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The Levels of Retinoic Acid-Inducible Gene I Are Regulated by Heat Shock Protein 90-α

Tomoh Matsumiya,‡ Tadaatsu Imaizumi,‡ Hideki Yoshida,‡ Kei Satoh,‡ Matthew K. Topham,*† and Diana M. Stafforini2*†

Retinoic acid-inducible gene 1 (RIG-I) is an intracellular pattern recognition receptor that plays important roles during innate immune responses to viral dsRNAs. The mechanisms and signaling molecules that participate in the downstream events that follow activation of RIG-I are incompletely characterized. In addition, the factors that define intracellular availability of RIG-I and determine its steady-state levels are only partially understood but are likely to play a major role during innate immune responses. It was recently reported that the antiviral activity of RIG-I is negatively regulated by specific E3 ubiquitin ligases, suggesting participation of the proteasome in the regulation of RIG-I levels. In this study, we used immunoprecipitation combined with mass spectrometry to identify RIG-I-interacting proteins and found that RIG-I forms part of a protein complex that includes heat shock protein 90-α (HSP90-α), a molecular chaperone. Biochemical studies using purified systems demonstrated that the association between RIG-I and HSP90-α is direct but does not involve participation of the CARD domain. Inhibition of HSP90 activity leads to the dissociation of the RIG-I-HSP90 complex, followed by ubiquitination and proteasomal degradation of RIG-I. In contrast, the levels of RIG-I mRNA are unaffected. Our studies also show that the ability of RIG-I to respond to stimulation with polyinosinic:polycytidylic acid is abolished when its interaction with HSP90 is inhibited. These novel findings point to HSP90-α as a chaperone that shields RIG-I from proteasomal degradation and modulates its activity. These studies identify a new mechanism whose dysregulation may seriously compromise innate antiviral responses in mammals. The Journal of Immunology, 2009, 182: 2717–2725.

Viral infection leads to the initiation of complex innate immune responses that result from recognition of the viral nucleic acid by cellular receptors including TLRs (1), followed by host cell secretion of antiviral factors such as cytokines (2, 3). Additional mechanisms are responsible for the activation of the IFN response during viral infections (4). We recently reported that a cytoplasmic RNA helicase, retinoic acid-inducible gene I (RIG-I),† is an essential regulator of dsRNA-induced signaling (5). RIG-I is also known as Ddx58 owing to the fact that this protein belongs to the DEXH box-containing helicase family; it harbors two caspase recruitment domains (CARD) at the amino-terminal end and an RNA helicase motif at the carboxyl terminus (5). The CARD domains are responsible for activating subsequent downstream signaling events through interactions with special E3 ubiquitin ligases, suggesting participation of the proteasome in the regulation of RIG-I levels. In this study, we used immunoprecipitation combined with mass spectrometry to identify RIG-I-interacting proteins and found that RIG-I forms part of a protein complex that includes heat shock protein 90-α (HSP90-α), a molecular chaperone. Biochemical studies using purified systems demonstrated that the association between RIG-I and HSP90-α is direct but does not involve participation of the CARD domain. Inhibition of HSP90 activity leads to the dissociation of the RIG-I-HSP90 complex, followed by ubiquitination and proteasomal degradation of RIG-I. In contrast, the levels of RIG-I mRNA are unaffected. Our studies also show that the ability of RIG-I to respond to stimulation with polyinosinic:polycytidylic acid is abolished when its interaction with HSP90 is inhibited. These novel findings point to HSP90-α as a chaperone that shields RIG-I from proteasomal degradation and modulates its activity. These studies identify a new mechanism whose dysregulation may seriously compromise innate antiviral responses in mammals. The Journal of Immunology, 2009, 182: 2717–2725.

IPS-I/MAVS/VISA/Cardif, resulting in the induction of IFNs and the activation of antiviral responses (6–9). In previous work, we reported that, aside from its recognized role as a viral sensor, RIG-I has additional functions. We found that RIG-I is a transcriptional activator of the cyclooxygenase 2 gene and that its expression is enhanced following stimulation of endothelial cells with inflammatory agents (10).

RIG-I is the subject of active investigations aimed at dissecting its precise function and mechanism of action in viral immunity. Factors likely to play a key role in these responses include those that affect expression levels, location, stability, and posttranslational modifications, all of which can potentially modulate the ability of RIG-I to affect cellular functions. Zhao et al. (11) recently reported that RIG-I becomes conjugated to IFN-regulated gene 15 (ISG15)/ubiquitin cross-reacting protein, a 15-kDa ubiquitin-like protein expressed following cellular stimulation with IFN. ISG15 becomes conjugated to a wide array of cellular proteins. Several of the targets, including RIG-I, are IFN-α/β-induced antiviral proteins, but most are constitutively expressed proteins that function in diverse cellular pathways, including RNA splicing, chromatin remodeling/polymerase II transcription, cytoskeletal organization and regulation, stress responses, and translation (11). The precise functional consequences of ISG15 modification remain to be established but, unlike ubiquitin, ISG15 does not appear to target proteins for proteasomal degradation (12, 13). In addition to ISG15-mediated derivatization, RIG-I has been shown to undergo two types of ubiquitin-dependent modifications that result in remarkably different effects on functional proteins. Gack et al. (14) recently showed that RIG-I is robustly ubiquitinated at its amino-terminal CARD domains by interacting with the E3 ubiquitin ligase TRIM, a member of the tripartite motif protein family, and that this process is necessary for RIG-I-mediated IFN-β production and antiviral activity in response to infection. This finding...
indicates that ubiquitination is a RIG-I modification that can modulate its signaling properties. Conversely, Arimoto et al. (15) reported that conjugation of RIG-I with ubiquitin targets RIG-I for degradation by the proteasome, thus effectively acting as a negative regulator of RIG-I. These combined observations point to the existence of at least three tightly regulated protein conjugation mechanisms that control both the stability and signaling functions of RIG-I.

The goal of the present study was to obtain additional insights on the mechanisms that contribute to the regulation of RIG-I function and expression levels. We found that in resting cells RIG-I is a component of a protein complex that also includes the molecular chaperone heat shock protein (HSP) 90-α. In addition, our studies showed that the association between RIG-I and HSP90 is direct and that inhibiting the interaction between these proteins leads to ubiquitination and proteasomal degradation of RIG-I. Importantly, disrupting the integrity of the RIG-I-HSP90 complex also inhibits key functional responses mediated by RIG-I. We conclude that partnership between RIG-I and HSP90 is likely to affect the function of RIG-I in vivo.

Materials and Methods

Cycloheximide, a protease inhibitor mixture, FLAG-agarose, anti-FLAG Abs, the expression vector p3XFLAG-CMV7.1, polynomials-polyctydilic acid (poly(IC)), His-tagged human high-mobility group protein 1 (HMGI-1), creatine kinase, and creatine phosphate were purchased from Sigma-Aldrich. We obtained a metal-affinity purification system (TALON), the transfer vector pBacPAK8, and the pBacPAK6 viral DNA from Clontech Laboratories. The β-galactosidase expression vector pSVβ-gal was obtained from Promega and the bacterial expression vector pGEX-5X-1 and glutathione-Sepharose 4B were from Amersham Biosciences. Factor Xa was from Qiagen and Oligo (dT) 30. DNase I, Platinum Pfx DNA polymerase, and TRIZol were from Invitrogen. Polyvinylidene fluoride membranes and chemiluminescence detection reagents were from PerkinElmer Life Sciences. Protein content was assessed using a Pierce protein assay kit. Reagents for cDNA synthesis were obtained from Fermentas and iQ SYBR Green Supermix was from Bio-Rad. Valeant Pharmaceuticals provided an anti-actin mouse mAb. Anti-HSP90, anti-pS6, and protein A/G-agarose were purchased from Santa Cruz Biotechnology. Cell Signaling Technology provided anti-JNK1/2 Abs. We obtained HRP-conjugated secondary Abs from BioSource International; the anti-RIG-I antiserum was previously described (10) and the anti-ubiquitin Ab was from Novagen. Geldanamycin was from BIOMOL and MG-132 and HSP90 were provided an anti-actin mouse mAb. Anti-HSP90, anti-RIG-I Abs, and Alexa Fluor 488-conjugated anti-rabbit and Texas Red-conjugated anti-mouse IgGs were from Molecular Probes. We obtained rabbit reticulocyte lysates from Hanko.

Cellular studies

HeLa and HEK293T cells were maintained at 5% CO2 atmosphere at 37°C in DMEM supplemented with 10% FBS. HT-29 cells were maintained in a 5% CO2 atmosphere at 37°C in McCoy’s 5A medium supplemented with 1% FBS. HSP90 inhibition was accomplished by treating monolayers for 3 days at 37°C, collected the virus-containing supernatant, and plated this fraction on a fresh monolayer. To assess geldanamycin-related effects on ubiquitination of RIG-I, we mixed 1 pmol each of recombinant HSP90 and RIG-I and then cloned into the pXFLAG-CMV7.1 expression vector, and analyzed by automated sequencing.

For studies that required the use of purified, full-length RIG-I, we amplified cDNA isolated from HeLa cells using primers RIG-I-xhol for BacPAK8-F (5’-GC etc gac GCA GAG CCC ATG ACC ACC GAG-3’) and RIG-I-6xHis-NotI for BacPAK8-4R (5’-AAG cgg cgc ctc/AGT GAT GGT GAT GAT GAT GTT TGG ACA TTT TGG CTG CA-3’); the reverse primer included a 6xHis tag for purification purposes. The product was digested with Xhol and NotI and then cloned into the BacPAK8 expression vector to generate pBacPAK8-6xHis-RIG-I. We co-transformed pBacPAK8 viral DNA and pBacPAK8-6xHis-RIG-I into Sf21 insect cells, cultured the cells for 3 days at 27°C, collected the virus-containing supernatant, and plated this fraction on a fresh monolayer. To generate individual plaques that were subsequently amplified in Sf21 cells. We harvested the cellular proteins using equilibration/wash buffer (50 mM sodium phosphate and 300 mM NaCl (pH 7.0)) containing proteasome inhibitors and purified RIG-I by metal-affinity chromatography on a TALON resin according to the manufacturer’s instructions. We eluted 6xHis-tagged RIG-I with 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 75 mM imidazole, dialyzed the preparation against 10 mM Tris-HCl (pH 8.0), and subjected it to immunoblot analysis.

We also expressed various domains of RIG-I and the full-length recombinant protein in the bacterial expression vector pGEX-5X-1 per the instructions provided by the manufacturer. We amplified cDNA isolated from HeLa cells using primers BamHI-Card-F (5’-CGT gatt ccc CAT GAC CAC CGA GCA GCG A-3’) and Xhol-Card-R (5’-CG ctc gag GTG ATG ATG GTT TGG ACA TTT TGG CTG CA-3’); the reverse primer included a 6xHis tag for purification purposes. The product was digested with Xhol and NotI and then cloned into the pBacPAK8 expression vector to generate pBacPAK8-6xHis-RIG-I. We co-transformed pBacPAK8 viral DNA and pBacPAK8-6xHis-RIG-I into Sf21 insect cells, cultured the cells for 3 days at 27°C, collected the virus-containing supernatant, and plated this fraction on a fresh monolayer. To generate individual plaques that were subsequently amplified in Sf21 cells. We harvested the cellular proteins using equilibration/wash buffer (50 mM sodium phosphate and 300 mM NaCl (pH 7.0)) containing proteasome inhibitors and purified RIG-I by metal-affinity chromatography on a TALON resin according to the manufacturer’s instructions. We eluted 6xHis-tagged RIG-I with 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 75 mM imidazole, dialyzed the preparation against 10 mM Tris-HCl (pH 8.0), and subjected it to immunoblot analysis.

In vitro binding and ubiquitination assays

To assess whether direct interaction(s) accounted for the association between RIG-I and HSP90, we amplified cDNA isolated from HeLa cells using primers BamHI-Card-F (5’-CGT gatt ccc CAT GAC CAC CGA GCA GCG A-3’) and Xhol-Card-R (5’-CG ctc gag GTG ATG ATG GTT TGG ACA TTT TGG CTG CA-3’); the reverse primer included a 6xHis tag for purification purposes. The product was digested with Xhol and NotI and then cloned into the pXFLAG-CMV7.1 expression vector, and analyzed by automated sequencing.

To assess geldanamycin-related effects on ubiquitination of RIG-I, we incubated 1 pmol of His-tagged RIG-I in 50 mM Tris-HCl (pH 7.4), 20 mM MgCl2, 2 mM ATP, 10 mM creatine phosphate, and 10 IU/ml of factor Xa with either His-tagged RIG-I or His-tagged HMG-I as a control and incubated the mixtures in binding buffer (200 mM NaCl, 50 mM HEPES, 1% BSA (pH 7.4)) for 30 min at 30°C in a total volume of 20 μl. We then added a suspension of TALON resin in PBS containing 0.5% Triton X-100 (final volume: 500 μl) and rocked the mixtures for 60 min at 4°C. The beads were extensively washed with PBS containing 0.5% Triton X-100 and resuspended with 2% SDS sample buffer. We then purified the proteins to electrophoresis on SDS-PAGE, followed by immunoblot analysis using anti-HSP90 and anti-RIG-I Abs.

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analyzed by electrophoresis on SDS-PAGE, followed by immunoblot analyses using anti-ubiquitin and anti-RIG-I Abs.

**Immunoprecipitation studies**

HeLa cells subjected to various treatments were washed twice with ice-cold PBS and then lysed in immunoprecipitation buffer (20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1.5 mM MgCl₂, and 1% Triton X-100) containing 0.5% protease inhibitor mixture for 30 min on ice. The lysates were centrifuged at 12,000 × g for 10 min at 4°C, and the pellets were discarded. The supernatants were precleared by incubation with normal IgG and protein A/G-plus for 2 h. Immunoprecipitations were performed by overnight rocking of the precleared supernatants with 1 μl of the primary Ab indicated in each case at 4°C. The Ag-Ab complexes were harvested by incubation with protein A/G-agarose for 1 h at 4°C. FLAG-tagged proteins were harvested by overnight incubation with FLAG-agarose beads at 4°C. After extensive washing with ice-cold PBS, the beads were boiled for 5 min in 2× SDS-PAGE sample buffer to dissociate the immunoprecipitated proteins. These fractions were analyzed by electrophoresis on SDS-PAGE and immunoblot analyses as described below. Studies involving coimmunoprecipitation of endogenous proteins were conducted on the human colon cancer cell line HT-29 using essentially the same approach, except that immunoprecipitations were performed by overnight rocking of the precleared supernatants with 1 μl of an anti-RIG-I antisera at 4°C. The complexes were harvested by incubation with protein A/G-plus for 1 h at 4°C. After extensive washing with ice-cold PBS containing 0.5% Triton X-100, the beads were boiled for 5 min in 2× SDS-PAGE sample buffer and the eluted proteins were subjected to SDS-PAGE and immunoblot analyses.

**Immunoblot analyses**

We subjected the protein extracts indicated in each case to electrophoresis on 8.5% SDS-PAGE gels and then transferred the proteins to polyvinylidene difluoride membranes that were blocked for 60 min at room temperature in 1× TBST buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 0.1% Tween 20) containing either 3% nonfat dry milk or 3% BSA. The membranes were incubated for 60 min at room temperature with the primary Ab indicated in each case. After five washes with blocking solution, we added a HRP-conjugated secondary Ab, incubated the membranes for 1 h, washed the membranes with TBST, and then visualized the immunoreactive bands using chemiluminescence detection reagents.

**Immunofluorescence analyses**

HeLa cells grown on glass coverslips were rinsed in PBS, fixed with 4% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% BSA for 1 h. All solutions were prepared in PBS. The cells were then incubated for 1 h with a polyclonal anti-RIG-I Ab (1/250-fold dilution) and a monoclonal anti-HSP90 Ab (1/100). After a washing step, we incubated the coverslips with Alexa Fluor 488-conjugated secondary Ab, incubated the membranes for 60 min at room temperature, repeated the washes using TBST, and then visualized the immunoreactive bands using chemiluminescence detection reagents.

**Mass spectrometry**

HeLa cells protein extracts that coimmunoprecipitated with RIG-I were resolved on SDS-PAGE and identified by staining, and individual lanes were subjected to in-gel trypsin digestion at the Mass Spectrometry Core Facility (University of Utah). The tryptic peptides were identified by mass spectrometry (MS/MS) studies and analyzed using Mascot software (Matrix Science).

**Reporter assays**

To generate an IFN-β reporter vector that spanned nt −299 to +19, we amplified HeLa cell genomic DNA with a sense primer harboring a 5′-XhoI site (XhoI-IFN-β-p299, 5′-GGG ctc gag AGT GTG TTG AGG TTC TGT A-3′) and an antisense primer designed with a 5′-HindIII site (HindIII-IFN-β-19+19, 5′-GCC aag ctt AAG GTT GTA GCA GTA ATG T-3′). We cloned the product in the pGL3-basic vector and cotransfected 300 ng of the plasmid with 50 ng of pSV40-β-gal and 100 ng of a p3XFLAG7.1 expression vector (RIG-I full length or RIG-CARD) using Lipofectamine. The following day we transfected the cells with 200 ng of poly(IC) using Lipofectamine and incubated the cells in the presence or absence of 200 nM geldanamycin for 24 h. We harvested the cells in 200 μl of reporter lysis buffer and assayed luciferase and β-galactosidase activities in the extracts (n = 4).

**Results**

**Identification of heat shock proteins as partners of RIG-I**

Our initial goal was to identify protein partners that associated with RIG-I under basal conditions. We used HT-29, a human colonic adenocarcinoma cell line that expresses high levels of RIG-I (our unpublished observations). We subjected HT-29 lysates to immunoprecipitation using anti-RIG-I polyclonal Abs or control IgG. After electrophoretic separation of the immunoprecipitated proteins, we noted the presence of multiple species that specifically associated with RIG-I (Fig. 1A). We initially focused our attention on the identification of RIG-I-interacting proteins that ranged in size between 70 and 110 kDa, as these represented a considerable proportion of the immunoprecipitated material (Fig. 1A). We analyzed various species using mass spectrometry and identified the two largest proteins (100 and 110 kDa) as RIG-I (data not shown). These results validated the specificity of our immunoprecipitation approach and suggested that the smaller, less abundant 100-kDa species was a proteolytic fragment derived from full-length RIG-I (M_r = 110 kDa). We next analyzed the composition of the immunoprecipitated 90- and 70-kDa proteins (Fig. 1A, arrow and asterisk, respectively). We found that the 90-kDa RIG-I-interacting protein harbored peptides that precisely matched sequences present in human HSP90-α (Table 1). Our studies also revealed the presence of a 70-kDa protein in immunoprecipitates generated using both control IgG and anti-RIG-I Abs (Fig. 1A). The amount of 70-kDa protein in the latter appeared to be much higher than that observed in the control lane, suggesting that RIG-I may specifically interact with this protein. Additional studies revealed that the 70-kDa protein was heat shock 70-kDa protein 8 (also known as HSPA8 and HSP70; Table I). This conclusion was drawn based on the sequence of seven peptides identified by mass spectrometric analyses and that precisely matched sequences found in the 70-kDa human chaperone (Table I). Our data supported the existence of in vivo partnerships between RIG-I and select heat shock proteins.

**Molecular characterization of RIG-I-HSP90 interaction**

We next focused on the characterization of the interaction between HSP90 and RIG-I. To address this issue, we used molecular, immunohistological, and biochemical approaches. First, transfection of HEK293T cells with FLAG-tagged RIG-I followed by immunoprecipitation using anti-FLAG or anti-HSP90 Abs revealed that endogenous HSP90 interacted with exogenous RIG-I (Fig. 1, B and C). We next considered whether the interactions observed in transfected cells were the consequence of forced expression of RIG-I and did not represent a naturally occurring association between endogenous proteins. To address this issue, we tested the ability of endogenous RIG-I and HSP90, both of which have been reported to be expressed in the cytoplasm, to colocalize under basal conditions (14, 17). Although no particular cellular structure was highlighted, we observed that in HeLa cells HSP90 and RIG-I were expressed in the same cytoplasmic region, suggesting that the proteins formed part of the same complex (Fig. 1D). In addition, immunoprecipitation studies confirmed that endogenous RIG-I interacted with endogenous HSP90 (Fig. 1E), indicating that the association between these proteins occurs in intact cells and is not an artifact of overexpression systems.

We next asked whether RIG-I and HSP90 interacted directly or whether participation of an additional protein(s) was required for association. To test this, we conducted in vitro experiments
FIGURE 1. RIG-I associates with HSP90. A, Cell lysates from HT-29 cells were immunoprecipitated with control IgG (left lane) or with an anti-RIG-I Ab (right lane) and proteins associated with the RIG-I immune complex were resolved by SDS-PAGE (7.5%). The arrow and asterisk indicate the relative mobilities of HSP90 and HSF70, respectively. B and C, A plasmid encoding FLAG-tagged RIG-I was transfected into HEK293T cells. Protein-protein interactions were monitored by coimmunoprecipitation using anti-FLAG-conjugated agarose followed by immunoblotting with anti-HSP90 Ab (B) and by coimmunoprecipitation using anti-HSP90 Abs followed by immunoblotting with an anti-FLAG Ab (C). D, HeLa cells were immunostained with anti-RIG-I or control rabbit IgG (green; left) or with anti-HSP90 or control mouse IgG (red; center). The left and center panels represent sequential confocal images of the same field. The right panel depicts an overlay of the images. E, Interaction between endogenous RIG-I and HSP90 was assessed by subjecting lysates from HT-29 cells to immunoprecipitation with anti-RIG-I or control IgG. The immune complexes were isolated by incubation with protein A/G-plus and eluted from the beads with 2× SDS-PAGE buffer. The eluted proteins were subjected to electrophoresis on SDS-PAGE and immunoblot analyses using anti-HSP90 and anti-His-tag (center panel), or His-tagged HMG-1 as a control (right panel). The resulting mixtures were subjected to affinity purification, and the bound proteins were eluted and subjected to immunoblot analyses using anti-HSP90 and anti-His-tag Abs.

Identification of domains required for interaction between HSP90 and RIG-I

Our next goal was to identify RIG-I domains involved in its interaction with HSP90. In previous studies, it was demonstrated that the ability of RIG-I to interact with the adaptor protein IFN-β promoter stimulator 1 (IPS-1) and the ubiquitin ligases RNF125 and TRIM25 depended on the presence of the amino-terminal CARD domain of RIG-I (5, 6, 14, 15). Second, it was recently shown that the CARD domain is the primary binding site of HSP90-β on Apaf-1 (18). To systematically analyze individual contributions, we generated four FLAG-tagged deletion mutants harboring various domains of RIG-I (Fig. 2A). We transfected HEK293T cells with each deletion construct and found robust expression of all species (Fig. 2B, left panel). Next, we subjected cell lysates from transfected cells to immunoprecipitation using anti-HSP90 Abs and found that the CARD domain lacked the ability to associate with HSP90 (Fig. 2B, right panel). The remaining deletion constructs interacted with HSP90 in an efficient manner, suggesting that a domain common to these constructs was necessary for the interaction. An examination of the deletion mutants tested in this study allowed us to conclude that the region comprised between aa 452–641 was involved in the association between RIG-I and HSP90.

To further explore this issue and to assess whether additional RIG-I domains also contributed to its interaction with HSP90, we expressed His-tagged constructs harboring various regions of RIG-I (Fig. 3A). We found that the constructs were expressed to various extents (Fig. 3B, bottom panel). The CARD domain, which was efficiently expressed, lacked the ability to interact with HSP90. Moreover, deletion of the CARD domain did not affect association with HSP90. These combined studies are in complete agreement with our results using FLAG-tagged constructs (Fig. 2B) and they firmly establish that the CARD domain does not participate in the interaction between RIG-I and HSP90. Interestingly, our studies also showed that all of the remaining RIG-I domains retained the ability to bind HSP90 (Fig. 3B), suggesting that more than one domain participates in the interaction between the proteins.

HSP90 affects the levels of RIG-I protein

HSP90 is primarily known as a molecular chaperone owing to its ability to ensure the stability and correct conformation, activity, intracellular localization, and proteolytic cleavage of a range of proteins involved in cell growth, differentiation, and survival (19). These include key signaling molecules such as ERBB2, AKT, Raf, hypoxia inducible factor 1α and steroid hormone receptors (20). To investigate the functional consequences of HSP90/RIG-I association, we destabilized the interaction using pharmacological approaches. Geldanamycin is a naturally occurring ansamycin antibiotic and antitumor agent that binds tightly to the HSP90 ATP/ADP pocket and prevents association of client proteins (20, 21). Treatment with geldanamycin prevented association between transfected RIG-I and HSP90 (Fig. 4A) and significantly reduced the levels of endogenous RIG-I protein in a time-dependent manner (Fig. 4B). To
assess the efficacy of geldanamycin treatment, we took advantage of a study reported by Piatelli et al. (22) who showed that geldanamycin selectively depletes cellular Raf-1, thus interrupting activation of MEK1/2 and ERK. In addition, these investigators showed that geldanamycin does not affect the levels of total ERK. We found that geldanamycin decreased basal levels of phospho-ERK in cells grown in the presence of serum, consistent with the notion that geldanamycin selectively depletes cellular Raf-1, thus interrupting activation of MEK1/2 and ERK. In addition, these investigators showed that geldanamycin did not affect the levels of total ERK. We found that geldanamycin decreased basal levels of phospho-ERK in cells grown in the presence of serum, consistent with the notion that geldanamycin selectively depletes cellular Raf-1, thus interrupting activation of MEK1/2 and ERK.

**Table I. Mass spectrometric identification of HSP90-α and HSP70 protein 8**

<table>
<thead>
<tr>
<th>Protein Size</th>
<th>Tryptic Peptide Sequences Identified by MS/MS</th>
<th>Identified Protein</th>
<th>Location in Table</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 kDa</td>
<td>ADLNNLGT1AKVILHK EIQYFNNYKGGVDSELPLN ISR</td>
<td>HSP90-α (HSP90AA1, HSP90AA2, HSP90A, HSP90N, HSPC1, HSPCA, HSPCAL1, HSPCAL3, HSPCN, HSPN, HSF86, HSF90, HSF90(LAP2))</td>
<td><strong>B</strong></td>
</tr>
<tr>
<td>70 kDa</td>
<td>TTPSYAVPDTGFSSSYPE ESSMVLTKVTNVAVTVT VAYFNSQDRDAGTIALG NVRLLINPEEAPAAIALG DKPEELAIALDLRFGQI HDIVLVGGR</td>
<td>HSP70 protein 8 (HSPA8, HSC54, HSC70, HSC71, HSP71, HSPA10, LAPI, NIP71)</td>
<td><strong>A</strong></td>
</tr>
</tbody>
</table>

HSP90 protects RIG-I from ubiquitination and proteasomal degradation

Our next goal was to identify the protease system that participates in the degradation of RIG-I following disruption of the RIG-I-HSP90 complex. We hypothesized that degradation of RIG-I might involve the proteasome because most proteins known to be stabilized by HSP90 are degraded through this mechanism once the interaction is inhibited (23). In control experiments, we found that treatment with MG-132, an agent known to specifically inhibit proteasomal activity, led to substantial accumulation of RIG-I (Fig. 5A). To specifically assess the contribution of HSP90 to the stability of RIG-I, we investigated whether the effects of geldanamycin on RIG-I levels were affected when proteasomal activity was inhibited with MG-132. We found (Fig. 5B) that MG-132 prevented geldanamycin-mediated RIG-I proteolysis, thus providing evidence for a role of HSP90 activity in the protection of RIG-I from proteasomal degradation.

Protein degradation through the proteasome commonly involves conjugation with ubiquitin, and HSP90 has been shown to utilize this mechanism in the stabilization of a variety of proteins. In addition, the degradation of RIG-I requires derivatization with ubiquitin. This led to substantial accumulation of RIG-I (Fig. 5A). To specifically assess the contribution of HSP90 to the stability of RIG-I, we investigated whether the effects of geldanamycin on RIG-I levels were affected when proteasomal activity was inhibited with MG-132. We found (Fig. 5B) that MG-132 prevented geldanamycin-mediated RIG-I proteolysis, thus providing evidence for a role of HSP90 activity in the protection of RIG-I from proteasomal degradation.

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forms of RIG-I using anti-RIG-I Abs, even after treatment with geldanamycin, suggesting that our RIG-I Abs were unable to recognize ubiquitinated forms of the protein. Our combined data are consistent with a model whereby blockade of RIG-I-HSP90 interactions results in ubiquitination and proteasomal degradation of RIG-I.

The interaction between HSP90 and RIG-I is functionally important

Our final goal was to assess whether the interaction between HSP90 and RIG-I had functional consequences on known biological activities mediated by RIG-I. It was previously shown that RIG-I acts as an intracellular dsRNA receptor that regulates IFN regulatory factor 3 activation and IFN-β induction at the transcriptional level (24). Thus, we investigated whether the ability of RIG-I to transcriptionally activate the IFN-β gene required association with HSP90. We transfected 293T cells with a reporter construct harboring nt -299 to +19 of the IFN-β gene combined with either empty vector, a FLAG-tagged construct harboring full-length RIG-I, or a FLAG-tagged CARD construct. We stimulated the cells with poly(I:C) which has been shown to be recognized by RIG-I (25) in the absence and presence of geldanamycin. We found that geldanamycin significantly inhibited both basal and poly(I:C)-stimulated IFN-β promoter activation (Fig. 6), suggesting that the association between RIG-I and HSP90 was necessary for this response. In addition, we found robust basal IFN-β promoter activation mediated by the CARD domain of RIG-I that was not affected by geldanamycin treatment (Fig. 6). These results indicate that blocking the ability of RIG-I to interact with HSP90 inhibits its biological activity. The fact that IFN-β promoter activation mediated by the CARD domain was not affected by geldanamycin demonstrates the specificity of the interaction between RIG-I and HSP90.
anti-RIG-I Abs.

on SDS-PAGE, followed by immunoblot analyses using anti-ubiquitin and beads. We eluted the bound proteins and subjected them to electrophoresis at 30°C and then isolated RIG-I using affinity chromatography on TALON somal protein S3 involves interaction with two independent do-

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demonstrated that HSP90 associates with 40S ribosomal compo-

in the absence and presence of MG-132 (0–10 μM) for 16 h. The cells were harvested and the lysates were subjected to SDS-PAGE and immunoblot analyses using Abs against RIG-I, HSP90, and actin. The numbers below this panel depict quantitative assessments of band intensity. B, We incubated His-tagged RIG-I with a rabbit reticulocyte lysate in the presence (right panel) or absence (left panel) of geldanamycin (400 nM) for 60 min at 30°C and then isolated RIG-I using affinity chromatography on TALON beads. We eluted the bound proteins and subjected them to electrophoresis on SDS-PAGE, followed by immunoblot analyses using anti-ubiquitin and anti-RIG-I Abs.

Our work did not directly address whether HSP70 facilitates the second chaperone that interacts with RIG-I under basal conditions. In humans, two distinct genes, HSP90-α and HSP90-β, encode closely related cytoplasmic proteins that display minor sequence differences at the amino termini (32–34). The isoforms differ in their ability to activate certain client proteins and regulate cellular functions of immune cells (34–38). The α and β isoforms of HSP90 often cooperate with other proteins to generate a cytosolic multichaperone machinery that includes HSP70, peptidyl-prolyl isomerases, and other chaperones to achieve adequate protein folding (39). Certain cytosolic client polypeptides have been shown to first bind HSP70 and then interact with dimeric HSP90 (39–42). Interestingly, our studies suggest that HSP70 may be a second chaperone that interacts with RIG-I under basal conditions. Our work did not directly address whether HSP70 facilitates the formation or stability of the RIG-I-HSP90 complex, but it is tempting to speculate that HSP70 contributes to this process and that a cochaperone(s) may be involved as well.

HSP90 protects RIG-I from proteasomal degradation and ubiquitination. A, HeLa cells were treated with geldanamycin (400 nM) in the absence and presence of MG-132 (0–10 μM) for 16 h. The cells were harvested and the lysates were subjected to SDS-PAGE and immunoblot analyses using Abs against RIG-I, HSP90, and actin. The numbers below this panel depict quantitative assessments of band intensity. B, We incubated His-tagged RIG-I with a rabbit reticulocyte lysate in the presence (right panel) or absence (left panel) of geldanamycin (400 nM) for 60 min at 30°C and then isolated RIG-I using affinity chromatography on TALON beads. We eluted the bound proteins and subjected them to electrophoresis on SDS-PAGE, followed by immunoblot analyses using anti-ubiquitin and anti-RIG-I Abs.

FIGURE 5. HSP90 protects RIG-I from proteasomal degradation and ubiquitination. A, HeLa cells were treated with geldanamycin (400 nM) in the absence and presence of MG-132 (0–10 μM) for 16 h. The cells were harvested and the lysates were subjected to SDS-PAGE and immunoblot analyses using Abs against RIG-I, HSP90, and actin. The numbers below this panel depict quantitative assessments of band intensity. B, We incubated His-tagged RIG-I with a rabbit reticulocyte lysate in the presence (right panel) or absence (left panel) of geldanamycin (400 nM) for 60 min at 30°C and then isolated RIG-I using affinity chromatography on TALON beads. We eluted the bound proteins and subjected them to electrophoresis on SDS-PAGE, followed by immunoblot analyses using anti-ubiquitin and anti-RIG-I Abs.

Discussion

RIG-I has been described as a pattern recognition receptor that detects intracellular dsRNA through its helicase domain in a TLR-independent fashion (5, 6). A series of well-characterized downstream signaling events that require the CARD domains of RIG-I lead to the activation of a family of IFN-induced genes, such as IFN regulatory factor 3, IFN regulatory factor 7, and NF-kB, through the signaling adaptor protein IPS-1 (6). The ability of RIG-I to elicit downstream signaling events requires the formation of a macromolecular protein complex necessary for gene activation (26). This complex is most likely in dynamic equilibrium depending on the state of cellular activation, viral stimulation, and other factors. To identify novel proteins that associated with RIG-I under basal conditions, we used biochemical approaches that, when combined with mass spectrometric measurements, resulted in the discovery of HSP90, and perhaps HSP70, as partners of RIG-I. HSP90 is a ubiquitous, constitutively expressed, highly conserved molecular chaperone involved in the folding, activation and assembly of key mediators of signal transduction, cell cycle control, and transcriptional regulation (27). This chaperone interacts with native and denatured partners such as protein kinases, transcription factors, viral replication proteins, and a range of intracellular receptors, affecting their turnover, trafficking, cellular localization, and activity (28, 29). Recent studies by Kim et al. (23) demonstrated that HSP90 associates with 40S ribosomal components and prevents ubiquitin-dependent ribosomal protein degradation. Our data point to a similar mechanism in which partnership between HSP90 and RIG-I serves to stabilize this protein. In addition, we provide novel evidence showing that the interaction between HSP90 and RIG-I is direct and independent of the CARD domain. Interestingly, we found that the DexH Box, the helicase, and other domains of RIG-I efficiently associated with HSP90. These results suggest that the interaction between these proteins may involve multiple domains. Similar results were reported by Kim et al. (23) who showed that interaction of HSP90 with ribosomal protein S3 involves interaction with two independent do-

In humans, two distinct genes, HSP90-α and HSP90-β, encode closely related cytoplasmic proteins that display minor sequence differences at the amino termini (32–34). The isoforms differ in their ability to activate certain client proteins and regulate cellular functions of immune cells (34–38). The α and β isoforms of HSP90 often cooperate with other proteins to generate a cytosolic multichaperone machinery that includes HSP70, peptidyl-prolyl isomerases, and other chaperones to achieve adequate protein folding (39). Certain cytosolic client polypeptides have been shown to first bind HSP70 and then interact with dimeric HSP90 (39–42). Interestingly, our studies suggest that HSP70 may be a second chaperone that interacts with RIG-I under basal conditions. Our work did not directly address whether HSP70 facilitates the formation or stability of the RIG-I-HSP90 complex, but it is tempting to speculate that HSP70 contributes to this process and that a cochaperone(s) may be involved as well.

As shown in Figures 5A and 5B, geldanamycin is consistent with our studies (Figs. 2 and 3) showing that the CARD domain is not required for association of RIG-I with HSP90.

FIGURE 6. Inhibition of HSP90/RIG-I association abolishes RIG-I-mediated transcriptional activation of the IFN-β promoter. HEK293T cells were cotransfected with a reporter construct harboring nt −299/+19 of the IFN-β gene, a β-galactosidase expression vector, and either full-length RIG-I or the CARD domain. The following day we transfected one-half of the cells with poly(I:C) in the presence or absence of geldanamycin (GA) for 24 h; the remaining cells were treated with vehicle or geldanamycin. We assessed luciferase and β-galactosidase activity in the extracts (n = 4).
HSP90 PROTECTS RIG-I FROM PROTEASOMAL DEGRADATION

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