 from its inhibitors Trx1 and 14-3-3, a critical step in ASK1 activation. BAEC were transfected with the constitutively active form of HIPK1 (HIPK1-KR5) and AIP1 (AIP1-N) individually or together; phosphorylation of ASK1 at Ser-967 (the 14-3-3-binding site) and ASK1 activation (phosphorylation at Thr-845) were determined by Western blot with phospho-specific antibodies. Association of ASK1 with 14-3-3 or Trx1 with was also determined by immunoprecipitation with anti-14-3-3 or anti-Trx1 followed by Western blot with anti-ASK1. Expression of HIPK1-KR5 did not alter the ASK1 protein level (Fig. 7a). However, expression of HIPK1-KR5 significantly increased ASK1 activation (Thr(P)-845) concomitant with reduction of Ser(P)-967 (Fig. 7b) and 14-3-3 binding to ASK1 (Fig. 7c). Consistent with our previous findings (17), AIP1-N specifically facilitated the release of 14-3-3 binding to ASK1 (Fig. 7). In contrast, HIPK1-KR5 also significantly reduced Trx1 binding to ASK1, suggesting that HIPK1 is involved in the dissociation of ASK1 from both Trx1 and 14-3-3 (Fig. 7c). As expected, HIPK1-KR5 and AIP1-N together enhanced reduction of ASK1 Ser(P)-967 and 14-3-3 binding as well as Trx1 binding to ASK1 leading to synergistic activation of ASK1 (Thr(P)-845) (Fig. 7b and c). We further determined whether HIPK1 and AIP1 synergistically enhanced ASK1-mediated EC apoptosis. BAEC were transfected with HIPK1-KR5 and AIP1-N alone or together in the presence or absence of ASK1. EC apoptosis was determined by DAPI staining for nuclei fragmentation. AIP1-N or HIPK1-
KR5 only weakly induced EC apoptosis. However, AIP1-N and HIPK1-KR5 together synergistically increased both basal and ASK1-induced EC apoptosis. (Fig. 7d). Taken together, these data suggest that HIPK1 in the nucleus (upon expression of HIPK1-KR5) forms a complex with AIP1-ASK1 and enhances the release of Trx and 14-3-3 from ASK1, leading to ASK1 activation and EC apoptosis.

**DISCUSSION**

Based on our data, we propose the following model for the role of HIPK1 in ASK1-JNK signaling (Fig. 8). HIPK1 is localized in the nucleus in resting EC. TNFα induces translocations of AIP1 (from membrane to cytoplasm) and HIPK1 (from nucleus to cytoplasm) leading to formation of HIPK1-AIP1-ASK1 ternary complex. HIPK1 transduces TNFα-induced ASK1-JNK/p38 activation involving three steps: desumoylation of HIPK1 by undefined mechanism; HIPK1 cytoplasmic translocation and association of HIPK1 with AIP1-ASK1 complex; and enhanced dissociation of Trx1 and 14-3-3 from ASK1 leading to activation of ASK1-JNK/p38 signaling.

**FIG. 8.** A model for regulation of ASK1-JNK/p38 signaling by SENP1-HIPK1. HIPK1 is localized in the nucleus in resting EC. TNFα induces translocations of AIP1 (from membrane to cytoplasm) and HIPK1 (from nucleus to cytoplasm) leading to formation of HIPK1-AIP1-ASK1 ternary complex. HIPK1 transduces TNFα-induced ASK1-JNK/p38 activation involving three steps: desumoylation of HIPK1 by undefined mechanism; HIPK1 cytoplasmic translocation and association of HIPK1 with AIP1-ASK1 complex; and enhanced dissociation of Trx1 and 14-3-3 from ASK1 leading to activation of ASK1-JNK/p38 signaling.
the first molecule implicated in TNFα-induced ASK1 activation. TRAF2 binds to the C-terminal domain of ASK1 to facilitate ASK1 oligomerization, which in turn leads to ASK1 autophosphorylation/activation (11, 14). TRAF2 also plays a role in endoplasmic reticulum stress-induced ASK1-JNK signaling by recruiting ASK1 to an endoplasmic reticulum transmembrane sensor IRE1 to form IRE1-TRAP2-ASK1 complex (40). It is conceivable that different adaptor molecules are responsible for transducing stress signals derived from distinct intracellular compartments to converge on the ASK1 complex.

The most important finding of our present study is that we have identified HIPK1 as a novel signal transducer blocked from nucleus to cytoplasm where it associates with and activates ASK1 (3). Moreover, Daxx-induced ASK1 activation is mediated by interaction of Daxx to the N-terminal domain of ASK1, and the kinase activity of ASK1 is not critical for Daxx-ASK1-mediated apoptosis (41). We did not observe Daxx cytoplasmic translocation in response to TNFα. Although HIPK1 has been recently shown to modulate Daxx localization, the translocation occurs within the nuclear compartments (36). Thus, Daxx cytoplasmic translocation might be specific to Fas activation.

The mechanism by which TNFα induces desumoylation and translocation of HIPK1 is not clear. Our data suggest that the SENP, SUMO-specific proteases may be critical mediators in TNFα-induced desumoylation of HIPK1 leading to subsequent translocation of HIPK1 from nucleus to cytoplasm. Furthermore, inhibition of JNK (by inhibitor SP600125) or p38 (by inhibitor SB203580) had no effects on the initial translocation of HIPK1. Together with the functional studies (see below), it is likely that TNFα-induced initial translocation of HIPK1 precedes TNFα-induced ASK1-JNK/p38 activation. Interestingly, we show that SP600125 (but not SB203580) specifically blocked the return of HIPK1 from cytoplasm to nucleus at 60 min. It has been recently shown that JNK can phosphorylate HIPK1 in stress signaling.

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31. Kondo, S., Lu, Y., Debbas, M., Lin, A. W., Sarosi, I., Itie, A., Wakeham, A.,
Tuan, J., Saris, C., Elliott, G., Ma, W., Benchimol, S., Lowe, S. W., Mak,
T. W., and Thukral, S. K. (2003) Proc. Natl. Acad. Sci. U. S. A. 100,
5431–5436
32. Zhang, Q., Yoshimatsu, Y., Hildebrand, J., Frisch, S. M., and Goodman, R. H.
(2003) Cell 115, 177–186
33. Li, X., Wang, Y., Debatin, K. M., and Hug, H. (2000) Biochem. Biophys. Res.
Commun. 277, 513–517
34. Rochat-Steiner, V., Becker, K., Micheau, O., Schneider, P., Burns, K., and
Tschopp, J. (2000) J. Exp. Med. 192, 1165–1174
35. Kim, Y. H., Choi, C. Y., and Kim, Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96,
12350–12355
36. Esedey, J. A., Michaelson, J. S., and Leder, P. (2003) Mol. Cell. Biol. 23,
950–960
37. Zhang, H., Zhang, R., Luo, Y., D’Alessio, A., Peber, J. S., and Min, W. (2004)
J. Biol. Chem. 279, 44955–44965
38. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S.
(2002) Genes Dev. 16, 948–958
39. Nishitoh, H., Matsuzawa, A., Tsuchimine, K., Saegusa, K., Takeda, K., Inoue, K.,
Hori, S., Kakizuka, A., and Ichijo, H. (2002) Genes Dev. 16, 1345–1355
40. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and
Ron, D. (2000) Science 287, 664–666
41. Charette, S. J., Lambert, H., and Landry, J. (2001) J. Biol. Chem. 276,
36071–36074
42. Song, J. J., and Lee, Y. J. (2003) J. Biol. Chem. 278, 47245–47252
43. Huang, T. T., Wuerzberger-Davis, S. M., Wu, Z. H., and Miyamoto, S. (2003)
Cell 115, 565–576
44. Hay, R. T. (2004) Nat. Cell Biol. 6, 89–91