RIP1-mediated AIP1 Phosphorylation at a 14-3-3-binding Site Is Critical for Tumor Necrosis Factor-induced ASK1-JNK/p38 Activation*

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Previously, we have shown that ASK1-interacting protein 1 (AIP1, also known as DAB2IP), a novel member of the Ras-GAP (Ras-GTPase-activating protein) protein family, opens its conformation in response to tumor necrosis factor (TNF), allowing it to form a complex with TRAF2-ASK1 that leads to activation of ASK1-JNK/p38 signaling in endothelial cells (EC). In the present study, we show that a TNF-inducible 14-3-3-binding site on AIP1 is critical for the opening of its conformation and for the AIP1-mediated TNF signaling. Ser-604, located in the C-terminal domain of AIP1, was identified as a 14-3-3-binding site. TNF treatment of EC induces phosphorylation of AIP1 at Ser-604 as detected by a phospho-specific antibody, with a similar kinetics to ASK1-JNK/p38 activation. 14-3-3 associates with an open, active state of AIP1 assessed by an in vitro pulldown assay. Mutation of AIP1 at Ser-604 (AIP1-S604A) blocks TNF-induced complex formation of AIP1 with 14-3-3. TNF treatment normally induces association of AIP1 with TRAF2-ASK1. The interactions with TRAF2 and ASK1 do not occur with AIP1-S604A, suggesting that phosphorylation at this site not only creates a 14-3-3-binding site but also opens up AIP1, allowing binding to TRAF2 and ASK1. Overexpression of AIP1-S604A blocks TNF-induced ASK1-JNK activation. We further show that RIP1 (the Ser/Thr protein kinase receptor-interacting protein) associates with the GAP domain of AIP1 and mediates TNF-induced AIP1 phosphorylation at Ser-604 and JNK/p38 activation as demonstrated by both overexpression and small interfering RNA knockdown of RIP1 in EC. Furthermore, RIP1 synergizes with AIP1 (but not AIP1-S604A) in inducing both JNK/p38 activation and EC apoptosis. Our results demonstrate that RIP1-mediated AIP1 phosphorylation at the 14-3-3-binding site Ser-604 is essential for TNF-induced TRAF2-RIP1-AIP1-ASK1 complex formation and for the activation of ASK1-JNK/p38 apoptotic signaling.

Vascular endothelial cells (EC) are among the principal physiological targets of TNF (1). 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), that are devoid of intrinsic catalytic activity (2). TNFR1-initiated signaling pathways have been extensively investigated. Upon TNF binding to the extracellular domain, TNFR1 recruits TNFR-associated death domain protein (TRADD) to its cytosolic death domain (3, 4). TRADD in turn functions as a platform adaptor to recruit both receptor-interacting protein-1 (RIP1) and TNFR-associated factor-2 (TRAF2), assembling a multiprotein complex that activates downstream signaling pathways. Specifically, RIP1 and TRAF2 recruit MEKK3 and TAK-1, mitogen-activated protein kinase kinase kinases (MAP3Ks), leading to activation of NF-κB (5–10). Recently, it has been shown that important signaling components of the TNF pathway are found in different complexes that are sequentially formed after TNF stimulation. An initial membrane-bound complex (complex I) composed of TNFR1-TRADD-RIP1-TRAF2 specifically activates the NF-κB pathway. Activated NF-κB promotes the transcription of anti-apoptotic genes such as cFLIP (cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein) (11). The role of complex I in the JNK/p38 MAPK cascade has not been defined (8). The TNFR1-associated signaling complex is rapidly internalized and dissociated from complex I. After a lag of some several hours, internalized TRADD-RIP1-TRAF2 may recruit Fas-associated death domain protein (FADD), procaspase-8, and procaspase-10 to form a cytoplasmic complex (complex II). Complex II promotes autocalytic activation of pro-caspase-8 to initiate apoptosis unless the NF-κB-induced long isoform of cFLIP (FLIPL) is also present in the complex.

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§ The abbreviations used are: EC, endothelial cells; HAEC, human aortic EC; BAEC, bovine aortic EC; ASK, apoptosis signal-regulating kinase-1; AIP, ASK1-interacting protein; RIP, receptor-interacting protein-1; GAP, Ras-GTPase-activating protein; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated death domain protein; TRAF, TNFR-associated factor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAP3K, mitogen-activated protein kinase kinase; FADD, Fas-associated death domain protein; GST, glutathione S-transferase; siRNA, small interfering RNA; CHX, cycloheximide; PARP, poly(ADP-ribosel) poly-merase-2; WT, wild type; KD, kinase domain; ID, intermediate domain; PR, proline-rich region; PER, period-like domain; PH, pleckstrin homology; C2, protein kinase C-conserved domain 2; C, C-terminal domain; N, N-terminal domain; Z, benzylxoycarbonyl.

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However, the events between the disassembly of complex I and the formation of complex II are not well established. TRAF2 and RIP1 are present in both TNF signaling complex I and TNF signaling complex II. Indeed, overexpression of either TRAF2 or RIP1 results in JNK, p38, and NF-κB activation, suggesting that RIP1 and TRAF2 mediate these pathways. TRAF2 is a member of a family of signal-transducing proteins containing C-terminal TRAF domains and N-terminal RING and zinc fingers (12). 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-terminal (87–501) behaves as a dominant negative mutant for both NF-κB and JNK activation (13). RIP1 contains a N-terminal kinase domain, an intermediate domain, and a C-terminal death domain (DD). RIP1 via its DD binds to the DD of TRADD. RIP1 also interacts with TRAFs (TRAF1, -2, and -3) via its intermediate domain. The essential function of RIP1 in TNF-induced activation of NF-κB and p38 MAPK pathways has been demonstrated in RIP1-deficient cells (9, 14, 15). RIP1 is also essential for Toll-like receptor-3-induced NF-κB activation (16). The role of RIP1 in JNK activation remains controversial (9, 17). Overexpression of RIP1 in many cell types results in induction of both apoptotic and necrotic cell death (9, 14, 15, 18).

Among MAP3Ks, apoptosis signal-regulating kinase-1 (ASK1) is an enzyme that specifically activates a cascade ending with JNK/p38 MAPK (but not NF-κB) activation. However, the mechanism for ASK1 activation by TNF remains unclear. Structurally, ASK1 is a 170-kDa protein containing an inhibitory N-terminal domain, an internal kinase domain, and the C-terminal domain (19, 20). ASK1 via its C-terminal domain binds to TRAFs, and this association is required for ASK1 activation (20). The current model for ASK1 activation by TNF involves several critical steps including release of inhibitors (thioredoxin and 14-3-3) (21, 22), TRAF-dependent homodimerization/polymerization (23), and ASK1 autophosphorylation at Thr-845 (24). We have recently identified ASK-interacting protein 1 (AIP1, a novel member of the Ras-GAP family; also called DAB2IP for DAB2-interacting protein) as a novel transducer of TRAF2 in TNF-induced ASK1-JNK signaling. AIP1 in a closed form is localized to the plasma membrane of EC, where it associates with TNFR1. In response to TNF, AIP1 dissociates from TNFR1 with concomitant cytoplasmic translocation and opens its closed conformation to allow formation of a complex composed of TRADD, TRAF2, RIP, and AIP1 (AIP1 complex). The AIP1 complex is distinct from the complex I and complex II and initiates specific activation of the ASK1-JNK/p38 MAPK pathway (25–27). However, the mechanism by which TNF induces the opening of AIP1 conformation and formation of AIP1 complex (TRAF2-RIP1-AIP1-ASK1) has not been defined.

In the present study, we show that a TNF-inducible 14-3-3-binding site (p-Ser-604) on AIP1 is critical for the opening of its closed conformation and for the AIP1-mediated TNF signaling. We also show that RIP1 in the AIP1 complex mediates TNF-induced AIP1 phosphorylation at Ser-604. Furthermore, mutation of AIP1 at Ser-604 (AIP1-S604A) blocks TNF-induced activation of NF-κB and p38 MAPK pathways.
complex formation of AIP1 with TRAF2, RIP1, and ASK1, as well as TNF-induced ASK1-JNK/p38 MAPK activation. Our results demonstrate that RIP1-mediated AIP1 phosphorylation at the 14-3-3-binding site Ser-604 plays a critical role in TNF-induced ASK1-JNK signaling.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Expression plasmids for TRAF2, ASK1, and AIP1 have been described previously (25, 27). FLAG-tagged RIP1 was from Dr. James J. Chen (University of Texas Southwestern Medical School, Dallas, TX) (28), and Myc-tagged RIP1 expression plasmids were from Dr. Michelle A. Kelliher (University of Massachusetts Medical School, Worcester, MA) (9). AIP1-S604A and T811A were generated by site-directed mutagenesis using a QuickChange™ site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer. Constructs were confirmed by DNA sequencing and by immunoblotting for protein expression.

Cells and Cytokines—Human and bovine aortic EC (HAEC and BAEC) were purchased from Clonetics (San Diego, CA). Human umbilical vein EC were purchased from Yale University cell culture Core Facility. Human recombinant TNF-α was from R&D Systems (Minneapolis, MN) and used at 10 ng/ml.

Transfection and Reporter Assay—BAEC were transfected by Lipofectamine 2000 (Invitrogen). 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 (25, 27).

Generation of Antibodies against Phospho-AIP1-specific Antibody—Polyclonal antibodies directed against specific phospho-AIP1 (p-Ser-604) were produced from BIOSOURCE by immunizing rabbits with a synthetic phospho-peptide corresponding to residues surrounding AIP1 Ser-604. The peptide sequence is AC-CPARSSpS YEAN-amide, where pS indicates phospho-Ser-604 and AC indicates acetylated. The peptide was synthesized with N-terminal cysteine residues and coupled to keyhole limpet hemocyanin for immunization. The antibodies were affinity-purified from rabbit antisera by affinity chromatography steps using protein A columns to purify immunoglobulins followed by specific phospho-peptide (immunogen) columns.

Immunoprecipitation and Immunoblotting—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 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA) for 20 min on ice. Immunoprecipitation and immunoblotting were performed as described previously (25, 27). Anti-TRAF2, anti-RIP1, anti-ASK1, anti-14-3-3 (mouse monoclonal and rabbit polyclonal), anti-PARP, and anti-GST antibodies were purchased from Santa Cruz Biotechnology. Anti-Myc monoclonal

FIGURE 2. TNF induces phosphorylation of AIP1 at Ser-604 concomitant with an association with 14-3-3. a, verification of p-Ser-604 (p-S604)-specific antibody. A polyclonal antibody specific to p-Ser-604 (AIP1) was generated (see “Experimental Procedures”). 293T cell lysates expressing FLAG-tagged AIP1-C or AIP1-C-S604A were used to determine the specificity of anti-p-Ser-604 by Western blot (IB). Anti-FLAG was used as a control. b and c, p-Ser-604 and 14-3-3 binding of AIP1 are induced by TNF. BAEC were transfected with AIP1-F or AIP1-F-S604A followed by treatment with TNF (10 ng/ml for 15 min). Phosphorylation of AIP1 was detected by Western blot with anti-p-Ser-604 (b), and 14-3-3 binding of AIP1 was determined by an in vitro GST-14-3-3 pulldown assay (c). p-AIP-F, phosphorylated AIP-F. d and f, phosphorylation of endogenous AIP1 in EC. BAEC were treated with TNF (10 ng/ml) for various times (0–60 min). d, phosphorylation of endogenous AIP1 was determined by Western blot with a p-Ser-604-specific antibody. Association of AIP1 with 14-3-3 was determined by immunoprecipitation (IP) with anti-14-3-3 antibody (mouse monoclonal) followed by Western blot with anti-AIP1 or anti-14-3-3 antibodies (rabbit polyclonal). p-AIP1, phosphorylated AIP1. e, a mouse IgG isotype was used as a negative control for immunoprecipitation. f, TNF-induced activation of ASK1-JNK/p38 signaling was determined by Western blot with phospho-specific antibodies against active ASK1 and JNK.
antibody was purchased from Roche Applied Science, and anti-FLAG monoclonal antibody (M2) antibody was from Sigma. RNA Interference for RIP1 — Two pairs of siRNA oligonucleotides for human RIP1 (sense strand, 5'-GGA GCA AAC UGA AUA AUG AAG AGC A-3' (siRIP1-A); 5'-GGG UGA UGA GGG AAG GCA UAA AGG G-3' (siRIP1-B); and a pair of control siRNA oligonucleotide: 5'-CAG AGA GGA GGA AAG GAG ACG CAG G-3') were synthesized by Integrated DNA Technologies (Coralville, IA). 20 nm siRNA was transfected into cells using Oligofectamine (Invitrogen) following protocol provided by the manufacturer. 72 h after transfection, cells were harvested for protein analyses as described.

RESULTS

AIP1 Contains a 14-3-3-binding Site at Ser-604 of the C-terminal Domain — We have previously shown that the C-terminal half of AIP1 maintains AIP1 in a closed inactive state in resting EC by binding to its N-terminal half and that TNF treatment opens this closed conformation, leading to an association of AIP1 with TRAF2 and ASK1 (25). To understand the mechanism by which TNF induces a dissociation of the C-terminal domain from the N-terminal domain, we searched for potential functional motifs located in the C-terminal domain by the ScanSite program. The C-terminal domain of AIP1 (AIP1-C) contains two potential non-classic 14-3-3-binding sites (29) surrounding Thr-811 (RGRTTP-RXXp(S/T)XP) and Ser-604 (RSS-SXX RX1-2PSX2-3S) (bold letters indicate phosphorylation sites) (Fig. 1a). We first determined whether AIP1-C binds to 14-3-3. AIP1-F (the full-length), AIP1-N (amino acid 1-522), and AIP1-C (amino acids 523-1065) were transfected into BAEC (which are easily transfectable), and the 14-3-3 binding of AIP1 was determined by an in vitro GST-14-3-3 pulldown assay. As a control, the C-terminal domain of ASK1 (ASK1-C) containing a 14-3-3-binding site was pulled down by GST-14-3-3. AIP1-C, but not AIP1-N, bound to GST-14-3-3 (Fig. 1b). Interestingly, AIP1-F was not pulled down by GST-14-3-3. The likely explanation is that AIP1-F protein exists in a closed conformation in resting cells through an intramolecular interaction between its N-terminal and C-terminal domains as we had demonstrated previously (25). To further define the 14-3-3-binding site, we generated a mutation at Ser-604 (AIP1-C-S604A) or Thr-811 (AIP1-C-T811A) on AIP1-C by site-specific mutagenesis. AIP1-C (WT), AIP1-C-T811A, or AIP1-C-S604A was each transfected into BAEC, and 14-3-3 binding was determined. Mutations at Ser-604, but not at Thr-811, abolished the 14-3-3 binding (Fig. 1c), suggesting that the Ser-604 of AIP1 is critical for 14-3-3 binding.

TNF Treatment Induces Phosphorylation of AIP1 at Ser-604 Concomitant with an Association with 14-3-3 — Since TNF induces a disruption of the closed conformation of AIP1 (25), we determined whether phosphorylation of AIP1 (p-Ser-604) as well as its 14-3-3 binding to AIP1-F is induced by TNF treatment. To this end, we first developed a rabbit polyclonal antibody against p-Ser-604 of AIP1 (anti-p-Ser-604) using a phospho-peptide compassing the p-Ser-604 of AIP1 as an antigen (see “Experimental Procedures”). To determine the specificity of anti-p-Ser-604, cell lysates containing AIP1-C and AIP1-C-S604A expressed from 293T transfectants were used for Western blot. Consistent with its binding to 14-3-3 (Fig. 1), AIP1-C was basally phosphorylated at Ser-604 as detected by anti-p-Ser-604 (Fig. 2a). In contrast, AIP1-C-S604A was not recognized by anti-p-Ser-604, confirming the specificity of the antibody.

To determine whether p-Ser-604 and the 14-3-3 binding of AIP1 are induced by TNF, BAEC were transfected with AIP1-F or AIP1-F-S604A followed by treatment with TNF (10 ng/ml for 15 min). Phosphorylation of AIP1 was first detected by Western blot with anti-p-Ser-604. Phosphorylation of Ser-604 in AIP1-F was not detected in resting EC and was highly
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induced in response to TNF (Fig. 2b). In contrast, AIP1-F-S604A did not show detectable phosphorylation under either resting or TNF-treated state (Fig. 2b). The 14-3-3 binding of AIP1 was then determined by an in vitro GST-14-3-3 pulldown assay. Results showed that AIP1-F from TNF-treated EC but not from resting EC bound to GST-14-3-3 (Fig. 2c). Similarly, AIP1-F-S604 failed to bind to GST-14-3-3 (Fig. 2c). These results support the conclusion that TNF induces phosphorylation of AIP1 at Ser-604 and a subsequent 14-3-3 binding.

To determine whether endogenous AIP1 in EC shows a similar pattern of phosphorylation at the 14-3-3-binding site (p-Ser-604), BAEC were treated with TNF (10 ng/ml) for various times (0–60 min), and endogenous AIP p-Ser-604 was determined by Western blot with a phospho-specific antibody. Association of AIP1 with 14-3-3 was determined by immunoprecipitation with anti-14-3-3 antibody followed by Western blot with anti-AIP1. Phosphorylation of AIP1 (p-Ser-604) was undetectable in resting EC but strongly induced in response to TNF. TNF-induced p-Ser-604 peaked at 15 min and declined by 60 min (Fig. 2d). Similarly, association of AIP1 with 14-3-3 was not observed in resting EC but dramatically induced in response to TNF at 15 min followed by a decline at 60 min (Fig. 2e). As a control, activation of ASK1-JNK signaling was determined by Western blot with phospho-specific antibodies against active ASK1 and JNK (Fig. 2f). AIP1 phosphorylation/14-3-3 binding and ASK1-JNK activation showed a similar kinetics in response to TNF (Fig. 2f). Similar results were obtained for endogenous AIP1 in HAEC (not shown).

Critical Roles of AIP1 p-Ser-604 in TNF-induced Formation of AIP1 Signaling Complex—To define the role of AIP1 phosphorylation in TNF signaling, we determined the effect of the non-phosphorylatable form of AIP1 on TNF-induced associations of AIP1 with TRAF2, RIP1, and ASK1. BAEC were transfected with AIP1-F or AIP1-F-S604A followed by TNF treatment (10 ng/ml for 15 min). Association of AIP1 with endogenous TRAF2, RIP1, and ASK1 was determined by immunoprecipitation with anti-TRAF2, anti-RIP1, or anti-ASK1 antibody followed by Western blot with anti-FLAG. Similar to our previous findings on endogenous AIP1 in EC (27), AIP1-F associated with TRAF2 (Fig. 3a), RIP1 (Fig. 3b), and ASK1 (Fig. 3c) in response to TNF. However, AIP1-F-S604A failed to bind to TRAF2, RIP1, or ASK1 (Fig. 3, a–c). These data suggest that phosphorylation of AIP1 at the 14-3-3-binding site (p-Ser-604) plays a critical role in TNF-induced association of AIP1 with TRAF2, RIP1, and ASK1.

The role of AIP1 p-Ser-604 in TNF signaling was further analyzed for its effects on TNF-induced activation of ASK1-JNK by a reporter gene assay. BAEC were transfected with a JNK/p38-dependent reporter gene in the presence of a vector control, AIP1-F or AIP1-F-S604A. BAEC were treated with TNF (10 ng/ml for 6 h), and TNF-induced luciferase activity was determined as described previously (25). As shown in Fig. 3d, AIP1-F augmented, whereas AIP1-F-S604A blunted, TNF-induced JNK reporter gene activity. Previously, we had shown that AIP1 specifically induces ASK1-JNK activation while inhibiting TNF-induced NF-κB signaling (27). The effect of AIP1 phosphorylation on a κB-dependent reporter gene was determined. As expected, AIP1-F expression inhibited TNF-induced κB reporter gene activity. However, AIP1-F-S604A augmented TNF-induced activation of κB reporter gene (Fig. 3e). Taken together, these data suggest that AIP1 phosphorylation at the 14-3-3-binding site (p-Ser-604) is critical not only for TNF-induced formation of AIP1-containing signaling complex (TRAF2-RIP1-AIP1-ASK1) but also for the ability of AIP1 to switch from NF-κB to JNK/p38 MAPK activation.

A Critical Role of RIP1 in TNF-induced AIP1 Phosphorylation and JNK/p38 Activation—Given that TNF induces an association of AIP1 with TRAF2-RIP1 signaling complex (27) and that RIP1 is a Ser/Thr kinase implicated in TNF-induced JNK activation (17), we reasoned that RIP1 might be the kinase responsible for AIP1 phosphorylation at Ser-604. To test this hypothesis, we used both overexpression and siRNA knockdown approaches to determine the role of RIP1 in AIP1 phosphorylation. BAEC were transfected with AIP1-F or AIP1-F-S604A in the presence or absence of RIP1, and AIP1 p-Ser-604 was determined by phospho-specific antibody and by a GST-14-3-3 pulldown assay. Co-expression of RIP1 increased both the phosphorylation of AIP1 and the 14-3-3 binding to AIP1-F but not to AIP1-F-S604A (Fig. 4, a and b). To determine the role of endogenous RIP1 in AIP1 phosphorylation, a siRNA against human RIP1 (siRIP1-A) was transfected to HAEC. AIP1 phosphorylation...
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**Figure 5.** RIP associates with the GAP domain of AIP1. a, schematic diagram of AIP1 and RIP1 domains and expression constructs. Truncated N-terminal (N, PHC2, PH, and ΔPH) and C-terminal domains (C, C-PR, and C-ΔPR) were FLAG-tagged. Truncated RIP1 domains (WT, ΔKI, and KD) were Myc-tagged. LZ, leucine-zipper.

b, both the kinase and the intermediate domains on RIP1 are required for AIP1 binding. BAEC were transfected with RIP1 truncates in the presence of AIP1 (indicated by Flag). Association between RIP1 and AIP1 was determined by immunoprecipitation (IP) with anti-Myc followed by Western blot (WB) with anti-FLAG. c and d, RIP1 binds to the GAP and PER domains of AIP1. BAEC were transfected with AIP1-N and AIP1-C truncates in the presence of RIP1 (indicated by Flag). Association between RIP1 and AIP1-N or AIP1-C truncates was determined by immunoprecipitation with anti-RIP1 followed by Western blot with anti-FLAG.

AIP1-mediated phosphorylation at Ser-604 and activation of downstream JNK/p38 MAPK was determined by a GST-14-3-3 pulldown assay. RIP1 siRNA, but not a control siRNA, significantly knocked down endogenous RIP1 in EC. TNF failed to induce AIP1 phosphorylation (measured by the 14-3-3 binding) in RIP1-knockdown EC (Fig. 4c). TNF-induced JNK/p38 MAPK activation was also determined by Western blot with phospho-specific antibodies. Knockdown of RIP1 resulted in significant inhibition of TNF-induced activation of JNK/p38 MAPK (Fig. 4d). Similar results were obtained from another pair of siRNA against RIP1 (siRIP1-B). These data suggest a critical role of RIP1 in TNF-induced AIP1 phosphorylation at Ser-604 and activation of downstream JNK/p38 MAPK.

**RIP1 Associates with the GAP Domain of AIP1**—To define the molecular interaction between RIP1 and AIP1, we mapped the critical domains of RIP1 and AIP1 for their interactions. RIP1 contains a N-terminal kinase domain (KD, amino acids 1–300), an intermediate domain (ID, amino acids 301–558), and a C-terminal DD (amino acids 559–671) (Fig. 5a). To map the critical domain in RIP1 for AIP1 binding, Myc-tagged RIP1-WT (amino acids 1–671), RIP1-ΔKI (deletion of amino acids 132–322 containing part of the KD and ID), and RIP1-KD immunoprecipitation with anti-RIP1 followed by Western blot with anti-FLAG. RIP1 associated with AIP1-N and ΔPH, but not PHC2 or PH, suggesting that the GAP domain of AIP1 is critical for RIP1 binding (Fig. 5c). Using the same approach, we found that RIP1 bound to AIP1-C, PR, and ΔPR, suggesting that the PER domain is critical for RIP1 interaction (Fig. 5c). Previously, we have shown that the N-terminal C2 domain and the C-terminal PER domain of AIP1 are critical for the binding of ASK1 and TRAF2, respectively (27) (Fig. 5d). Taken together, these data suggest that AIP1 uses different domains to interact with TRAF2, RIP1, and ASK1 and may function as a scaffolding protein to facilitate a formation of TNF-induced TRAF2-RIP1-ASK1 signaling complex.

**Critical Roles of RIP1-mediated AIP1 p-Ser-604 in TNF Signaling**—To determine the functional significance of RIP1-mediated AIP1 phosphorylation, we examined whether RIP1 augments AIP1-mediated JNK/p38 activation and EC apoptosis. To this end, we first examined the effects of RIP1- and AIP1-mediated JNK/p38 activation in a JNK/p38 MAPK reporter gene assay. BAEC were transfected with the reporter gene in the presence of RIP1, AIP1-F, or AIP1-F-S604 alone or
combinations. Cells were then treated with TNF for 12 h, and the JNK/p38 reporter gene activity was determined. Consistent with previous findings (25), expression of RIP1 or AIP1 induced JNK/p38 reporter gene activity. Co-expression of RIP1 with AIP1, but not with AIP1-F-S604, synergistically induced the JNK/p38 reporter gene activation (Fig. 6 a). Moreover, AIP1-F-S604A blunted RIP1-induced JNK/p38 activity. As a control, RIP1 strongly activated a \( \beta \)-H9260B reporter gene. Consistent with the effect of AIP1 on TNF-induced \( \beta \)-H9260B reporter gene activity (Fig. 3 e), AIP1-F inhibited, but AIP1-F-S604A augmented, RIP1-induced \( \beta \)-H9260B reporter gene activity (Fig. 6 b). These data suggest that RIP1-mediated phosphorylation of AIP1 at Ser-604 is critical for RIP1/AIP1-mediated JNK/p38 activation.

We then determined the cooperative function of RIP1 and AIP1 in TNF-induced EC apoptosis. BAEC were transfected with vector control, RIP1, AIP1-F, or AIP1-F-S604 alone or combinations. The cells were untreated or treated with TNF (10 ng/ml) plus cycloheximide (CHX, 10 mg/ml) for 6 h, and EC apoptosis was determined by 4',6-diamidino-2-phenylindole staining for nuclei fragmentation. In the absence of TNF + CHX, overexpression of AIP1 or RIP1, but not AIP1-F-S604A, induced EC apoptosis. RIP1 enhanced the effect of AIP1-F but not of AIP1-F-S604A. TNF + CHX alone caused EC apoptosis, which was increased by RIP1 or by AIP1 or, to the greater extent, by a combination of RIP1 and AIP1. More importantly, AIP1-F-S604A blocked TNF + CHX-induced EC apoptosis (Fig. 6 c). The RIP1/AIP1-induced EC death could be blocked by a pan-caspase inhibitor Z-VAD, suggesting that RIP1/AIP1 induce a caspase-dependent apoptotic death pathway (Fig. 6 c, +zVAD). Poly-ADP-ribose-polymerase (PARP) is a 113-kDa protein that binds specifically to DNA strand breaks, and cleavage of PARP (to 89 kDa) by caspases has been used to serve as an early marker of apoptosis. We determined the effects of AIP1-F-S604A on TNF(+CHX)-induced PARP cleavage. Consistent with the DNA fragmentation assay, overexpression of AIP1-F-S604A strongly blocked TNF(+CHX)-induced PARP cleavage in BAEC (Fig. 6 d). The addition of Z-VAD completely blocked TNF(+CHX)-induced PARP cleavage (not shown). Taken together, these data strongly support the critical roles of AIP1 phosphorylation in TNF-induced JNK activation and in EC apoptosis.

**DISCUSSION**

In this report, we present evidence for a critical role of AIP1 phosphorylation (p-Ser-604) in TNF signaling. TNF treatment induces phosphorylation of AIP1 at Ser-604 with a similar kinetics as ASK1-JNK/p38 activation. More importantly, mutation of AIP1 at Ser-604 (AIP1-S604A) blocks TNF-induced
complex formation of AIP1 with TRAF2, RIP1, and ASK1, suggesting that AIP1-S604A remains in a closed, inactive state. These data demonstrate that AIP1 phosphorylation is essential for opening of its closed conformation, a crucial step for AIP1 function in TNF signaling. Supporting this conclusion, AIP1-S604A blocks TNF-induced ASK1-JNK/p38 activation. As p-Ser-604 is tightly associated with 14–3–3 binding to AIP1, we speculate that p-Ser-604-mediated 14–3–3 binding may facilitate AIP1 conformation change. Whether or not 14–3–3 binding is required for AIP1 opening will require further investigation.

Tschopp’s group (11) has demonstrated that the initial plasma membrane-bound complex I (TNFR1-TRADD-RIP1-TRAF2) is responsible for NF-κB activation and a cytoplasmic complex (complex II) in which the internalized TRADD, RIP, or TRAF2 recruits FADD and pro-caspase-8 for apoptotic signaling. However, it has not been determined whether either complex I or complex II is active in the initiation of JNK/p38 MAPK signaling. It has been proposed that that complex I recruits one or more MAP3Ks to induce JNK/p38 MAPK activation (8, 11). Our previous report (27) and the present data demonstrate that AIP1 signaling complex might represent such an intermediate complex, which is structurally and functionally distinct both from complex I and from complex II in TNF signaling. Specifically, an AIP1 signaling complex (TRADD-RIP1-TRAF2-ASK1) lacks TNFR1 (a complex I component) and FADD (a component of complex II). AIP1 specifically recruits MAP3K ASK1 to initiate ASK1-JNK/p38 cascade; furthermore, AIP1 inhibits TNF-induced NF-κB pathway. In the present study, we provide further characterization and regulation of AIP1 signaling complex. We show that the Ser/Thr protein kinase RIP1 associates with the GAP domain of AIP1. Furthermore, RIP1 mediates TNF-induced AIP1 phosphorylation at Ser-604, resulting in activation of the downstream JNK/p38 MAPK cascade. This was demonstrated by both overexpression and siRNA knockdown of RIP1 in EC. Thus, AIP1 utilizes a C-terminal PER domain, an N-terminal GAP domain, and an N-terminal C2 domain to bind to TRAF2, RIP1, and ASK1, respectively. Similarly, TRAF2 binds to AIP1 via its N-terminal RING finger (27), whereas RIP1 binds to AIP1 via its kinase/intermediate domains (this study). Moreover, these interactions induce an opening of AIP1 closed conformation, resulting in formation of AIP1 signaling complex. Previous studies indicate that TRAF2 binds to TRADD via a C-terminal TRAF domain, whereas RIP1 binds to TRADD via its C-terminal DD (5–8). Taken together, it is conceivable that TRADD functions as a platform adaptor to initiate assembly of TRADD-RIP1-TRAF2 complex, whereas AIP1 functions as a scaffold protein to facilitate the activation of the AIP1 signaling complex (TARDD-RIP1-TRAF2-AIP1-ASK1).

The role of RIP1 in NF-κB activation has been extensively investigated. RIP1, either via MEKK3 and/or via TAK1 or directly, recruits IkB kinases complex to complex I, resulting in activation of NF-κB pathway (9, 10). Interestingly, RIP1 does not require its kinase activity for the activation of NF-κB because reintroduction of a kinase-dead form of RIP1 into RIP1-deficient cells completely restores NF-κB activation. In contrast, the kinase activity of RIP1 seems to be critical for TNF-induced MAPK cascades (9, 10). However, the precise mechanism by which RIP1 mediates JNK/p38 MAPK is unclear. Previous data suggest that RIP1 directly associates with MEKK3, but not ASK1. Here, our results suggest that RIP1 may act through AIP1 to form a complex with ASK1. Furthermore, RIP1 enhances ASK1-JNK/p38 MAPK signaling by mediating AIP1 phosphorylation. Thus, RIP1 synergizes AIP1 (but not AIP1-S604A)-mediated JNK/p38 MAPK activation and EC apoptosis. Our study provides a link between RIP1 to the downstream JNK/p38 MAPK pathway. It needs to be determined whether RIP1 directly or through other kinases phosphorylates AIP1.

Based on these results, we propose the following model for the function of AIP1 phosphorylation in TNF signaling. AIP1 exists in an inactive form complexed with TNFR1 in resting EC. In response to TNF, AIP is unfolded, dissociates from TNFR1, and binds to RIP1. In TNF-treated EC, RIP1 mediates AIP1 phosphorylation at a 14–3–3-binding site (Ser-604), and this phosphorylation (and thus the 14–3–3 binding) on AIP1 appears to be critical for an opening of its closed conformation. Once in the open conformation, AIP1 associates with TRAF2, RIP1, and ASK1, stabilizing the ASK1 signaling complex. This complex activates the ASK1-JNK/p38 MAPK cascade. Our study uncovers a novel mechanism by which AIP1 mediates TNF signaling.

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