Previously we have shown that ASK-interacting protein 1 (AIP1, also known as DAB2IP), a novel member of the Ras-GAP family, mediates TNF-induced activation of ASK1-JNK signaling pathway. However, the mechanism by which TNF signaling is coupled to AIP1 is not known. Here we show that AIP1 is localized on the plasma membrane in resting endothelial cells (EC) in a complex with TNFR1. TNF binding induces release of AIP1 from TNFR1, resulting in cytoplasmic translocation and concomitant formation of an intracellular signaling complex comprised of TRADD, RIP1, TRAF2, and AIP1. A proline-rich region (amino acids 796–807) is critical for maintaining AIP1 in a closed form, which associates with a region of TNFR1 distinct from the death domain, the site of TNFR1 association with TRADD. An AIP1 mutant with deletion of this proline-rich region constitutively binds to TRAF2 and ASK1. A PERIOD-like domain (amino acids 591–719) of AIP1 binds to the intact RING finger of TRAF2, and specifically enhances TRAF2-induced ASK1 activation. At the same time, the binding of AIP1 to TRAF2 inhibits TNF-induced IKK-NF-κB signaling. Taken together, our data suggest that AIP1 is a novel transducer in TNF-induced TRAF2-dependent activation of ASK1 that mediates a balance between JNK versus NF-κB signaling.

Cytokines of the TNF1 superfamily utilize receptors that are devoid of intrinsic catalytic activity (1). Vascular endothelial cells (EC) are among the principal physiological targets of TNF (2). In EC, as in other cell types, TNF elicits a broad spectrum of biological effects including proliferation, differentiation, and apoptosis. TNF signals are initiated by binding to either of two different cell surface receptors, known as TNFR1 (CD122a) and TNFR2 (CD122b). The best-characterized signaling pathways are initiated by TNFR1. A current model postulates that in the absence of TNF, TNFR1 is kept in an inactive state through binding of an adaptor protein called silencer of death-domains (SODD). The interaction between TNFR1 and SODD is mediated by association of death domains on both proteins. TNF induces dissociation of SODD from TNFR1 with concomitant recruitment of TNFR-associated death-domain protein (TRADD) (3, 4). TRADD functions as a platform adaptor to initiate assembly of a multiprotein complex that activates downstream signaling pathways. Specifically, TRADD recruits both receptor-interacting protein-1 (RIP1) and TNFR-associated factor (TRAF2), which in turn recruit the IkB kinase complex (IKK) and a mitogen-activated protein kinase kinase kinase (MAP3K), leading to activation of NF-κB and JNK pathways, respectively (5–8). Finally it has been proposed that TNFR1-TRADD-RIP-TRAF2 complex functions as an initial membrane bound complex (complex I) to specifically activate the NF-κB cascade. The role of the complex I in the JNK cascade has not been addressed (8). Complex I is rapidly internalized, and TRADD rapidly dissociates from TNFR1. After a lag of several hours, internalized TRADD may recruit Fas-associated death domain protein (FADD) and procaspase-8 to form a cytoplasmic complex (complex II). Complex II promotes autocatalytic activation of pro-caspase-8 to initiate apoptosis unless the NF-κB-induced long isoform of the component known as FLICE inhibitory protein (FLIPL) is also present in the complex (9). The events between the dissociation of complex I and the formation of complex II are not well established.

TRAF2 is a member of a family of signal transducing proteins. Six members of TRAF family have been extensively studied (10) and TRAF7 has been recently described (11). JNK and NF-κB pathways can be activated by overexpression of TRAF 2, 5, and 6, but not of TRAF 1, 3, and 4, suggesting that different TRAFs, despite their structural homology, might perform very different functions. Studies from various cell types have identified TRAF2 as the bifurcation of two different kinase cascades leading to activation of NF-κB and JNK. TRADD-TRAF2/RIP-IKK for NF-κB activation, and TRADD-TRAF2-MAP3K-MAP2K for JNK activation (5, 6). Recent studies of TRAF2 knockout mice or transgenic mice expressing dominant negative TRAF2 mutant protein have established that TRAF2 is absolutely required for activation of JNK by TNF, although it may be redundant in some cell types for NF-κB activation (12, 13) due to presence of TRAF5 (14). Studies in transfected cells
have shown that the intact RING finger of TRAF2 is critical for TRAF2 function since a TRAF2 mutant with deletion of the N terminus (87–501) behaves as a dominant negative mutant for both NF-κB and JNK activation (15). The TRAF2 gene may code for an alternatively spliced form, designated as TRAF2-A, which contains a seven amino acids insertion in its RING domain (16). TRAF2-A fails to activate NF-κB pathway while still retains an ability to activate the JNK pathway (17). These results suggest that NF-κB and JNK pathways may be differentially regulated by minor alterations in the effector domain of TRAF2 or by modulators that bind to different sites on the TRAF2 RING finger.

The mechanism by which TRAFs activate MAP3Ks remains unclear, and there is some disagreement about which MAP3K is critical for JNK activation. It has been shown that the TRAF2 domain of TRAF2 and TRAF6 interact with apoptosis signal-regulating kinase-1 (ASK1), a member of the MAP3K family that specifically activates a cascade ending with JNK (but not NF-κB) activation. The association of TRAFs with ASK1 is required for ASK1 activation (18). ASK1 is a 170-kDa protein containing an inhibitory N-terminal domain, an internal kinase domain, and the C-terminal domain interacting with TRAFs (18, 19). The current model for ASK1 activation by TNF involves several critical steps including release of inhibitors (thioredoxin and 14-3-3) (20, 21), TRAF-dependent homodimerization/polymerization (22), and ASK1 autophosphorylation at Thr-845 (23). We have recently shown that TNF can induce a TRAF2-dependent association of ASK1 with ASK-interacting protein 1 (AIP1, also called DAB2IP for DAB2-interacting protein), a novel member of the Ras-GAP family (24, 25). AIP1 causes 14-3-3 release from ASK1, initiating ASK1-dependent JNK activation (24).

In the present study, we show that AIP1 is localized to the plasma membrane of EC where it associates with TNFR1 through a site distinct from the death domain involved in SODD and TRADD binding. In response to TNF, AIP1 dissociates from TNFR1 with concomitant cytoplasmic translocation and formation of a complex comprised of TRADD, RIP1, TRAF2, and AIP1, which is distinct from the complex I and complex II and which initiates specific activation of the ASK1-JNK pathway. Furthermore, we demonstrate that AIP1 specifically interacts with the effector domain RING finger of TRAF2 to enhance TNF-induced ASK1-JNK but to inhibit IKK-NF-κB signaling. Our data suggest that AIP1 is a novel transducer of TRAF2 in TNF-induced ASK1-JNK signaling.

MATERIALS AND METHODS

Plasmid Construction—Expression plasmids for truncated TNFR1 was generously provided by Dr. Martin Krone (Institute for Medical Microbiology, Immunology and Hygiene, Medical Center of the University of Cologne, Cologne, Germany) (26). Expression plasmids for TRAF2, ASK1, and AIP1 were described previously (24, 27). Deletion constructs of TRAF2 and AIP1 were generated by PCR and cloning into FLAG vectors. TRAF2-CA was generated by site-directed mutagenesis with expression constructs for FLAG-tagged AIP1 containing ectopic (Invitrogen, Life Technologies) containing 10% fetal calf serum, 2% (w/v) HAT (hypoxanthine/aminopterin/thymidine (Sigma), 20 μM l-glutamine, 100 units/ml penicillin/streptomycin) (Invitrogen, Life Technologies) at 37 °C in a 5% CO₂ humidified atmosphere. Human rTNF was from R&D Systems (Minneapolis, MN) and used at 10 ng/ml.

The Kinase Assays for JNK, ASK1, and IKK—JNK, ASK1, and IKK assay was performed by kinase assays using GST-c-Jun (1–80), GST-MKK4, GST-1,1-ba fusion proteins as a substrate, respectively (24, 27).

Transfection and Reporter Assay—Transfection of HUVEC was performed by DEAE-Dextran method as described previously (27). BAEC and EAhy926 were transfected by LipofectAMINE 2000 (Invitrogen, Life Technologies). Luciferase activity followed by Renilla activity was measured twice in duplicate using a Berthold luminometer. All data were normalized as relative luciferase light units/Renilla unit.

Isolation of Caveolae-enriched Membranes—Purification of caveolae-enriched membrane fractions was performed as described (29) with minor modifications. In brief, EAhy926 cells were treated with 10 ng/ml TNF for the indicated time point, washed twice with ice-cold Dulbecco’s phosphate-buffered saline and scraped into 1 ml of MBS (25 mM MES, pH 6.5, 0.15 μM NaCl, 5 mM EDTA, and 0.2% Triton X-100) containing 15 μM inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM Pefabloc) and left 20 min on ice. The suspension was subjected to 10–15 strokes in a Dounce homogenizer and centrifuged for 10 min at 2000 rpm at 4 °C to remove high density debris. Clarified post-nuclear supernatants were combined with 90% (w/v) sucrose prepared in MES, transferred to the bottom of a Beckman 12.5 ml ultracentrifuge tubes and overlayed gently with 6 ml of 35% and 3 ml of 5% sucrose respectively. The resulting 5–40% discontinuous sucrose gradients were centrifuged 18–20 h at 40,000 rpm in a SW41 Beckman rotor at 4 °C to allow the separation of the low density membranes. After centrifugation, a floating ligand corresponding to the Triton X-100 insoluble material, was detectable at the interface between 35 and 5% of each gradient. Fractions were harvested from the top to the bottom of the gradients and analyzed either by SDS-PAGE followed by immunoblotting for cavelin-1, TRFN1, TRAF2, or AIP1 or for binding of cholesterol subunit B (CTxB) by dot blot analysis.

Immunoprecipitation and Immunoblotting—EAhy926 cells or BAEC were washed twice with cold PBS 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 fluoride, 1 mM EDTA) for 20 min on ice. Immunoprecipitation and immunoblotting were as described previously (24, 27). A rabbit polyclonal antibody against AIP1 was generated by immunizing rabbits with GST-AIP1 protein through Cocalico Biologicals Inc. (Reamstown, PA) (24). Anti-TRAF2, anti-TRADD, anti-RIP1, anti-ASK1, and anti-GST were purchased from Santa Cruz Biotechnology. Anti-TNFFR1 was purchased from R&D System. Anti-FLAG (M2) was from Sigma.

Indirect Immunofluorescence Confocal Microscopy—Fixation, permeabilization, and staining of cultured EC were performed as described previously (27). Alexa Fluor 488 (green) or 594 (red) conjugated-second antibody (Molecular Probes, Eugene, OR) were used. Confocal immunofluorescence microscopy was performed using an Olympus confocal microscope and acquired images were transferred to Photoshop 6.0 to generate the final figures.

RESULTS

TNF Induces a Translocation of AIP1 from Plasma Membrane to Cytoplasm in EC—We have recently shown that AIP1/DAB2IP is highly expressed in EC but not in several common tumor cell lines including human prostate cancer and breast cancer cell lines (24, 30). AIP1 contains a pleckstrin homology (PH) and a PKC-conserved region 2 (C2), two motifs implicated in plasma membrane targeting. To determine if AIP1 is localized on EC plasma membrane, we first examined AIP1 localization in resting BAEC by indirect immunofluorescence microscopy with an anti-AIP1 antibody. AIP1 showed a distinct plasma membrane staining (Fig. 1a, top panel). Control staining with a normal rabbit serum did not show any staining. To further improve AIP1 to plasma membrane localization, an EC membrane protein CD31 was used as a positive control. AIP1 and CD31 showed co-localization on EC (Fig. 1a, bottom panel). AIP1 also showed plasma membrane localization in cultured HUVEC (not shown). To determine the critical domain for AIP1 for membrane localization, BAEC were transiently transfected with expression constructs for FLAG-tagged AIP1 containing various domains (AIP1-F for the full-length, AIP1-N for the N-terminal domain containing the PH, C2, and GAP domains, AIP1-PHC2, AIP1-PH, AIP1-ΔPH for the mutant with a deletion of the PH domain, and AIP1-C for the C-terminal domain).
AIP1-F, AIP1-N, and PHC2 are predominantly localized on membrane vesicles (or microdomains). AIP1-PH showed plasma membrane as well as cytoplasm with no detectable vesicles, and AIP1-PH also caused elongation of cells. In contrast, AIP1-C and AIP1-ΔPH are detected in the cytoplasm, suggesting that the PH and C2 domain are critical for AIP1 localization. AIP1 as a Transducer of TRAF2

**Fig. 1.** TNF induces a translocation of AIP1 from plasma membrane to cytoplasm in EC. *a,* AIP1 is localized on EC plasma membrane. *Top panel,* endogenous AIP1 in BAEC was stained with anti-AIP1 followed by an Alexa Fluor 488-conjugated anti-rabbit secondary antibody. Cells were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for nucleus. *Bottom panel,* AIP1 and EC membrane protein CD31 were costained with anti-AIP1 (rabbit) and anti-CD31 (mouse) followed by an Alexa Fluor 488 conjugated anti-rabbit and an Alexa Fluor 594 conjugated anti-mouse secondary antibodies. Location of AIP1 and CD31 was visualized under a confocal fluorescence microscope. *b,* critical domain of AIP1 for membrane localization. AIP1 constructs containing different domains (AIP1-F, N, PHC2, PH, and C) were transfected into BAEC, and localization of AIP1 proteins was determined by indirect immunofluorescence microscopy with anti-FLAG followed by an Alexa Fluor 488 (green)-conjugated anti-mouse secondary antibody. *c,* TNF induces AIP1 translocation in a time-dependent manner. Human EC (HUVEC) were treated with TNF (10 ng/ml) for various time points (0, 5, 15, 30, 45, and 60 min). EC were stained with AIP1 followed by an Alexa Fluor 594-conjugated anti-rabbit secondary antibody. Localization of AIP1 at 0, 15, and 60 min are shown.
localization in membrane microdomains (Fig. 1b).

Previously we have shown that TNF induces association of AIP1 with ASK1, which is predominantly localized in cytoplasm, raising the possibility that TNF might induce a translocation of AIP1 from plasma membrane to cytoplasm. To test this hypothesis, HUVEC were treated with TNF (10 ng/ml) for indicated times. Protein expression was determined with respective antibodies. b, cell lysates were immunoprecipitated with indicated antibodies (TNFR1, TRADD, RIP1, TRAF2) followed by Western blot with anti-AIP1. As controls, TRAF2-ASK1 and AIP1-ASK1 complex were determined by immunoprecipitation with anti-TRAF2 or anti-AIP1 followed by Western blot with anti-ASK1. ASK1 activity was determined by an *in vitro* kinase assay using GST-MKK4 as a substrate. c, TNFR1-TRADD association precedes AIP1 complex. EAhy926 were treated with TNF for indicated time (0, 2, 5, 15, or 30 min) and association of TNFR1-TRADD was determined by immunoprecipitation with anti-TNFR1 followed by Western blot with anti-TRADD. d, AIP1 is not localized in caveolae. EAhy926 cells were either untreated or treated with TNF (10 ng/ml for 15 min) and fractionated by sucrose gradient as described. No protein was detected in fractions 1–2 and 10 μl of cell lysates from fractions 3–10 were subjected to Western blot with anti-TNFR1, anti-TRAF2 and anti-AIP1. TRADD, RIP1 and ASK1 were also determined. Anti-caveolin-1 was used as a control for caveolae.

**FIG. 2.** TNF induces release of AIP1 from TNFR1 with concomitant formation of AIP1 complex (TRADD-RIP1-TRAF2-AIP1-ASK1) in EC. a, human EC EAhy926 were treated with TNF (10 ng/ml) for indicated times. Protein expression was determined with respective antibodies. b, cell lysates were immunoprecipitated with indicated antibodies (TNFR1, TRADD, RIP1, TRAF2) followed by Western blot with anti-AIP1. As controls, TRAF2-ASK1 and AIP1-ASK1 complex were determined by immunoprecipitation with anti-TRAF2 or anti-AIP1 followed by Western blot with anti-ASK1. ASK1 activity was determined by an *in vitro* kinase assay using GST-MKK4 as a substrate. c, TNFR1-TRADD association precedes AIP1 complex. EAhy926 were treated with TNF for indicated time (0, 2, 5, 15, or 30 min) and association of TNFR1-TRADD was determined by immunoprecipitation with anti-TNFR1 followed by Western blot with anti-TRADD. d, AIP1 is not localized in caveolae. EAhy926 cells were either untreated or treated with TNF (10 ng/ml for 15 min) and fractionated by sucrose gradient as described. No protein was detected in fractions 1–2 and 10 μl of cell lysates from fractions 3–10 were subjected to Western blot with anti-TNFR1, anti-TRAF2 and anti-AIP1. TRADD, RIP1 and ASK1 were also determined. Anti-caveolin-1 was used as a control for caveolae.

EC were treated with TNF (10 ng/ml for 0, 15, and 60 min) and association of AIP1 with TNFR1 signaling components by co-immunoprecipitation assays. TNF treatment did not alter protein expression of TNFR1, TRADD, RIP1, TRAF2, AIP1, or ASK1 (Fig. 2a). AIP1-TNFR1 complex is readily detected in resting EC. In response to TNF, AIP1 is dissociated from TNFR1 at 15 min but reassociates with TNFR1 at 60 min (Fig. 2b), consistent with the kinetics of AIP1 translocation. Formation of AIP1-SODD complex shows a similar kinetics as that of AIP1-TNFR1 complex (not shown). In contrast, TNF treatment induced a strong interaction of AIP1 with TRADD, RIP1, and TRAF2 at 15 min, which declined by 60 min of treatment (Fig. 2b). Association of TRAF2 and AIP1 with ASK1 as well as ASK1 activation show a similar kinetics (Fig. 2c). These data suggest that TNF induces formation of a complex comprising of TRADD-RIP1-TRAF2-AIP1-ASK1 (so named AIP1 complex) in EC. Furthermore, we determined kinetics of TNFR1-TRADD complex, which peaks at 2 min, declines at 5 min and disappears by 15 min (Fig. 2d), consistent with previous studies of HUVEC (31). Interestingly AIP1, which associates with the unactivated TNFR1 molecule, is absent in the TNFR1-TRADD-RIP1-TRAF2 complex (complex 1), which assembles on the plasma membrane and has been shown to trigger activation of NF-κB pathway (9).
Ehhy296 displays an extensive caveolar system, which has been shown to be critical for signaling by TNFR1 and TRAF2 (32, 33). To determine if AIP1 complex is localized in caveolae, Ehhy296 were treated with TNF (10 ng/ml for 15) and cell fractions (fractions 1–10) by sucrose gradient centrifugation following Dounce homogenization in Triton X-100 buffer. In resting EC, the majority of TNFR1 is detected in fractions 10 containing heavy membranes and a small portion of TNFR1 can be detected in fractions 4–5 where caveolin-1 is typically distributed. TNF decreases distribution of TNFR1 in fractions 4–5 at 15 min (Fig. 2c). TRAF2 is also detected in caveolar fractions in both resting and TNF-treated EC. However, AIP1 is only detected in fractions 10, but not in other fractions, suggesting that AIP1 is not localized in caveolae (Fig. 2c). Similar to AIP1, TRADD, RIP1, and ASK1 were not detected in caveolar fractions (Fig. 2c). Taken together, these data suggest that AIP1 associates with TNFR1 in resting EC whereas associates with TRADD-RIP1-TRAF2-ASK1 in TNF-treated EC in different membrane microdomains other than caveolae.

**AIP1 in a Closed Inactive Form Associates with TNFR1 Whereas in an Open Active Form Binds to TRAF2/ASK1**—We have previously shown that in resting EC AIP1 is retained in a closed conformation by intramolecular interactions and AIP1 associates with ASK1 only after the disruption of these intramolecular interactions (24). To define the critical domains for AIP1 intramolecular loop formation, we first generated deletion constructs of AIP1 at the N terminus or the C terminus (AIP1-ΔPH with a deletion of the N-terminal PH, amino acids 81–1056), AIP1-ΔLZ with deletion of the C-terminal LZ motif, amino acids 1–910), and AIP1-ΔPR with a deletion of the PR region, amino acids 1–796) (Fig. 3a). EC were transfected with various AIP1 mutants, and association of ASK1 with various AIP1 was determined by co-immunoprecipitation assays. AIP1-ΔPR, but not AIP1-F, AIP1-ΔPH or AIP1-ΔLZ, binds to ASK1 in the absence of TNF (Fig. 3b, −TNF). However, TNF treatment (10 ng/ml for 15 min) induced association of ASK1 with these AIP1 proteins (Fig. 3b, +TNF). These data suggest that the PR region, but not the LZ motif or the PH domain, is a critical domain in retaining AIP1 in a closed inactive form.

Association of AIP1 mutants with endogenous TRAF2 and TNFR1 in EC were then examined by co-immunoprecipitation assays. AIP1-ΔPR, but not AIP1-F, AIP1-ΔPH, or AIP1-ΔLZ, binds to TRAF2 in the absence of TNF (Fig. 3b, −TNF). Like ASK1, TRAF2 associated with the AIP1 proteins in response to TNF (Fig. 3b, +TNF). In contrast, TNFR1 associates with AIP1-F, AIP1-ΔPH, and AIP1-ΔLZ, but not to AIP1-ΔPR (Fig. 3b). These data suggest that TNFR1 binds to a closed inactive whereas TRAF2/ASK1 bind to an open active form of AIP1. To further test this idea, we examined association of AIP1-F (the closed form) and AIP1-ΔPR (the open form) with TNFR1 and TRAF2 in response to TNF. BAEC were transfected with AIP1-F or AIP1-ΔPR in the absence or presence of TNF (10 ng/ml for 15 min). Association of AIP1 proteins with endogenous TNFR1 and TRAF2 were determined. AIP1-F (but not AIP1-ΔPR) binds to TNFR1 in resting EC, and AIP1-F/TNFR1 complex was significantly reduced in the presence of TNF (Fig. 3c). In contrast, association of AIP1-F with TRAF2 is only weakly detected in resting EC but is strongly induced in response to TNF. AIP1-ΔPR constitutively binds to TRAF2 and TNF has no effects on their interaction (Fig. 3c). These data strongly suggest that TNF induces an alteration of AIP1 conformation leading to release of AIP1 from TNFR1 with concomitant binding of AIP1 to the TRAF2/ASK1 complex.

**AIP1 Associates with a Non-death Domain Region of TNFR1**—We next determined the critical domain in TNFR1 for AIP1 binding. The intracellular domain of TNFR1 comprises of several conserved regions—a juxtamembrane (amino acids 205–307), a NSD (amino acids 308–319), which binds to an adaptor protein FAN responsible for neutral sphingomyelinase activation domain (34), and the death-domain (amino acids 346–426), which binds to SODD in resting state while recruits TRADD in response to TNF (Fig. 4a). To map the AIP1-binding domain (AID) in TNFR1, AIP1-F, and various TNFR1 truncates were transfected into BAEC. Expression of TNFR1 proteins was determined by an indirect fluorescence microscopy with anti-TNFR1 (Fig. 4b). Association of AIP1 with TNFR1 was determined by co-immunoprecipitation assay with anti-TNFR1 followed by Western blot with anti-FLAG (for AIP1-F). AIP1 associates TNFR1-WT and D212–308/346, but not with TNFR1-D205, D244, or D308–346), suggesting that the sequence between amino acids 309–346 of TNFR1 is critical for AIP1 interaction (Fig. 4c). AIP1 poorly binds to TNFR1-D320 with the intact NSD, which has been shown to be a FAN-binding motif, suggesting that AIP1 and FAN bind to different sequences of TNFR1. As a control, TRADD interacts with the death domain-containing TNFR1 (TNFR1-WT and D308–346) as determined by Western blot with anti-TRADD. The results were summarized in Fig. 4a. Taken together, these data suggest that AIP1 associates with TNFR1 at a site distinct from the TRADD-binding domain (DD) and the FAN-binding domain (NSD).

**AIP1 via a PERIOD-like Domain Binds to the Intact RING Finger of TRAF2**—TRAF2 associates with AIP1 in response to TNF (Fig. 2) and constitutively binds to an open form of AIP1 (AIP1-ΔPR) (Fig. 3). To further map the critical domain in AIP1 for TRAF2 binding, we determined association of TRAF2 with various truncated AIP1 proteins. Since we have previously shown that the C2 domain of AIP1 is critical for ASK1 association (24), and we first examined if TRAF2 binds to the N-terminal half of AIP1. Results show that TRAF2 did not interact with the N-terminal domains (AIP1-N, PHC2, and PH) (Fig. 5a), indicating that TRAF2 and ASK1 bind to different sites on AIP1. To determine TRAF2 binds to the C-terminal half of AIP1, we generated truncated AIP1-C (AIP1-C, C-ΔLZ with a deletion of the LZ motif, C-ΔPR with a deletion of the PR region). Results show that TRAF2 binds to AIP1-C-ΔPR, but not AIP1-C or C-ΔLZ (Fig. 5a). These data suggest that TRAF2 binds to an upstream sequence of the PR region in the C-terminal half of AIP1, and that the PR region is also critical to maintain AIP1-C in a closed conformation. Domain searching indicated that AIP1-C-ΔPR does not contain a TRAF2-binding consensus site but has a PERIOD-like domain (PER, amino acids 591–719). We further generated AIP1-C-PER (amino acids 522–719) and C-tPER (amino acids 522–620) with a truncation of PER domain. Association of TRAF2 with C-PR, C-PER, and C-tPER was then determined by a co-immunoprecipitation assay. TRAF2 binds to AIP1-C-ΔPR and C-PER, but not C-tPER (Fig. 5b), suggesting that the PER domain in the C-terminal-half is involved in TRAF2 binding.

To define the critical domain of TRAF2 for AIP1 binding, we generated a series of mutant forms of FLAG-tagged TRAF2. DN-TRAF2 (amino acids 80–531) contains a deletion of the whole RING finger whereas TRAF2-CA contains a single mutation at C31 within the RING finger leading to disruption of the RING structure (15). TRAF2-N (amino acids 1–249) contains the intact RING and the five zinc fingers of the N-terminal half of TRAF2 whereas TRAF2-C (amino acids 265–531) consists of the C-terminal TRAF domains of TRAF2 (Fig. 5c). AIP1-ΔPR was co-transfected with various TRAF2, and association of AIP1-ΔPR with TRAF2 molecules was determined. As
previously described, TRAF2-N was detected in an insoluble fraction (27) and we cannot determine association of AIP1 with TRAF2-N. Results show that only TRAF2-WT binds to AIP1/H9004PR. However, DN-TRAF2, TRAF2-CA, or TRAF2-C did not interact with AIP1/H9004PR (Fig. 5d). These data indicate that the intact RING finger (the effector domain) of TRAF2 is required for AIP1 association.

AIP1 Is a Transducer of TRAF2 and Specifically Induces ASK1-JNK Activation While It Inhibits NF-κB Activation—
TRAF2 has been shown to be a critical adaptor in TNF-induced activation of both JNK and NF-κB cascades. We first determined effects of AIP1 in TRAF2-induced JNK and NF-κB activation in reporter gene assays. BAEC were transfected with FLAG-tagged truncated AIP1 (AIP1-F, ΔPH, ΔLZ, ΔPR), and cells were untreated or treated with TNF (10 ng/ml for 15 min). Association of endogenous ASK1, TRAF2, or TNFR1 with AIP1 domains was examined by immunoprecipitation with a respective antibody followed by Western blot with anti-FLAG. c, TNF responses of AIP1-TNFR1 and TRAF2-AIP1 complexes. BAEC were transfected with AIP1-F or ΔPR and treated with TNF (10 ng/ml for 15 min). Association of AIP1 with TNFR1 and TRAF2 was determined as in b.

FIG. 3. AIP1 in a closed inactive form associates with TNFR1 whereas in an open active form binds to TRAF2/ASK1. a, schematic diagram of AIP1 domains and expression constructs (C2, protein kinase C-conserved domain 2; GAP, GTPase-activating protein; PER, period-like domain; PR, proline-rich; LZ, leucine-zipper; aa, amino acid). b, proline-rich region is critical for closed conformation of AIP1. BAEC were transfected with FLAG-tagged truncated AIP1 (AIP1-F, ΔPH, ΔLZ, ΔPR), and cells were untreated or treated with TNF (10 ng/ml for 15 min). Association of endogenous ASK1, TRAF2, or TNFR1 with AIP1 domains was examined by immunoprecipitation with a respective antibody followed by Western blot with anti-FLAG. c, TNF responses of AIP1-TNFR1 and TRAF2-AIP1 complexes. BAEC were transfected with AIP1-F or ΔPR and treated with TNF (10 ng/ml for 15 min). Association of AIP1 with TNFR1 and TRAF2 was determined as in b.
effector domain of TRAF2 to reciprocally regulate TNF-induced JNK and NF-κB cascades.

We then determined the critical domains of AIP1 in regulating TNF-induced JNK and NF-κB activation. BAEC were transfected with FLAG-tagged AIP1 and various TNFR1 deletion constructs. Expression of TNFR1 proteins was determined by indirect immunofluorescence microscopy with anti-TNFR1 (goat, recognizing the extracellular domain of TNFR1) followed by an Alexa Fluor 488-conjugated anti-goat secondary antibody (b). Association of AIP1 domains with TNFR1 was determined by immunoprecipitation with anti-TNFR1 followed by Western blot with anti-FLAG (c). TNFR1-associated TRADD was also determined by anti-TRADD. Associations of these TNFR1 proteins with TRADD and AIP1 are summarized on the right panel in a.

FIG. 4. AIP1 associates with a non-death domain region of TNFR1. a, schematic diagram of TNFR1 domains and deletion constructs (EM, extracellular domain; TM, transmembrane; JMD, juxtamembrane; NSD, neutral sphingomyelinase domain; DD, death domain; aa, amino acid). b and c, AIP1-interacting domain (AID) is located between NSD and DD. BAEC were transfected with FLAG-tagged AIP1 and various TNFR1 deletion constructs. Expression of TNFR1 proteins was determined by indirect immunofluorescence microscopy with anti-TNFR1 (goat, recognizing the extracellular domain of TNFR1) followed by an Alexa Fluor 488-conjugated anti-goat secondary antibody (b). Association of AIP1 domains with TNFR1 was determined by immunoprecipitation with anti-TNFR1 followed by Western blot with anti-FLAG (c). TNFR1-associated TRADD was also determined by anti-TRADD. Associations of these TNFR1 proteins with TRADD and AIP1 are summarized on the right panel in a.

binding abilities for ASK1 and TRAF2.

We have previously shown that a short hairpin RNA (shRNA) down-regulated endogenous AIP1 level leading to enhanced TNF-induced ASK1-JNK activation and apoptosis (24). To determine a physiological role of AIP1 in TNF-induced NF-κB signaling, we further examined TNF-induced ASK1 and IKK activation in AIP1-knockdown cells by in vitro kinase assays as described. Endogenous AIP1 expression was significantly (80%) reduced in BAEC expressing a short hairpin RNA of AIP1 (ShAIP1) while expression of TRAF2 or ASK1 was not altered (Fig. 6c). As we have shown previously (24), knockdown of AIP1 inhibited TNF-induced ASK1 activation. However, AIP1 knockdown cells showed enhanced TNF-induced IKK kinase activity (Fig. 6f). These data further support that physiological AIP1 differentially regulates TNF-induced ASK1-JNK and IKK-NF-κB pathways.
FIG. 5. AIP1 via a PERIOD-like domain binds to the intact RING finger of TRAF2. a and b, critical domains of AIP1 for TRAF2 binding. Truncated AIP1-N (N, PHC2, and PH) and AIP1-C (C, C-APR, C-ΔLZ) were transfected into BAEC. Association of endogenous TRAF2 with AIP1 proteins was determined by immunoprecipitation with anti-TRAF2 followed by Western blot with anti-FLAG.

Intermediate Complex—Tschopp and co-workers (9) have recently dissected TNFR1 signaling complexes in more details and have proposed a sequential signaling complex model: the initial plasma membrane bound complex (complex I comprising of AIP1 for TRAF2 binding. Truncated AIP1-ΔN (N, PHC2, and PH) and AIP1-C (C, C-APR, C-ΔLZ) were transfected into BAEC. Association of endogenous TRAF2 with AIP1 proteins was determined by immunoprecipitation with anti-TRAF2 followed by Western blot with anti-FLAG. These data suggest that AIP1 complex is also structurally different from complex II. Second, AIP1 complex is formed at 15 min in response to TNF and is dissociated by 60 min. However, complex II formation detected in certain cell types (HT1080) at 2–8 h post-treatment with TNF. Moreover, we could not detect association of AIP1 with FADD and pro-caspase-8 in response to TNF in EC (not shown). These data suggest that AIP1 complex is also structurally different from complex II. Most significantly, AIP1 overexpression enhances, whereas knockdown of AIP1 inhibits, TNF/TRAF2-induced ASK1-JNK activation. However, AIP1 has opposite effects on TNF/TRAF2-induced IKK-NF-κB pathway. These data suggest AIP1 complex is functionally different from complex I. In cell types including EC, ASK1-JNK activation induces apoptotic signaling which is dependent on intrinsic but not extrinsic pathways. We have recently shown that AIP1 enhances ASK1-mediated JNK activation and EC apoptosis. AIP1 suppressed IKK-NF-κB activation further support that AIP1 is proapoptotic. Thus, AIP complex is functionally different from complex II. Whether or not complex II is present in EC are under investigation.

AIP1 Specifies TRAF2 toward to ASK1-JNK Pathway—TRAF2 has been shown to be the bifurcation point in TNF-induced activation of NF-κB and activation of JNK (8, 35, 36). While the C-terminal TRAF domain is responsible for association with TNFR1-TRADD complex as well as the MAP3Ks, the N-terminal RING finger of TRAF2 is an effector domain in activation of both JNK and NF-κB. Deletion of the RING domain (TRAF2-(87–501)) completely loses the ability to activate JNK and NF-κB, and this mutant functions as a dominant negative. Specific downstream signaling events from TRAF2 are mediated by RIP/IKK and MAP3Ks. For example, IKK is specific for NF-κB signaling, while ASK1 appear to direct sig-
naling exclusively to JNK. In contrast, TAK1 and MEKK1 can activate both NF-κB and JNK cascades simultaneously. For example, sphingosine kinase binds to TRAF domain of TRAF2 and specifically activates NF-κB while inhibits JNK activation (39). In contrast, TNF-induced TRAF2 ubiquitination coincides with its translocation to an insoluble cellular fraction, which is critical for TNF-induced activation of JNK but not of NF-κB (40). These data suggest that TRAF2 modulation/translocation or interactions with other proteins may provide a new layer of regulation to determine specific downstream signaling (activation of JNK and NF-κB). Our study shows that AIP1 associates with TRAF2 in response to TNF and the intact RING finger of TRAF2 is required for TRAF2-AIP1 association. Moreover, TRAF2, AIP1, and ASK1 form a complex in response to TNF

FIG. 6. AIP1 as a transducer of TRAF2 and specifically induces ASK1-JNK activation while inhibits IKK-NF-κB activation. a, AIP1 specifically enhances TRAF2-induced JNK (but inhibits NF-κB) activation. AIP1 together with a JNK or NF-κB-dependent reporter gene was transfected into BAEC in the presence or absence of TRAF2 as indicated. A constitutive expression vector for Renilla was used as an internal control for transfection efficiency. Reporter gene activity was determined by luciferase assay as described. Data are presented as fold increase by expression of TRAF2 or AIP1 compared with the vector (as 1). Similar results were obtained from additional three independent experiments. b, AIP1 specifically enhances ASK1 whereas inhibits IKK activation by TNF. BAEC were transfected with a control vector or AIP1 and treated with TNF as indicated (10 ng/ml for 15 min). ASK1 and IKK activities were determined by an in vitro kinase assay using GST-MKK4 and GST-IκBα, respectively. Relative ASK1 and IKK activities are presented by taking TNF-treated VC as 1.0. Similar results were obtained from two additional independent experiments. c and d, critical domains of AIP1 in TNF-induced activation of JNK and NF-κB. BAEC were transfected with a JNK- or NF-κB-dependent reporter gene with various AIP1 (F, N, ΔPR, or ΔPER) as indicated and a constitutive expression vector for Renilla was used as an internal control for transfection efficiency. 24 h post-transfection, cells were treated with TNF (10 ng/ml) for 12 h. Reporter gene activity was determined by luciferase assay as described. Data are presented as fold increase by AIP1 expression by taking untreated vector as 1. Similar results were obtained from additional three independent experiments. e, knockdown of AIP1 in BAEC. BAEC were transiently transfected with pShag or pSh-AIP1 encoding a short-hairpin RNA of AIP1 (Sh-AIP1) as described previously (24). Total cell lysates were used to determine AIP1 expression by Western blot with anti-AIP1. As expected, expression of TRAF2 and ASK1 was not altered by Sh-AIP1. f, knockdown of AIP1 suppresses ASK1 activity whereas enhances IKK activation by TNF. BAEC expressing pShag or pSh-AIP1 were untreated or treated with TNF (10 ng/ml for 15 min). ASK1 and IKK activities were determined as described in b. Relative ASK1 and IKK activities are presented by taking untreated pShag as 1.0. Similar results were obtained from additional two experiments.
and overexpression of TRAF2 enhances association of AIP1 with ASK1, at least in part by unfolding both AIP1 and ASK1. Most importantly, AIP1 specifically mediates TNF/TRAf2-induced ASK1-JNK activation while inhibits IKK-NF-κB signaling as demonstrated by both overexpression and knockdown of AIP1. Although many TRAF-binding proteins have been identified to modulate TRAF2 function, AIP1 represents a first molecule, which may specify TRAF2 toward to ASK1-JNK pathway. It needs to further determine the effects of AIP1 on TRAF2 ubiquitination/translocation and TRADD-TRAf2-RIp1 complex formation.

REFERENCES

1. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) Cell 104, 487–501
2. Madge, L. A., and Pober, J. S. (2001) Exp. Mol. Pathol. 70, 317–325
3. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 769–778
4. Jiang, Y., Woronicz, J. D., Liu, W., and Goeddel, D. V. (1999) Science 283, 543–546
5. Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003) Cell Death Differ. 10, 45–65
6. Baud, V., and Karin, M. (2001) Trends Cell Biol. 11, 372–377
7. Ghosh, S., and Karin, M. (2002) Cell 108, suppl. 881–896
8. Varfolomeev, E. E., and Ashkenazi, A. (2004) Cell 116, 491–497
9. Miecznikowska, B., and Schoppmann, S. F. (2003) Cell 115, 181–189
10. Bradley, J. R., and Pober, J. S. (2001) Oncogene 20, 6492–6491
11. Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P. O., Bergamini, G., Crouthamer, K., Cruciati, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hofp, C., Hulte, B., Mangano, R., Michon, A. M., Schirle, M., Schlegl, J., Schwab, M., Stein, M. A., Bauer, A., Casarsi, G., Dreweas, G., Gavrieli, A. C., Jackson, D. B., Jobery, G., Neubauer, G., Rick, J., Kuster, B., and Superti-Furga, G. (2004) Nat. Cell Biol. 6, 97–105
12. Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C., and Choi, Y. (1997) Immunity 7, 703–713
13. Yeh, W. C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N., Ohashi, P., Rotte, M., Goeddel, D. V., and Mak, T. W. (1997) Immunity 7, 715–725
14. Tada, K., Okazaki, T., Sakon, S., Kobarai, T., Kurosawa, K., Yamaoka, S., Hashimoto, H., Mak, T. W., Yagita, H., Okumura, K., Yeh, W. C., and Nakano, H. (2001) J. Biol. Chem. 276, 36530–36534
15. Takeuchi, M., Rotte, M., and Goeddel, D. V. (1996) J. Biol. Chem. 271, 19935–19942
16. Brink, R., and Lodish, H. F. (1998) J. Biol. Chem. 273, 4129–4134
17. Badogostar, H., and Cheng, G. (1998) J. Biol. Chem. 273, 24775–24780
18. Nishizato, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rotte, M., Miyazato, K., and Ichijo, H. (1998) Mol. Cell 2, 389–395
19. Ichijio, H., Nishida, E., Irie, K., ten, D.-P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazato, K., and Gotto, Y. (1997) Science 275, 90–94
20. Saitoh, M., Nishizato, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawahata, M., Miyazato, K., and Ichijo, H. (1998) EMBO J. 17, 2596–2606
21. Zhang, L., Chen, J., and Fu, H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8511–8515
22. Gotto, Y., and Cooper, J. A. (1998) J. Biol. Chem. 273, 17477–17482
23. Tobiume, K., Saitoh, M., and Ichijo, H. (2002) J. Cell. Physiol. 191, 95–104
24. Zhang, R., He, X., Liu, W., Lu, M., Hsieh, J. T., and Min, W. (2003) J Clin. Invest. 111, 1893–1943
25. Guicciardi, M. E., and Gores, G. J. (2003) J. Clin. Invest. 111, 1813–1815
26. Adam, D., Wiegmann, K., Adam-Klages, S., Ruff, A., and Kronke, M. (1996) J. Biol. Chem. 271, 14617–14622
27. Min, W., Bradley, J. R., Galbraith, J. J., Jones, S. J., Ledgerwood, E. C., and Pober, J. S. (1998) J. Immunol. 161, 319–324
28. Edgell, C. J., McDonald, C. C., and Graham, J. B. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3734–3737

FIG. 7. A proposed model for AIP1 as a transducer of TRAF2 in TNF-induced ASK1-JNK activation. AIP1 via its PH and C2 domains anchors to EC plasma membrane where it associates with a non-death domain region of TNFRI. In response to TNF, AIP1 is dissociated from TNFRI with concomitant cytoplasmic translocation of AIP1 and formation of AIP1 complex comprising of TRADD-RIp2-AIP1, which specifically activates ASK1-JNK signaling. As demonstrated by Tschopp and co-workers (9), TNFR1 recruits TRADD, RIP1, and TRAF2 to form a membrane-bound complex I, which specifically activates NF-κB signaling. Since TNFRI-TRADD association precedes formation of AIP1 complex, it is likely that AIP1 complex is derived from complex I upon ubiquitination (●) and internalization of TRADD, RIP1, TRAF2, and AIP1. As we described previously (9), unfolded AIP1 binds to and activates ASK1-JNK at least in part by facilitating dephosphorylation of ASK1 at Ser967 and dissociation of ASK1 from its inhibitor 14-3-3.
29. Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993) *J. Cell Biol.* **122**, 789–807
30. Chen, H., Toyooka, S., Gazdar, A. F., and Hsieh, J. T. (2003) *J. Biol. Chem.* **278**, 3121–3130
31. Jones, S. J., Ledgerwood, E. C., Prins, J. B., Galbraith, J., Johnson, D. R., Pober, J. S., and Bradley, J. R. (1999) *J. Immunol.* **162**, 1942–1948
32. Legler, D. F., Micheau, O., Doucey, M. A., Tschopp, J., and Bron, C. (2003) *Immunity* **18**, 655–664
33. Arron, J. R., Pewzner-Jung, Y., Walsh, M. C., Kohayashi, T., and Choi, Y. (2002) *J. Exp. Med.* **196**, 923–934
34. Adam-Klages, S., Adam, D., Wiegemann, K., Struve, S., Kolanus, W., Schneider-Mergener, J., and Kronke, M. (1996) *Cell* **86**, 937–947
35. Song, H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V., and Rothe, M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 9792–9796
36. Natoli, G., Costanzo, A., Lanni, A., Templeton, D. J., Woodgett, J. R., Balsamo, C., and Levreto, M. (1997) *Science* **275**, 200–203
37. Ninomiya-Tsuji, J., Kishimoto, K., Hyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) *Nature* **398**, 252–256
38. Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, L., Janeway, C. A., and Ghosh, S. (1999) *Genes Dev.* **13**, 2059–2071
39. Xia, P., Wang, L., Moretti, P. A., Albanese, N., Chai, F., Pitsen, S. M., D’Andrea, R. J., Gamble, J. R., and Vadas, M. A. (2002) *J. Biol. Chem.* **277**, 7996–8003
40. Habelhah, H., Takahashi, S., Cho, S. G., Kadoya, T., Watanabe, T., and Ronai, Z. (2004) *EMBO J.* **23**, 322–332
