Triad3A Regulates Ubiquitination and Proteasomal Degradation of RIP1 following Disruption of Hsp90 Binding*

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Toll-like receptors (TLRs) play a crucial role in innate immunity by recognizing microbial pathogens. Triad3A is an E3 ubiquitin-protein ligase that interacts with the Toll/interleukin-1 receptor domain of TLRs and promotes their proteolytic degradation. In the present study, we further investigated its activity on signaling molecules downstream of TLRs and tumor necrosis factor (TNF) receptor 1. Triad3A promoted down-regulation of signaling molecules downstream of TLRs and tumor necrosis factor (TNF) receptor 1. Triad3A cooperatively maintain the homeostasis of RIP1. These results suggest that Triad3A is an E3 ubiquitin-protein ligase to RIP1 and that Hsp90 and Triad3A acted as a negative regulator in TNF-α signaling. Reduction of Triad3A expression by small interference RNAs rendered cells hyperresponsive to TNF-α stimulation. Conversely, overexpression of Triad3A in cells blocked TNF-α-induced cell activation. This negative regulation was effected independently of changes in the cellular protein level of RIP1. Further studies indicated that RIP1 formed a complex with Triad3A and heat shock protein 90 (Hsp90), which is a chaperone protein capable of maintaining the stability of its client proteins. Treatment of cells with geldanamycin to disrupt the Hsp90 complex led to proteasomal degradation of RIP1. Depletion of Triad3A by small interference RNA treatment inhibited geldanamycin-activated ubiquitination and proteolytic degradation of RIP1. These results suggest that Triad3A and Proteasomal Degradation of RIP1 following Disruption of Hsp90 Binding*

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C-terminal region contains a RIP1 homotypic interaction motif (RHIM). As to the other TIR domain-containing adapter proteins, TIRAP has been shown to be essential for MyD88-dependent TLR2 and TLR4 signaling and TIRP is essential for the TLR4-mediated MyD88-independent/TRIF-dependent pathway, whereas the role of SRAM in TLR signaling has not yet been studied (18–21).

Triad3A is a RING finger E3 ligase identified in a yeast two-hybrid screening using the TIR domain of TLR9 as bait (22). This E3 ligase promotes ubiquitination and proteolytic degradation of TLR4 and TLR9 and negatively regulates their activation by lipopolysaccharide (LPS) and CpG-DNA, respectively (22). Triad3A is the most abundant alternatively spliced form of the Triad3 family. The other two members are Triad3 and Triad3B (22). Triad3, also called ZIN (zinc finger protein inhibiting NF-κB), was shown to interact with RIP1 in a yeast two-hybrid screening (23). RIP1 contains an N-terminal kinase domain, a RHIM motif, and a C-terminal death domain and is essential for TNF-α-induced NF-κB activation (21, 24, 25). Given that Triad3A is capable of binding to the TIR domains of TLR4 and TLR9, we reasoned that Triad3A may also be able to regulate proteolytic degradation of the TIR domain-containing adapter molecules involved in TLR signaling and that RIP1 may contain a TIR-homologous (TIRH) domain with some conserved motifs that allow for interaction with Triad3 family members. In this study, we investigated the activity of Triad3A toward different adapter molecules in the TLR and TNF-α signaling pathways and further investigated its cellular functions, in cooperation with Hsp90, to control the ubiquitination and proteolytic degradation of RIP1.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—LPS (Re595) from Salmonella minnesota R595, anti-FLAG monoclonal antibodies, and geldanamycin were purchased from Sigma. TNF-α was purchased from PeproTech Inc. (Rocky Hill, NJ). Anti-Myc, anti-TNF-R1, and anti-actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ubiquitin and anti-RIP1 monoclonal antibodies were purchased from BD Biosciences. Anti-Hsp90 polyclonal antibody was purchased from Cell Signaling (Beverly, MA). Anti-TRIF and anti-Triad3A polyclonal antibodies were generated by using recombinant proteins purified from an *Escherichia coli* expression system (Novogen/EMD Bioscience, Madison, WI). Lactacystin and MG132 were purchased from EMD Biosciences (San Diego, CA). Pefabloc and E64 were purchased from Roche Applied Science.

**Plasmid Constructs**—Mammalian expression vectors for Triad3A and various adapter proteins were generated by PCR amplifications from first strand cDNA libraries prepared from poly(A⁺)-mRNA isolated from human placenta and spleen (purchased from Clontech, Palo Alto, CA) as previously described (26) and subcloned into PRK5 expression vectors containing a FLAG or Myc epitope tag. Full-length TNF-R1 cDNA was subcloned into PEF6 vector. Expression vectors for truncated and point-mutated RIP1 were constructed by subcloning cDNAs encoding the truncated or mutated proteins into the PRK5 mammalian expression vector with an N-terminal Myc tag. The numbers of each construct indicate the N-terminal and C-terminal amino acid residues or indicate the residue that was substituted.

**Computational Analysis**—Domain architecture of RIP1 and TIR domain-containing adapter proteins were analyzed by a SMART architecture research computer program (smart. EMBL-heidelberg.de/). Protein sequence alignments were performed with AlignX of the Vector NTI computer program (Informax, Inc., Bethesda, MD).

**RT-PCR Analysis**—Total RNAs were isolated from plasmid-transfected 293 cells using an RNA isolation kit (Qiagen, Valencia, CA). First strand cDNA was prepared from total RNA samples using a SuperScript™ preamplification kit (Invitrogen). PCR amplifications were performed using an Expand Hi Fid PCR kit (Roche Applied Science). The sequences of the gene-specific primers used are as follows: TIRAP (5′-TACCTCAGGACAACCCCTAACC-3′, 5′-AGATCAGATACGTCGATTCCCG-3′), TRIF (5′-AGATACCACCTCTCCAATTCAACGGC-3′, 5′-CATCTGAGCCCTGGTAGAGGAACAGTAG-3′), RIP1 (5′-GTCAATTCCGCCAAGAACAGCC-3′, 5′-CCCTTTATGCCTTCCCCTCATCCCC-3′), G3PDH (5′-TGAAGGTCCGGATCTAACGGATTGGTTGAT-3′, 5′-CATGTTGGCCATGAGGTCCAC-3′). PCR products were visualized by electrophoresis on a 1% agarose gel after staining with ethidium bromide.

**Cell Culture, Transfection, and NF-κB Reporter Assays**—Human embryonic kidney 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were plated in 6-well plates and transfected on the following day using Lipofectamine 2000 (Invitrogen) with differing amounts of expression vectors as indicated. For NF-κB reporter assay, ELAM-1 luciferase reporter plasmid (100 ng) and β-galactosidase plasmid (100 ng), supplemented with empty pBluescript vector to 1 μg, were co-transfected into the cells. Twenty-four hours later, the cells were treated with 10 ng/ml TNF-α for 8 h. The cells were lysed, and luciferase activity was determined using reagents from Promega Corp. (Madison, WI). Relative luciferase activities were calculated as -fold of induction compared with an unstimulated vector control. The data are presented as means ± S.E. (*n* = 3).

**RNA Interference**—Three siRNAs targeting different positions on the cDNA sequence as indicated by the numbers were designed to deplete endogenous Triad3A expression. Their target sequences are: siRNA(1067): AACCCAGAT-TATCCAAAGAGA, siRNA(1242): AAGTGCCTCAGTAGCAGGATTGTGAT-3′, 5′-CATGTGGGCCATGAGGTCCACCAC-3′). These siRNA oligonucleotides were synthesized by Qiagen. A control non-silencing siRNA with target sequence AATTCTCCGAACGTGTCACGT was purchased from Qiagen. 293 cells seeded in six-well plates were transfected with these siRNAs using Lipofectamine 2000 (Invitrogen). After 24 h, these cells were split 1 to 2.5 into another six-well plate. This procedure of transfection and splitting was repeated twice more. The cells were then treated with 10 ng/ml TNF-α or 1 μM geldanamycin. In some experiments, 100 ng of ELAM-1 luciferase reporter plasmid and 100 ng of β-galactosidase plasmid were co-transfected with the last siRNA transfection for the NF-κB reporter assay.
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Immunoblotting—Cell lysates were subjected to SDS-PAGE and transfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in phosphate-buffered saline for 30 min and then incubated with the indicated antibody in phosphate-buffered saline plus 0.5% nonfat milk for 2 h. After washes, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 50 min. After subsequent washes, the immunoreactive bands were visualized with ECLPlus Western blotting detection reagents (Amersham Biosciences).

Co-immunoprecipitation—Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.5% NonidetP-40, plus complete protease inhibitor mixture (Roche Applied Science)). For analysis of ubiquitinated proteins, the lysis buffer was supplemented with 0.1 mM ALLnL and 5 mM indoacetamide. Cell lysates were clarified by centrifugation and incubated with indicated antibody plus protein G-Sepharose (Amersham Biosciences) at 4°C overnight to form immunocomplexes. After extensive washing with lysis buffer, the immunocomplexes were analyzed by immunoblotting as described.

RESULTS

Triad3A Promotes Down-regulation of TIRAP, TRIF, and RIP1—We have shown previously that Triad3A regulates ubiquitination and proteolytic degradation of several TLRs (22). In addition, ZIN/Triad3 was reported to interact with RIP1 (23). To investigate whether Triad3A also promotes down-regulation of any of the TIR domain-containing adapter proteins and RIP1, we co-transfected 293 cells with expression vectors for Triad3A and various adapter proteins in the TLR and TNF-α signaling pathways. These adapter proteins included members of the MyD88 family (MyD88, TIRAP, TRIF, and TIRP), IRAK family members (IRAK, kinase inactive IRAK(K239A), IRAK-M, and IRAK4), TRAF2, TRAF6, RIP1, and RIP2. Eighteen hours after transfection, the cells were lysed and cell lysates were analyzed by immunoblotting for the expression levels of the adapter proteins. Triad3A promoted down-regulation of TIRAP, TRIF, and RIP1 (Fig. 1A, left panel), and TRIF (right panel) but had no effect on the other proteins tested. Compared with the others, full-length TRIF was more difficult to express in these cells. A series of degraded products rather than the intact protein were detected in the immunoblots of the overexpressed TRIF, suggesting that the expressed TRIF may have a rapid turnover rate in these cells. Nevertheless, down-regulation of full-length TRIF by Triad3A was detectable with longer exposures of the immunoblots (Fig. 1A, right panel). We further analyzed the mRNA levels of these three Triad3A-regulated proteins by reverse transcription PCR with gene-specific primers. The results indicated that their mRNA levels were unaffected by the expression of Triad3A (Fig. 1B). Thus, similar to the regulation of TLRs, down-regulation of these adapter proteins by Triad3A was controlled at the protein level.

Triad3A Interacts with TIRAP, TRIF, and RIP1—In general, an E3 ligase directly associates with its target proteins to promote their ubiquitination and proteolytic degradation (1–3). To investigate the interaction between Triad3A and its substrates, we co-transfected 293 cells with an expression vector for Triad3A and excess amount of vectors for its target proteins. The transfected cells were lysed 18 h later and cell lysates were subjected to co-immunoprecipitation analyses. The results showed that the two substrates, TIRAP and RIP1, co-immunoprecipitated with Triad3A, whereas MyD88 and TRIF did not (Fig. 2A). Because TRF was more difficult to express, we investigated the interaction between endogenous Triad3A and TRIF using THP-1 cells stably transfected with CD14 (THP-1/CD14) (27). These cells were stimulated with LPS for 10 and 30 min and then lysed. The cell lysates were subjected to co-immunoprecipitation with an antibody against TRIF and subsequently immunoblotted with anti-Triad3A antibody. The result indicated that Triad3A interacted with TRIF in both resting cells and LPS-stimulated cells (Fig. 2B). These results demonstrated a direct physical contact between Triad3A and its substrates. RIP1 Contains a TIRH Domain for Interaction with Triad3A—This observation of direct interaction between Triad3A and TRIF, as well as TRIF, is consistent with our previous finding that Triad3A interacts with TLRs because these proteins all contain a TIR domain and some conserved motif within this domain could be responsible for the interaction. However, RIP1 does not contain a TIR domain. RIP1 contains 671 amino acid residues with an N-terminal kinase domain, a RHIM motif, and a C-terminal death domain (Fig. 3A and Refs. 21, 24). The inter-
action between Triad3A and RIP1 suggested to us that RIP1 may have a domain with some conserved motif homologous to a TIR domain that allows for interaction with Triad3A. We performed protein sequence alignment analyses using amino acid sequences from the TIR domain of each of the MyD88 family members and RIP1. The results indicated that a C-terminal region in RIP1 comprising the RHIM motif and extending into the death domain can be aligned with the sequences of the TIR domains, suggesting that this region has significant homology with the TIR domains and thus could be the TIRH domain (Fig. 3A and B).

To investigate further the interaction between RIP1 and Triad3A, we co-transfected 293 cells with expression vector for RIP1 and increasing amounts of expression vector encoding Triad3A. After 18 h, the cells were lysed and analyzed by immunoblotting for the expression levels of RIP1. The results indicated that Triad3A promoted down-regulation of RIP1 in a concentration-dependent manner (Fig. 4). To determine whether the TIRH domain was involved in the protein interaction with Triad3A, a series of expression vectors with truncation mutants of RIP1 deleted progressively from either the N- or C-terminal were generated. Excess amount of Triad3A was co-expressed in 293 cells with these truncation mutants to determine its activity toward these mutants. Triad3A promoted down-regulation of N-terminal-deleted RIP1s, whereas the C-terminal-deleted proteins remained intact. Removal of the C-terminal region from amino acid residue 595 rendered RIP1 unresponsive to Triad3A, suggesting the importance of this C-terminal region for its interaction with Triad3A (Fig. 5A). Requirement of the C-terminal for RIP1 to bind Triad3A was further determined by co-immunoprecipitation of the truncated proteins with Triad3A from cell lysates generated from 293 cells co-transfected with excess amount of these truncated proteins and Triad3A. The results showed that the N-terminal deletion mutants were capable of binding to Triad3A, whereas the C-terminal TIRH domain-truncated mutants were not (Fig. 5B).

To further investigate the amino acid residues in the TIRH domain that are essential for binding to Triad3A, residues conserved in both TIRAP and TRIF and in the C-terminal of RIP1 were identified (Fig. 3B). Point mutants of RIP1 were generated by mutagenesis of these residues into the corresponding residues in MyD88 or into alanine. These point mutants were co-expressed with Triad3A in 293 cells and analyzed by immunoblotting and co-immunoprecipitation for their interaction with Triad3A. The results showed that mutagenesis of the conserved residues Ile615, Gly623, and Leu632 rendered RIP1 unresponsive to Triad3A. The results indicated that Triad3A promoted down-regulation of RIP1 in a concentration-dependent manner (Fig. 4). To determine whether the TIRH domain was involved in the protein interaction with Triad3A, a series of expression vectors with truncation mutants of RIP1 deleted progressively from either the N- or C-terminal were generated. Excess amount of Triad3A was co-expressed in 293 cells with these truncation mutants to determine its activity toward these mutants. Triad3A promoted down-regulation of N-terminal-deleted RIP1s, whereas the C-terminal-deleted proteins remained intact. Removal of the C-terminal region from amino acid residue 595 rendered RIP1 unresponsive to Triad3A, suggesting the importance of this C-terminal region for its interaction with Triad3A (Fig. 5A). Requirement of the C-terminal for RIP1 to bind Triad3A was further determined by co-immunoprecipitation of the truncated proteins with Triad3A from cell lysates generated from 293 cells co-transfected with excess amount of these truncated proteins and Triad3A. The results showed that the N-terminal deletion mutants were capable of binding to Triad3A, whereas the C-terminal TIRH domain-truncated mutants were not (Fig. 5B).

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**Triad3A Regulates TNF-α-induced NF-κB Activation through a RIP1 Degradation-independent Mechanism**—RIP1 is required for TNF-α-induced NF-κB activation, and ZIN/Triad3, an alternatively spliced form of Triad3A, has been shown to interact with RIP1 and inhibit TNF-α activation (23). Here we have shown that Triad3A interacts and promotes down-regulation of RIP1. Taken together, these data suggest that Triad3A may play a role in regulation of TNF-α-induced NF-κB activation. To investigate this function of Triad3A, we first developed siRNAs, targeting different positions of the TriadA gene, to reduce the expression of endogenous Triad3A in 293 cells. The efficiency of these siRNAs was determined by immunoblotting with specific antibody for the expression of Triad3A (Fig. 6A, lower panel). These siRNA-treated cells were subsequently transfected with an NF-κB-driven luciferase reporter gene and stimulated with TNF-α. Luciferase reporter assays demonstrated that reduction of Triad3A expression ren-

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**FIGURE 2. Interaction between Triad3A and adapter proteins.** A, co-immunoprecipitation of Triad3A and adapter proteins. Cell lysates from FLAG-tagged Triad3A and Myc-tagged adapter protein co-expressed 293 cells were subjected to immunoprecipitation with an anti-FLAG antibody and then analyzed by immunoblotting with anti-Myc antibody for the co-immunoprecipitation of Triad3A and adapter proteins. Cell lysates from FLAG-tagged Triad3A were subjected to immunoprecipitation with an anti-FLAG antibody and then immunoblotted with anti-Triad3A and anti-TRIF antibody.

B, endogenous interaction between Triad3A and TRIF. THP-1/Triad3A (H9251) and CD14 cells were stimulated with LPS for different periods of time as indicated. Cells were lysed, and lysates were subjected to immunoprecipitation with anti-TRIF antibody and then immunoblotted with anti-Triad3A and anti-TRIF antibodies.
dered cells hyperresponsive to TNF-α (Fig. 6A, upper panel). To further confirm that Triad3A acted as a negative regulator, 293 cells were co-transfected with an NF-κB luciferase reporter gene and increasing amount of expression vector for FLAG-tagged Triad3A and subsequently stimulated with TNF-α. These experiments showed that Triad3A blocked TNF-α-induced NF-κB activation in a concentration-dependent manner (Fig. 6B). Interestingly, at lower concentrations, expression of Triad3A itself somewhat activated NF-κB (Fig. 6B). The molecular mechanism for this activation is unclear but may be due to the interactions between Triad3A and its substrates, which, in turn, lead to NF-κB activation.

We further investigated the molecular mechanisms by which Triad3A controls TNF-α activation. To determine whether down-regulation of RIP1 expression was responsible for the negative regulation by Triad3A on TNF-α activation, 293 cells were treated with TNF-α for different periods of time and analyzed for the protein levels of RIP1 by immunoblotting with specific antibody (Fig. 6C). The results showed that the level of endogenous RIP1 was unaffected following TNF-α stimulation, suggesting that the negative regulatory function of Triad3A on TNF-α activation could be independent of its proteolytic degradation activity toward RIP1.

Because the death domain in RIP1 is required for homotypic interactions with upstream signaling molecules for its recruitment to the TNF-R1 complex (24, 28–30), we reasoned that binding of Triad3A to RIP1 through this region may sequester this protein from association with the TNF-R1 complex. To test this possibility, we co-transfected 293 cells with expression vectors for TNF-R1, RIP1, and varying amounts of Triad3A at expression levels that are insufficient to significantly down-regulate RIP1 expression. The cell lysates were analyzed by immunoprecipitation of TNF-R1 for binding of RIP1 to the TNF-R1 complexes (Fig. 7). The result showed that Triad3A is capable of sequestering RIP1 from binding to the TNF-R1 complex and suggests an alternative mechanism for this E3 ligase to regulate cellular signaling.

**RIP1 Forms a Complex with Triad3A and Hsp90**—We then turned our focus to the role of Hsp90 in modulation of Triad3A activity on RIP1, because Hsp90 is a chaperone protein that is important in maintaining the stability and function of its client proteins and RIP1 has been reported to be a client protein of Hsp90 (31). Disruption of the Hsp90 complex by GA treatment results in proteasomal degradation of RIP1 and abrogation of TNF-α-induced NF-κB activation (31, 32). We reasoned that whereas the binding of Triad3A to RIP1 may negatively regulate TNF-α signaling by preventing its association to TNF-R1 complex, the interaction with Hsp90 may protect RIP1 from the
proteolytic activity of Triad3A. To investigate this possibility, we first determined the interaction between RIP1, Triad3A, and Hsp90 in 293 cells. These cells were treated with TNF-α for various times, cell lysates were immunoprecipitated with antibodies against RIP1, and the immunocomplexes were analyzed by immunoblotting with antibodies against Myc, FLAG, or actin. To determine the interaction between Triad3A and various RIP1 truncation and point mutants excess amount of Myc-tagged truncated RIP1 (A) and excess amount of Myc-tagged point-mutated RIP1 (B) were co-expressed with FLAG-tagged Triad3A in 293 cells. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody and then analyzed by immunoblotting with anti-Myc antibody for the co-immunoprecipitated proteins. The expression levels of these proteins were analyzed by resolving cell lysates by SDS-PAGE and immunoblotting with anti-Myc or anti-FLAG antibodies.

**FIGURE 5.** Key amino acid residues in the TIRH domain for interaction between RIP1 and Triad3A. To determine the activity of Triad3A to various RIP1 truncation and point mutants Myc-tagged truncation mutants of RIP1 (A) and Myc-tagged point mutants of RIP1 (C) were co-expressed with excess amount of FLAG-tagged Triad3A in 293 cells. The cells were subsequently lysed, resolved by SDS-PAGE, and analyzed by immunoblotting with antibodies against Myc, FLAG, or actin. To determine the interaction between Triad3A and various RIP1 truncation and point mutants excess amount of Myc-tagged truncated RIP1 (B) and excess amount of Myc-tagged point-mutated RIP1 (D) were co-expressed with FLAG-tagged Triad3A in 293 cells. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody and then analyzed by immunoblotting with anti-Myc antibody for the co-immunoprecipitated proteins. The expression levels of these proteins were analyzed by resolving cell lysates by SDS-PAGE and immunoblotting with anti-Myc or anti-FLAG antibodies.

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**DISCUSSION**

The TIR domain, a motif of ~160 amino acid residues conserved in the cytosolic domains of TLRs, mediates homotypic interaction with downstream signaling molecules in the MyD88 family (16). Triad3A is a RING finger-type E3 ubiquitin-protein ligase that recognizes this domain in several TLRs and regulates their activation (22). To further explore the cellular functions of Triad3A, we determined its activity on proteins in the MyD88 family and other signaling molecules downstream of TLRs and TNF-R1. In this report, we show that Triad3A interacts and promotes down-regulation of three signaling molecules, TIRAP, TRIF, and RIP1. TIRAP and TRIF are two TIR domain-containing proteins that play important roles in mediating TLR signaling (18, 19). However, because the
binding and down-regulation of these two proteins were shown only in an overexpression system, the biological effects of Triad3A-mediated control of TIRAP and TRIF activities require further investigation. In the present study, we focused our investigation on the molecular bases and cellular function of the interaction between Triad3A and RIP1, which is not a TIR domain-containing protein.

RIP1 is a protein of 671 amino acid residues containing an N-terminal kinase domain, a RHIM motif, and a C-terminal death domain. The RHIM motif mediates homotypic interaction with TRIF in TLR3- and TLR4-induced NF-κB activation, and the death domain is required for homotypic interaction with upstream signaling molecules for recruitment to the TNF-R1 complex (21, 24, 28–30). The interaction between RIP1 and Triad3A suggested the existence of some common structural determinants among RIP1 and the other TIR domain-containing adapter proteins that would allow for Triad3A binding. At first glance, the RHIM motif seemed most likely to be the protein region that could mediate the interaction with Triad3A because this motif is conserved within RIP1 and several of the TIR domain-containing adapter proteins (21). Nevertheless, by using multiple sequence alignment analysis, a C-terminal region in RIP1 starting from amino acid residue 490 was identified to have substantial homology with the TIR domains in proteins of the MyD88 family. This TIRH domain comprised the RHIM motif and the C-terminal death domain. Further analysis of the Triad3A binding site with truncated and point-mutated RIP1 indicated that a protein region in the death domain containing the Ile615, Gly623, and Leu632 residues is critical for Triad3A interaction. The corresponding region of this Triad3A-interacting sequence is flanked by the conserved box2 and box3 sequences in the TIR domain. In TLRs, box2 forms a loop structure that mediates the interaction with downstream signaling molecules, whereas the function of box3 is not clear although it contains residues important for signaling (17, 33). These results suggested that the TIRH domain and TIR domain could be distantly related to one another. While the RHIM motif and the box2 region may mediate the interaction with Triad3A because this motif is conserved within RIP1 and several of the TIR domain-containing adapter proteins, the box3 region in the death domain containing the Ile615, Gly623, and Leu632 residues is critical for Triad3A interaction. The corresponding region of this Triad3A-interacting sequence is flanked by the conserved box2 and box3 sequences in the TIR domain. In TLRs, box2 forms a loop structure that mediates the interaction with downstream signaling molecules, whereas the function of box3 is not clear although it contains residues important for signaling (17, 33). These results suggested that the TIRH domain and TIR domain could be distantly related to each other. While the RHIM motif and the box2 region may mediate homotypic interaction between proteins with like domains, other conserved motifs within these domains, such as the Ile615, Gly623, and Leu632 triad, may be recognized by their molecular bases and cellular function of the interaction between Triad3A and RIP1, which is not a TIR domain-containing protein.

In terms of cellular function, RIP1 is essential for TNF-α-induced NF-κB activation (25). A previous report showed that Triad3/ZIN interacted with RIP1 and inhibited TNF-α-in-
duced NF-κB activation (23). Nevertheless, whether Triad3A was involved in this TNF-α signaling was not clear. We extended the investigation into the function and molecular mechanism of Triad3 family members in TNF-α-induced NF-κB activation to determine whether Triad3A could control this activation through modulation of RIP activity. Previously, we observed a marginal enhancement of TNF-α activation in a study with vector-based siRNA to silence the Triad3A gene expression levels of Triad3A were analyzed by immunoblotting with anti-Triad3A. Immunocomplexes were analyzed by immunoblotting with antibodies against ubiquitin or RIP1.

The immunocomplexes were analyzed by immunoblotting with antibodies against ubiquitin or RIP1. Control siRNA and siRNA1242-treated cells were treated with geldanamycin (1 μM) as indicated for 10 h. The cell lysates were subjected to immunoprecipitation with anti-RIP1 antibody, and the immunocomplexes were analyzed by immunoblotting with antibodies against ubiquitin or RIP1. A Cbl (34), which is a 120-kDa RING-type E3 ligase that promotes ubiquitination and down-regulation of several protein kinase receptors, cellular tyrosine kinases, and the TCR-CD3 complex (35–37). This E3 ligase negatively regulates the binding of a p85 subunit of phosphatidylinositol 3 kinase to cell surface CD28 or to the TCR-CD3 complex in a proteolysis-independent manner, probably through effects on protein-protein interactions (34). We reasoned that Triad3A may employ a similar mechanism to regulate TNF-α signaling because Triad3A binds to RIP1 through a motif within the death domain that is required for protein-protein interactions with its upstream signaling molecules (24, 28–30). Consistent with this hypothesis, our results showed that expression of Triad3A sequestered RIP1 from binding to the TNF-R1 complex. Co-immunoprecipitation of Triad3A and RIP1 from cell lysates further supported this notion. Triad3A might function as a gatekeeper type of constitutively negative regulator to maintain a threshold for TNF-α activation rather than a ligand induction-activated negative regulator, because Triad3A associated with RIP1 in resting cells and this association was only marginally increased in TNF-α-stimulated cells. Whether the expression level of Triad3A in cells can be induced by any inflammatory stimuli to increase the threshold of TNF-α activation remains to be investigated.

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**FIGURE 8. RIP1 forms a complex with Triad3A and Hsp90.** 293 cells were treated with TNF-α for various times as indicated. These cells were lysed, and cell lysates were subjected to immunoprecipitation with anti-RIP1 antibody. The immunocomplexes were analyzed by immunoblotting with antibodies against Triad3A and Hsp90 to determine the interaction among these proteins.

**FIGURE 9. Triad3A regulates ubiquitination and proteolytic degradation of RIP1 in geldanamycin-treated cells.** A, geldanamycin promotes down-regulation of RIP1. 293 cells were treated with geldanamycin (1 μM) for different periods of time as indicated. B, proteasome inhibitors block proteolytic degradation of RIP1. 293 cells were treated with out geldanamycin (1 μM) and different proteasomal and lysosomal inhibitors (30 μM) as indicated for 10 h. The cell lysates (A and B) were analyzed by immunoblotting with antibodies against RIP1 and actin. C, geldanamycin treatment promotes ubiquitination of RIP1. 293 cells were treated with geldanamycin (1 μM) plus MG132 (30 μM) for different periods of time as indicated. The cell lysates were subjected to immunoprecipitation with anti-RIP1 antibody, and the immunocomplexes were analyzed by immunoblotting with antibodies against ubiquitin or RIP1. D, reduction of Triad3A inhibits geldanamycin-promoted proteolytic degradation of RIP1. Control siRNA and siRNA1242-treated cells were treated with geldanamycin (1 μM) for different periods of time as indicated. E, reduction of Triad3A inhibits geldanamycin-promoted ubiquitination of RIP1. Control siRNA and siRNA1242-treated cells were treated with geldanamycin (1 μM) plus MG132 (30 μM) for different periods of time as indicated. The cell lysates were immunoprecipitated with anti-RIP1 antibody and then analyzed by immunoblotting with anti-ubiquitin or anti-RIP1 antibodies. The expression levels of Triad3A were analyzed by immunoblotting with anti-Triad3A.
Triad3A Regulates Ubiquitination of RIP1

We further investigated why Triad3A did not promote the proteolytic degradation of RIP1 during their interaction. We hypothesized that this may be due to the involvement of other modulators, such as chaperone proteins, because it was reported that RIP1 is a client protein of Hsp90 (31). This protein is one of the most abundant heat shock proteins in cells and distinct from other heat shock proteins in that it does not participate in general protein folding. Instead, it more specifically regulates the stability and function of several signaling proteins, including RIP1 (6). Disruption of the RIP1-Hsp90 complex by treatment of cells with GA results in RIP1 destabilization and its subsequent proteasome-mediated degradation (31). Our finding that RIP1 formed a complex with Triad3A and Hsp90 in cells supported this hypothesis. We further determined whether Triad3A could regulate ubiquitination and proteolytic degradation of RIP1 following GA activation. We first established that GA promoted ubiquitination and proteasomal degradation of RIP1 in 293 cells and then showed that reduction of Triad3A expression in cells by siRNA treatment inhibited GA-mediated ubiquitination and reduced its proteolytic degradation. Indeed, in addition to Triad3A, several other proteins have been shown to have E3 activity toward RIP1. cIAPs (cIAP1 and cIAP2) have been reported to promote ubiquitination and proteolytic degradation of RIP1 in vitro (38). TRAF2 and A20 were shown to participate in ubiquitination of RIP1 in the TNF-R1 complex in lipid rafts (39, 40). TRAF2 and A20 were shown to participate in ubiquitination of RIP1 in the TNF-R1 complex (38). At this time, whether these E3 ligases regulate the protein level of RIP1 in cells is not clear. Nevertheless, the observation that the RIP1 level was unaffected following TNF-α activation, whereas it was reduced after GA treatment, suggests the importance of Hsp90 in maintaining the stability of RIP1 in cells. The E3 ligases and Hsp90 could coordinately maintain the homeostasis of RIP1 in cells.

In conclusion, this study uncovered a proteolysis-independent mechanism by which Triad3A negatively regulates TNF-α activation. Triad3A and Hsp90 cooperatively regulate the homeostasis of RIP1. This may illustrate a general mechanism by which an E3 ligase and a chaperone protein cooperate to control the availability of an adapter protein for signal transduction.

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