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A Central Role for the Hsp90·Cdc37 Molecular Chaperone Module in Interleukin-1 Receptor-associated-kinase-dependent Signaling by Toll-like Receptors*

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Toll-like receptors (TLRs) serve crucial roles in innate immunity by mediating the activation of macrophages by microbial pathogens. The protein kinase interleukin-1 receptor associated kinase (IRAK-1) is a key component of TLR signaling pathways via its interaction with TRAF6, which subsequently leads to the activation of MAP kinases and various transcription factors. IRAK-1 is degraded following TLR activation, and this has been proposed to contribute to tolerance in macrophages by limiting further TLR-mediated signaling. Using a mass spectrometric-based approach, we have identified a cohort of chaperones and co-chaperones including Hsp90 and Cdc37, which bind to IRAK-1 but not IRAK-4 in 293T cells. Pharmacologic inhibition of Hsp90 led to a rapid decline in the expression level of IRAK-1, whereas overexpression of Cdc37 enhanced the activation and oligomerization of IRAK-1 in 293T cells. Significantly, the inhibition of Hsp90 in macrophages resulted in the destabilization and degradation of IRAK-1 but not IRAK-4. Concomitant with the loss of IRAK-1 expression was a reduction in the activation of p38 MAP kinase and Erk1/2 following stimulation with the bacterially derived TLR ligands, lipopolysaccharide and CpG DNA. Moreover, TLR ligand-induced expression of proinflammatory cytokines was also reduced. Thus we conclude that the level of on-going support provided to IRAK-1 by the Hsp90-Cdc37 chaperone module directly influences the magnitude of TLR-mediated macrophage activation. In addition, because further TLR signaling depends on the synthesis of new IRAK-1, the Hsp90-Cdc37 chaperone module could also contribute to tolerance in macrophages by controlling the rate at which nascent IRAK-1 is folded into a functional conformation.

The innate immune system represents the first line of defense of the host against infection by microbial pathogens. Macrophages are a key component of the innate immune system as they have the capacity to secrete inflammatory cytokines (*e.g.* TNFα[1] and IL-1), as well as phagocytose, and degrade microbial pathogens. Subsequent presentation of pathogen-derived peptides to T-helper cells by macrophages is important for the adaptive immune response (1).

The detection of microbial pathogens by macrophages is largely mediated by Toll-like receptors (TLRs) (2, 3). Different TLRs are activated by specific components of microbial pathogens. For example, TLR4 is activated by bacterial lipopolysaccharide (LPS) (4, 5), whereas unmethylated CpG DNA triggers the activation of TLR9 (6). Recently, a number of host factors (*e.g.* fibrin(ogen) and hyaluronan) have been proposed to function as endogenous ligands for TLRs (7–10). Ligation of TLRs triggers the activation of a complex network of intracellular signal transduction pathways. Prominent components of these pathways include IL-1 receptor-associated kinases (*e.g.* IRAK-1 and IRAK-4), TRAF6, MAP kinases (*e.g.* p38 MAP kinase and Erk1/2), and the transcription factors, NF-κB and AP-1 (11). Expression of various genes (*e.g. TNFα*, *IL-1*, *IL-8*, *CD80*, and *CD86*) mediates the ensuing innate and adaptive immune responses (2, 3).

The generation of IRAK-1 knock-out mice has revealed an important role for this protein kinase in signaling by TLR4 as well as the IL-1 receptor (12–14). Cells lacking IRAK-1 exhibit an impaired ability to activate p38 MAP kinase, c-Jun N-terminal kinase, and NF-κB and to secrete inflammatory cytokines (*e.g.* TNFα and IL-6) when stimulated with LPS or IL-1 (12–14). Notably, IRAK-1-deficient mice are less susceptible to the lethal effects of LPS than their wild-type counterparts (14). IRAK-1 also participates in signaling by TLR2, TLR5, TLR7, and TLR9 (6, 15, 16).

IRAK-1 is rapidly phosphorylated, ubiquitinated, and then degraded via the proteasome following its activation by TLR ligands or IL-1 (17–19). Such degradation of IRAK-1 may represent a negative feedback mechanism to prevent prolonged activation of cells in response to TLR ligands or IL-1. IRAK-1 is also subject to negative regulation via its interaction with Tollip and IRAK-M (20, 21). Tollip forms a complex with IRAK-1 in resting cells and prevents its activation in the absence of an appropriate stimulus (*e.g.* TLR ligand) (20). IRAK-M is a catalytically inactive kinase that suppresses IRAK-1 function by inhibiting phosphorylation of IRAK-1 or preventing its release from the receptor complex (21).

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[1] The abbreviations used are: TNFα, tumor necrosis factor α; IL, interleukin; MAP, mitogen-activated protein; BMMs, bone marrow-derived macrophages; GA, geldanamycin; IRAK, interleukin-1 receptor associated kinase; LPS, lipopolysaccharide; RAD, radicicol; TLR, Toll-like receptor; KD, kinase-dead; HA, hemagglutinin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Hsc, heat shock cognate protein; IFN-γ, interferon-γ.

Here we report that the stable expression of functional IRAK-1 in cells was dependent on its interaction with Hsp90. Hsp90 is a molecular chaperone that acts in concert with heat shock cognate protein (Hsc)/Hsp70 and various co-chaperones (*e.g.* Hop and Cdc37) to fold client proteins (*e.g.* steroid receptors and protein kinases) into functional conformations (22). Hop serves to coordinate interactions between Hsp90 and Hsc/Hsp70 during client protein folding and is typically associated with "early/intermediate" folding complexes consisting of the client protein, Hsp70 and Hsp90 (22, 23). Cdc37 specifically stabilizes the interaction of Hsp90 with client protein kinases, thereby promoting the formation of "mature" complexes containing the client protein kinase, Hsp90, and Cdc37 (24–28). The client protein kinase is subsequently released from the chaperone complex following its folding into a functional conformation. Inhibition of Hsp90 in macrophages resulted in the rapid loss of IRAK-1 expression and culminated in the impaired ability of TLR4 and TLR9 ligands to activate p38 MAP kinase and Erk1/2 and to stimulate the expression of inflammatory cytokines. Thus Hsp90 may play an important role in controlling the inflammatory response of macrophages by directly modulating the stability and hence signal transducing capacity of IRAK-1.

EXPERIMENTAL PROCEDURES

Reagents—Geldanamycin, LPS (*Escherichia coli* 0111:B4) and anti-FLAG, horseradish peroxidase-conjugated anti-FLAG, and agarose-coupled anti-FLAG antibodies were obtained from Sigma. Radicicol was obtained from Calbiochem, and the murine CpG oligonucleotide ODN1860 was from InvivoGen. The anti-Hsp90 antibody was from Affinity BioReagents, Inc. The anti-IRAK-1 antibody was obtained from Santa Cruz Biotechnology, Inc., and the anti-IRAK-4 antibody was from UBI, Inc. Anti-phospho-p38 MAP kinase, anti-p38 MAP kinase, anti-phospho-Erk1/2, and anti-Erk1 antibodies were from Cell Signaling Technology. The rabbit polyclonal anti-Cdc37 antibody (used for immunoprecipitation) was generated in this laboratory, whereas the mouse polyclonal anti-Cdc37 antibody (used for Western blotting) was a gift from Dr. Steven Hartson (Oklahoma State University). The anti-Hsp70 and anti-Hop antibodies were gifts from Dr. David Toft (Mayo Clinic, Rochester, MN).

Plasmids—Expression vectors encoding FLAG-tagged versions of wild-type IRAK-1 (pIC-FLAG-IRAK-1) and kinase-dead IRAK-1 (pIC-FLAG-IRAK-1 KD) were generous gifts of Dr. Sankar Ghosh (Yale University) (20). An expression vector encoding FLAG-tagged IRAK-4 (*i.e.* FLAG-IRAK-4) was constructed by PCR using *Pfu* DNA polymerase and the plasmid pTriplEx-mIRAK-4 (from Dr. Wen-Chen Yeh, University of Toronto) as template. The resulting 1.4-kb PCR product was digested with MluI and subcloned into the corresponding site in pEF-BOS-FLAG (29). An expression vector encoding HA-tagged Cdc37 (*i.e.* HA-Cdc37) was created by excising the cDNA for Cdc37 from pEF-FLAG-Cdc37 (29) with MluI and subcloning it into the corresponding site in pEF-BOS-HA.

Cell Culture and Transient Transfections—Human 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and transiently transfected using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions (29). Bone marrow-derived macrophages were prepared from 8-week-old female C57BL/6 mice as described previously (30).

Cell Lysis, Western Blotting, and Immunoprecipitation—Cells were lysed directly in tissue culture dishes with Nonidet P-40 lysis buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, 1.0% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, and Complete™ protease inhibitors) for 60 min on ice. Lysates were clarified by centrifugation at 13,000 × *g* for 10 min at 4 °C, and then protein concentrations were measured with a Bio-Rad protein assay kit. Western blotting and immunoprecipitation of cell lysates were performed by standard techniques (29, 31).

Mass Spectrometry—Protein bands were excised from gels stained with colloidal Coomassie Blue G-250 and digested *in situ* with trypsin. The peptides then were extracted, and peptide mass fingerprinting was performed using a Voyager DE™ MALDI-TOF mass spectrometer (Applied Biosystems) in linear mode or an Ultraflex™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in reflectron mode. The mass spectra were analyzed by searching the Mascot search engine (www.matrixscience.com) based on the NCBI and SwissProt protein databases.

In Vitro Kinase Assays—Anti-FLAG immunoprecipitates were incubated at 37 °C for 20 min in 20 μl of kinase buffer (20 mM Hepes, pH 7.4, 25 mM $MgCl_2$, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 20 μM ATP) containing 10 μCi of [γ-^{32}P]ATP. The ability of immunoprecipitated IRAK-1 to phosphorylate an exogenous substrate was tested by supplementing the kinase reactions with 2.5 μg of myelin basic protein. Reactions were terminated with SDS-PAGE sample buffer and heated for 5 min at 95 °C. The reactions then were subjected to SDS-PAGE followed by transfer to a nitrocellulose filter and exposure to x-ray film.

High Pressure Liquid Chromatography Size-Exclusion Chromatography—Transfected 293T cells were lysed by Dounce homogenization in lysis buffer containing 0.1% Nonidet P-40. The lysates (500 μl) then were applied to a Superose-6 column (HR 10/30, Amersham Biosciences) equilibrated with column buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM NaF, 2 mM EGTA, and 10% glycerol), and elution was performed at a flow rate of 0.4 ml/min with fractions collected at each minute. The column was calibrated with thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa).

Real-Time PCR Analysis of Gene Expression—Total RNA was isolated from bone marrow-derived macrophages (BMMs) with an RNeasy Mini kit (Qiagen) and then reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was performed using an ABI PRISM 7900HT sequence detection system and predeveloped TaqMan probe/primer combinations for *TNFα*, *IL-1β*, and 18 S rRNA (ABI). Threshold cycle numbers were transformed using the $_{\Delta\Delta}$Ct and relative value method as described by the manufacturer and expressed relative to 18 S rRNA.

RESULTS

IRAK-1 Forms a Complex with a Cohort of Molecular Chaperones and Co-chaperones in 293T Cells—To identify novel regulators or effectors of IRAK-1, FLAG-tagged versions of wild-type and kinase-dead IRAK-1 (*i.e.* FLAG-IRAK-1 and FLAG-IRAK-1 KD, respectively) were transiently expressed in human 293T cells and then affinity-purified using anti-FLAG antibodies coupled to agarose beads. IRAK-1-binding proteins were detected by subjecting the immunoprecipitates to SDS-PAGE followed by staining of the gel with colloidal Coomassie Blue G-250. As shown in Fig. 1*A*, five distinct proteins (designated "1", "2", "3" "4," and "5") were detected in anti-FLAG immunoprecipitates derived from 293T cells expressing FLAG-IRAK-1 (*lane 2*) or FLAG-IRAK-1 KD (*lane 3*) but not from empty vector control-transfected cells (*lane 1*). These five protein bands were excised from the gel and digested *in situ* with trypsin and the resulting peptides subjected to mass spectrometry (MALDI-TOF or MALDI-TOF/TOF). The identities of the proteins (in order 1–5) were established as Hsp90, IRAK-1, Hsc70, Hsp70, and Hsp90-organizing protein (Hop).

To confirm the mass spectrometric-based identification of the IRAK-1-binding proteins, anti-FLAG immunoprecipitates of FLAG-IRAK-1 were also subjected to Western blotting. As shown in Fig. 1*B*, Hsp90, Hsp70, and Hop were detected in anti-FLAG immunoprecipitates derived from 293T cells expressing either wild-type or kinase-dead FLAG-IRAK-1 (*lanes 2* and *3*, respectively) but not in immunoprecipitates from cells transfected with empty control vector (*lane 1*). Cdc37 is a 50-kDa protein that recruits a subset of protein kinases to Hsp90 to facilitate their folding into active conformations (24, 25, 28, 29). Although a 50-kDa protein was not detected in the anti-FLAG immunoprecipitates, the heavy chain of the anti-FLAG antibody could have obscured the presence of Cdc37 (Fig. 1*A*). Indeed, Western blotting of the immunoprecipitates with an anti-Cdc37 antibody revealed co-immunoprecipitation of Cdc37 with both wild-type and kinase-dead FLAG-IRAK-1 (Fig. 1*B*, *lanes 2* and *3*). Reciprocal immunoprecipitation experiments with an anti-Cdc37 antibody demonstrated co-immunoprecipitation of FLAG-IRAK-1 (kinase-active and kinase-dead) and Hsp90 with Cdc37 (Fig. 1*C*, *lanes 2* and *3*). However, neither

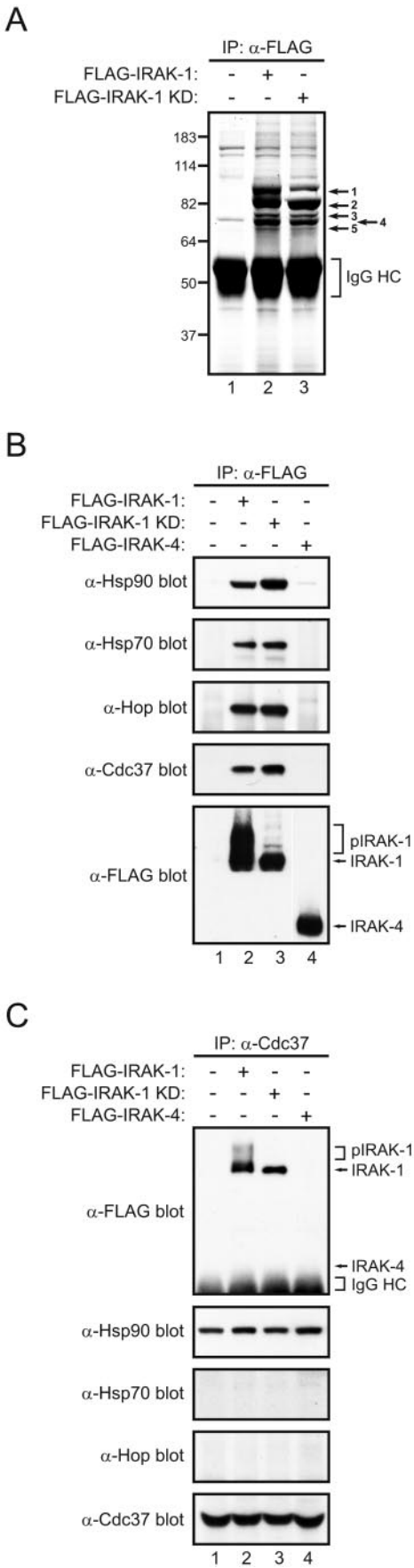

FIG. 1. **Identification of IRAK-1-binding proteins in 293T cells.** *A*, lysates of 293T cells transiently expressing FLAG-tagged versions of wild-type (*FLAG-IRAK-1*) or kinase-dead (*FLAG-IRAK-1 KD*) IRAK-1 were immunoprecipitated (*IP*) with anti-FLAG antibodies. The immunoprecipitates then were subjected to SDS-PAGE followed by staining with colloidal Coomassie Blue G-250. Proteins specifically immunoprecipitated by the anti-FLAG antibodies (*numbered arrows*), positions of molecular mass standards (in kDa), and the heavy chain of the anti-FLAG antibody (*IgG HC*) are indicated. *B* and *C*, lysates of cells transiently expressing FLAG-IRAK-1 (*lane 2*), FLAG-IRAK-1 KD (*lane 3*), or FLAG-IRAK-4 (*lane 4*) were immunoprecipitated with anti-FLAG (*B*) or anti-Cdc37 antibodies (*C*). The immunoprecipitates then were subjected to SDS-PAGE and Western blotted with the indicated antibodies. Phosphorylated (*pIRAK-1*) and non-phosphorylated (*IRAK-1*) forms of FLAG-IRAK-1 and FLAG-IRAK-4 are indicated.

Hsp70 nor Hop was found to co-immunoprecipitate with Cdc37 (Fig. 1*C*, *lanes 1–4*). These findings suggest that FLAG-IRAK-1 is present in at least two distinct heterocomplexes, one consisting of FLAG-IRAK-1, Hsp90, Hsp70, and Hop and another consisting of FLAG-IRAK-1, Hsp90, and Cdc37. To examine the specificity of the interaction of IRAK-1 with Hsp90, Hsp70, Hop, and Cdc37, the ability of IRAK-4 to form a complex with these proteins was also determined. IRAK-4 is structurally related to IRAK-1 and has been proposed to mediate the activation of IRAK-1 in response to engagement of Toll-like receptors by their ligands (32). No interaction of FLAG-tagged IRAK-4 with Hsp90, Hsp70, Hop, or Cdc37 was observed (Fig. 1, *B* and *C*, *lane 4*). Thus the interaction of IRAK-1 with Hsp90, Hsp70, Hop, and Cdc37 appears to be specific and not the consequence of its overexpression in 293T cells.

Stable Expression of IRAK-1 in 293T Cells Is Dependent on Hsp90 Activity—The importance of Hsp90 for IRAK-1 function was directly addressed by treating 293T cells expressing FLAG-IRAK-1 with two structurally dissimilar Hsp90 inhibitors, geldanamycin (GA) and radicicol (RAD) (33–37). Consistent with an earlier report by Li *et al.* (38), several electrophoretically distinct forms of FLAG-IRAK-1 were observed upon its overexpression in 293T cells (Fig. 2*A*). Yamin and Miller (19) have previously shown that the more slowly migrating forms represent phosphorylated IRAK-1. Thus overexpression of FLAG-IRAK-1 in 293T cells results in ligand-independent autoactivation of the protein kinase (38). Treatment of the cells with either GA or RAD resulted in a dose-dependent decrease in the expression level of phosphorylated (*i.e.* activated) FLAG-IRAK-1 (Fig. 2*A*). Time course experiments with GA revealed a decrease in the level of phosphorylated IRAK-1 within 2 h of treating the cells with the Hsp90 inhibitor, which diminished significantly further by 8 h (Fig. 2*B*, *lanes 3–6*). To directly assess the effect of GA on the enzymatic activity of FLAG-IRAK-1, the capacity of immunoprecipitated FLAG-IRAK-1 to autophosphorylate as well as phosphorylate an exogenous substrate (*i.e.* myelin basic protein) was measured. These *in vitro* kinase assays confirmed a time-dependent loss of catalytically active FLAG-IRAK-1 in 293T cells treated with GA (Fig. 2*C*). To investigate the effect of GA on the composition of IRAK-1 heterocomplexes, FLAG-tagged IRAK-1 was immunoprecipitated from lysates of transfected 293T cells and subjected to Western blotting. The data presented in Fig. 2*D* clearly revealed that treatment of the transfected cells with GA reduced but did not abolish the association of Hsp90 with FLAG-IRAK-1. By contrast, the levels of Hsp70 and Hop associated with FLAG-IRAK-1 increased in response to GA treatment, whereas the association of Cdc37 with FLAG-IRAK-1 was abolished within 60 min of treating the cells with GA (Fig. 2*D*, *lane 2 versus lane 3*). These findings clearly indicate that Hsp90 activity is required for the stable expression of catalytically active IRAK-1 in 293T cells. Furthermore, they suggest that the interaction of Cdc37 with IRAK-1 is necessary for the stabilization of IRAK-1 by Hsp90.

Hsp90 Is Required for the Stable Expression of IRAK-1 in Macrophages—To establish whether a functional relationship

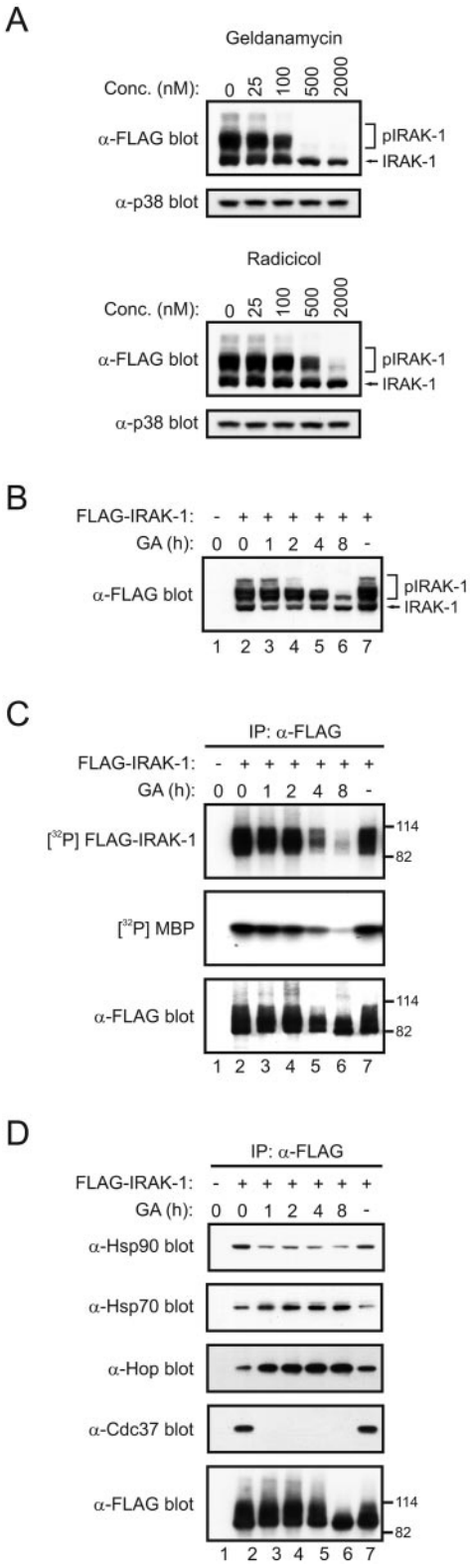

FIG. 2. **Inhibition of Hsp90 promotes the degradation of IRAK-1 in 293T cells.** *A*, human 293T cells transiently expressing FLAG-IRAK-1 were treated 24 h post-transfection with the indicated concentrations of GA or RAD for 8 h. The cells were then lysed, and the lysates were Western blotted with the indicated antibodies. *B–D*, 293T cells transiently expressing FLAG-IRAK-1 were treated 24 h post-transfection with 2 μM GA for the indicated times or with 0.1% Me_2SO for 8 h (−). The cells then were lysed, and the lysates were either Western blotted (*B*) or immunoprecipitated (*IP*) (*C* and *D*) with anti-FLAG antibodies. The immunoprecipitates then were subjected to either *in vitro* kinase assays in the presence of [γ-^{32}P]ATP and myelin basic protein (*MBP*) (*C*) or blotting with the indicated antibodies (*D*).

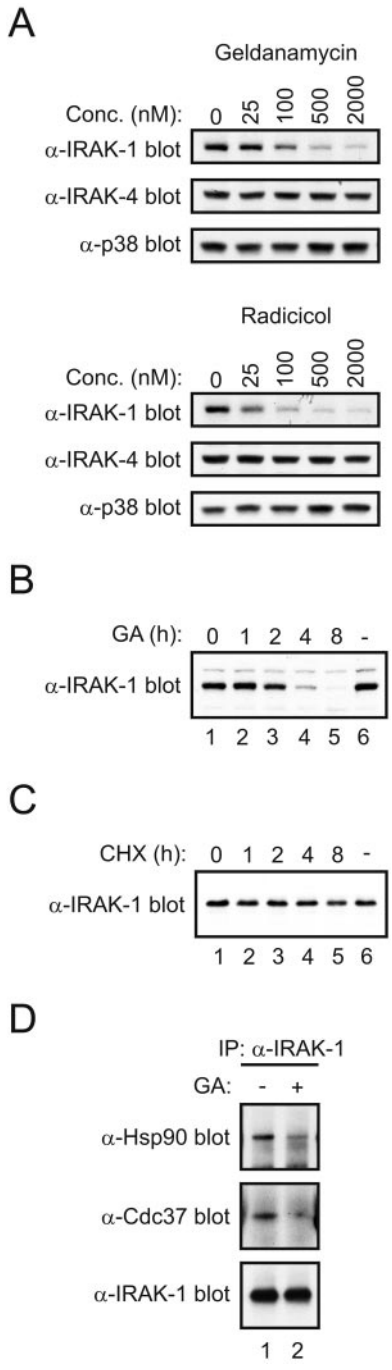

FIG. 3. **Hsp90-dependent expression of endogenous IRAK-1 in macrophages.** *A*, BMMs were treated with the indicated concentrations of GA or RAD for 8 h, lysed, and then Western blotted with the indicated antibodies. *B*, BMMs were treated with 2 μM GA (*lanes 1–5*) for the indicated times or with 0.1% Me_2SO (*lane 6*) for 8 h (−), lysed, and then subjected to Western blotting. *C*, BMMs were treated with 10 μg/ml cycloheximide (*CHX*; *lanes 1–5*) for the indicated times or with 0.1% ethanol (*lane 6*) for 8 h. The cells then were lysed, and the lysates were blotted with the indicated antibodies. *D*, BMMs were treated for 1 h with 2 μM GA (*lane 2*) or left untreated (*lane 1*). The cells then were lysed, and IRAK-1 was immunoprecipitated (*IP*) with an anti-IRAK-1 antibody. The immunoprecipitates were Western blotted with the indicated antibodies.

also exists between endogenous IRAK-1 and Hsp90 in macrophages, primary mouse BMMs were treated with different concentrations of GA or RAD for 8 h and the expression levels of IRAK-1 were evaluated by Western blotting. As shown in Fig. 3*A*, the treatment of BMMs with either GA or RAD induced a dose-dependent decrease in the expression level of endogenous

IRAK-1. Neither GA nor RAD had any significant effect on the expression levels of endogenous IRAK-4 or p38 MAP kinase (Fig. 3*A*). The expression levels of Hsp90 and Cdc37 were essentially unaltered in GA- or RAD-treated BMMs (data not shown). GA promoted the relatively rapid degradation of IRAK-1 in BMMs with the half-life of the protein kinase estimated to be ~3 h in GA-treated macrophages (Fig. 3*B*). To establish whether the loss of IRAK-1 in GA-treated BMMs was the result of degradation of nascent and/or mature (*i.e.* folded) IRAK-1, the half-life of IRAK-1 in macrophages containing functional Hsp90 was established. BMMs were treated with the protein synthesis inhibitor cycloheximide, and IRAK-1 levels were monitored by Western blotting. These experiments revealed that the half-life of mature IRAK-1 in macrophages is greater than 8 h (Fig. 3*C*). Likewise, the half-lives of IRAK-4, p38 MAP kinase, and Erk1/2 in BMMs were also greater than 8 h (data not shown). Thus our finding that the half-life of IRAK-1 is reduced in GA-treated BMMs (3 h *versus* >8 h) suggests that, even following its folding into a mature and functional conformation, IRAK-1 is still dependent on Hsp90 activity for its continued stable expression in macrophages. The functional dependence of IRAK-1 on Hsp90 and Cdc37 in macrophages was further illustrated by demonstrating co-immunoprecipitation of Hsp90 and Cdc37 with endogenous IRAK-1 (Fig. 3*D*). Consistent with the data presented in Fig. 2*D*, treatment of BMMs with GA for 1 h led to a reduction in the association of Hsp90 and Cdc37 with endogenous IRAK-1 (Fig. 3*D*).

Cdc37 Enhances Autoactivation of IRAK-1 in 293T Cells—The finding that GA-induced degradation of IRAK-1 correlated with the loss of Cdc37 from IRAK-1 heterocomplexes suggested that Cdc37 played an important role in the stabilization of IRAK-1 by Hsp90. To address this issue, the effect of Cdc37 overexpression on IRAK-1 expression and activity was investigated. The data presented in Fig. 4*A* revealed that the proportion of total FLAG-IRAK-1 that was hyperphosphorylated (and thus activated) was higher in 293T cells expressing HA-Cdc37 compared with cells expressing FLAG-IRAK-1 alone (*lane 4 versus lane 2*). Notably, the enhanced activation of FLAG-IRAK-1 in 293T cells co-expressing HA-Cdc37 was still dependent on Hsp90 activity (Fig. 4*A*, *lane 5 versus lane 4*). Examination of the composition of FLAG-IRAK-1 heterocomplexes revealed that HA-Cdc37 competed with endogenous Cdc37 for binding to FLAG-IRAK-1 (Fig. 4*B*, *lane 4 versus lane 2*). Expression of HA-Cdc37 did not appear to affect the level of Hsp90 associated with FLAG-IRAK-1 (Fig. 4*B*, *lane 4 versus lane 2*). However, the association of Hsp70 and Hop with FLAG-IRAK-1 was markedly reduced upon co-expression of the kinase with HA-Cdc37 (*lane 4 versus lane 2*), although treatment of identically transfected cells with GA resulted in the interaction of Hsp70 and Hop with FLAG-IRAK-1 (Fig. 4*B*, *lane 5 versus lane 4*). Thus the expression level of Cdc37 appears to be a critical determinant of the ability of Hsp90 to maintain IRAK-1 in a stable and functional conformation.

Cdc37 Enhances the Oligomerization of IRAK-1 in 293T Cells—The nature of IRAK-1·Hsp90·Cdc37 heterocomplexes was defined further by subjecting lysates of transfected 293T cells to size-exclusion chromatography. Both phosphorylated (*i.e.* active) and non-phosphorylated (*i.e.* inactive) forms of FLAG-IRAK-1 were primarily eluted from the Superose-6 column over a molecular mass range of ~160–400 kDa (Fig. 5*A*). The ~220-kDa anti-FLAG immunoreactive species detected in fractions 21–24 by Western blotting was most probably a ubiquitinated form of FLAG-IRAK-1 (19) (Fig. 5*A*). The co-expression of FLAG-IRAK-1 with HA-Cdc37 resulted in the majority of the kinase eluting from the column in fractions corresponding to a protein complex with a molecular mass of ~400–4000 kDa (Fig. 5*B*). However, treatment of 293T cells co-expressing FLAG-IRAK-1 and HA-Cdc37 with GA for 2 h prior to cell lysis resulted in a dramatic reduction in the level of phosphorylated FLAG-IRAK-1 in the high molecular mass fractions with the elution profile resembling that of FLAG-IRAK-1 expressed alone (Fig. 5, *A* and *C*). In all three cases, Hsp90 and endogenous Cdc37 primarily eluted from the Superose-6 column in fractions 31–34, which corresponds to a molecular mass of ~450 kDa (Fig. 5, *A–C*). A much smaller peak of endogenous Cdc37 was detected in fractions 39 and 40 (Fig. 5*A*), whereas transfected HA-Cdc37 was detected rather uniformly throughout fractions 31–42 (Fig. 5, *B* and *C*).

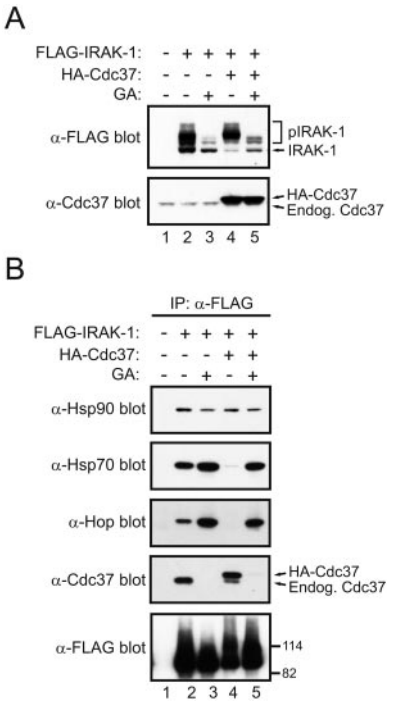

FIG. 4. **Overexpression of Cdc37 enhances autoactivation of IRAK-1.** 293T cells transiently expressing FLAG-IRAK-1 alone (*lanes 2* and *3*) or both FLAG-IRAK-1 and HA-Cdc37 (*lanes 4* and *5*) were treated 24 h post-transfection with 2 μM GA (+) or 0.1% Me_2SO (−) for 8 h. The cells were lysed, and the lysates were either Western blotted (*A*) or immunoprecipitated (*IP*) (*B*) with anti-FLAG antibodies and then subjected to blotting with the indicated antibodies.

Hsp90 and Cdc37 Form a Complex with Inactive IRAK-1—To determine whether the endogenous Hsp90, Cdc37, and transfected HA-Cdc37 present in the various column fractions were complexed with co-eluted FLAG-IRAK-1, anti-FLAG immunoprecipitates derived from fractions 21, 28, 32, and 36 were subjected to Western blot analysis. As shown in Fig. 5*A*, FLAG-IRAK-1·Hsp90·Cdc37 heterocomplexes were essentially restricted to fraction 32. Intriguingly, Hsp90 was also found to be complexed with FLAG-IRAK-1 in fraction 21 and, to a lesser extent, fraction 28 when the kinase was expressed in the absence of HA-Cdc37 but not when co-expressed with HA-Cdc37 (Fig. 6*A*). *In vitro* kinase assays revealed that FLAG-IRAK-1 in fraction 21 was catalytically active, whereas FLAG-IRAK-1 in fractions 28, 32, and 36 exhibited negligible kinase activity (Fig. 6*B*). Moreover, the co-expression of FLAG-IRAK-1 with HA-Cdc37 increased the level of FLAG-IRAK-1 kinase activity in fraction 21 (Fig. 6*B*). However, the level of FLAG-IRAK-1 kinase activity in fraction 21 from GA-treated cells was profoundly less than that in the corresponding fraction from untreated cells (Fig. 6*B*). Together these findings suggest that Hsp90 and Cdc37 primarily associate with a catalytically inactive form(s) of IRAK-1.

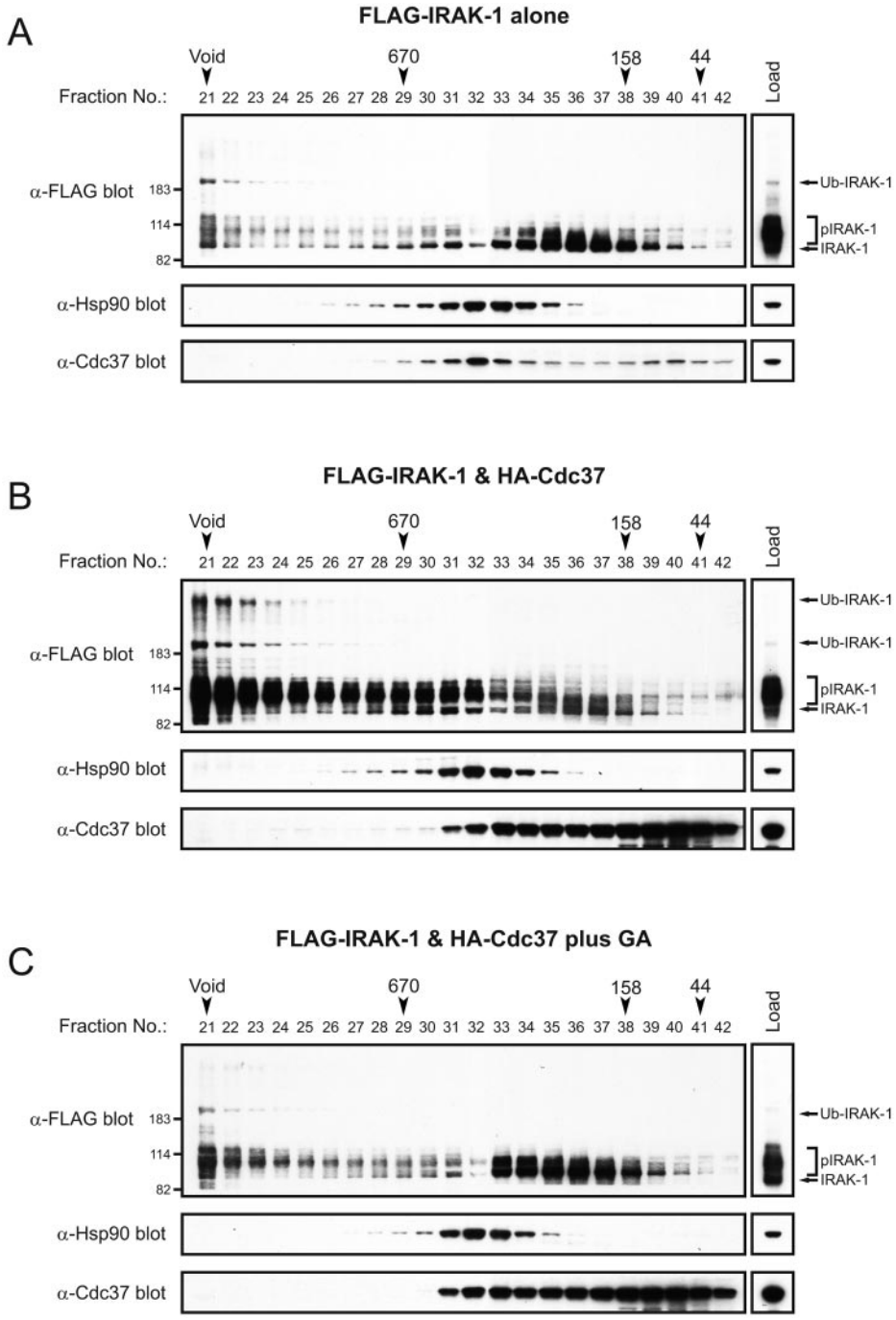

FIG. 5. **Separation of IRAK-1 heterocomplexes by size-exclusion chromatography.** Cell lysates derived from 293T cells expressing FLAG-IRAK-1 alone (*A*), FLAG-IRAK-1 and HA-Cdc37 (*B*), or FLAG-IRAK-1 and HA-Cdc37 but treated with 2 μM GA for 2 h prior to lysis (*C*) were applied to a Superose-6 size-exclusion column. Aliquots of column fractions were subjected to blotting with the indicated antibodies. The elution positions of column calibration standards (in kDa) are shown at the *top*. Ubiquitinated (*Ub-IRAK-1*), phosphorylated (*pIRAK-1*), and non-phosphorylated (*IRAK-1*) forms of IRAK-1 are indicated on the *right*.

Degradation of IRAK-1 in Response to Hsp90 Inhibition Abrogates TLR-mediated Macrophage Activation—Given the above findings, we assessed the impact of GA and RAD on the TLR-mediated activation of p38 MAP kinase and Erk1/2, both of which function downstream of IRAK-1 in TLR signaling. Consistent with other studies (17, 18), LPS (TLR4 ligand) induced the activation of p38 MAP kinase and Erk1/2 with concomitant degradation of IRAK-1 in BMMs (Fig. 7*A*). Significantly, however, prior treatment of BMMs with GA profoundly suppressed the ability of LPS to activate p38 MAP kinase (Fig. 7*A*). The reduction in LPS-induced activation of p38 MAP kinase correlated with the extent of GA-induced degradation of IRAK-1 (Fig. 7*A*). Noticeably, LPS-induced activation of Erk1/2 was more severely compromised by GA treatment than p38 MAP kinase activation (Fig. 7*A*). The ability of a CpG-containing oligonucleotide (TLR9 ligand) to activate p38 MAP kinase and Erk1/2 was also significantly perturbed by prior treatment of BMMs with GA (Fig. 7*B*). As was the case for LPS, the ability of the CpG-containing oligonucleotide to activate Erk1/2 in GA-treated cells was affected to a greater extent than p38 MAP kinase activation (Fig. 7*B*). No differences were observed in the kinetics of p38 MAP kinase or Erk1/2 activation in response to LPS or CpG DNA stimulation between GA-treated and untreated BMMs (data not shown). Pretreatment of BMMs with RAD also dramatically suppressed the ability of LPS and CpG DNA to induce the activation of p38 MAP kinase and Erk1/2 without having any effect on the expression level of either protein kinase (Fig. 7, *C* and *D*).

To further assess the impact of GA- and RAD-induced degradation of IRAK-1 on macrophage activation by TLR ligands, quantitative real-time PCR was used to measure the expression levels of inflammatory cytokines following stimulation of

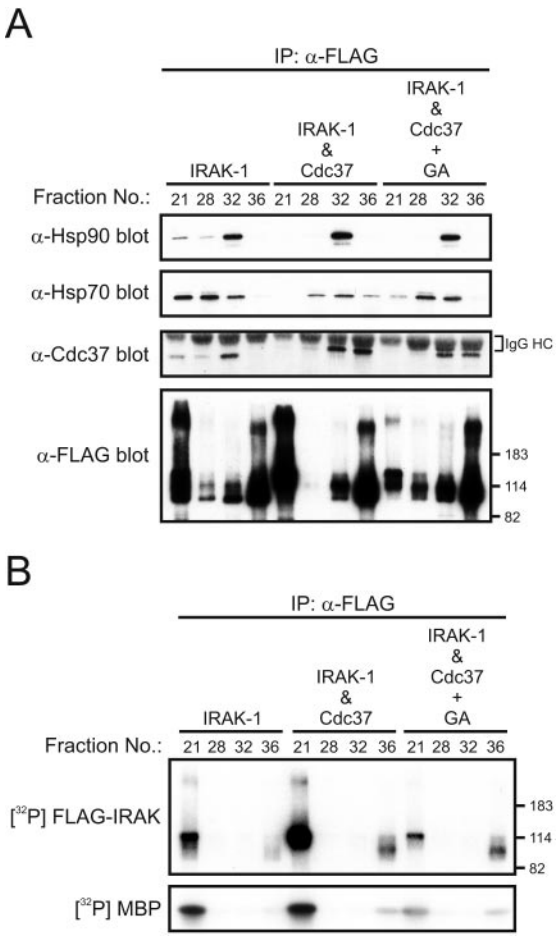

FIG. 6. **Analysis of IRAK-1 complexes separated by size-exclusion chromatography.** FLAG-IRAK-1 was immunoprecipitated (*IP*) from aliquots of the indicated column fractions shown in Fig. 4 using anti-FLAG antibodies and either Western blotted (*A*) or subjected to *in vitro* kinase assays (*B*) in the presence of [γ-^{32}P]ATP and myelin basic protein (*MBP*). *IgG HC*, heavy chain of IgG antibody.

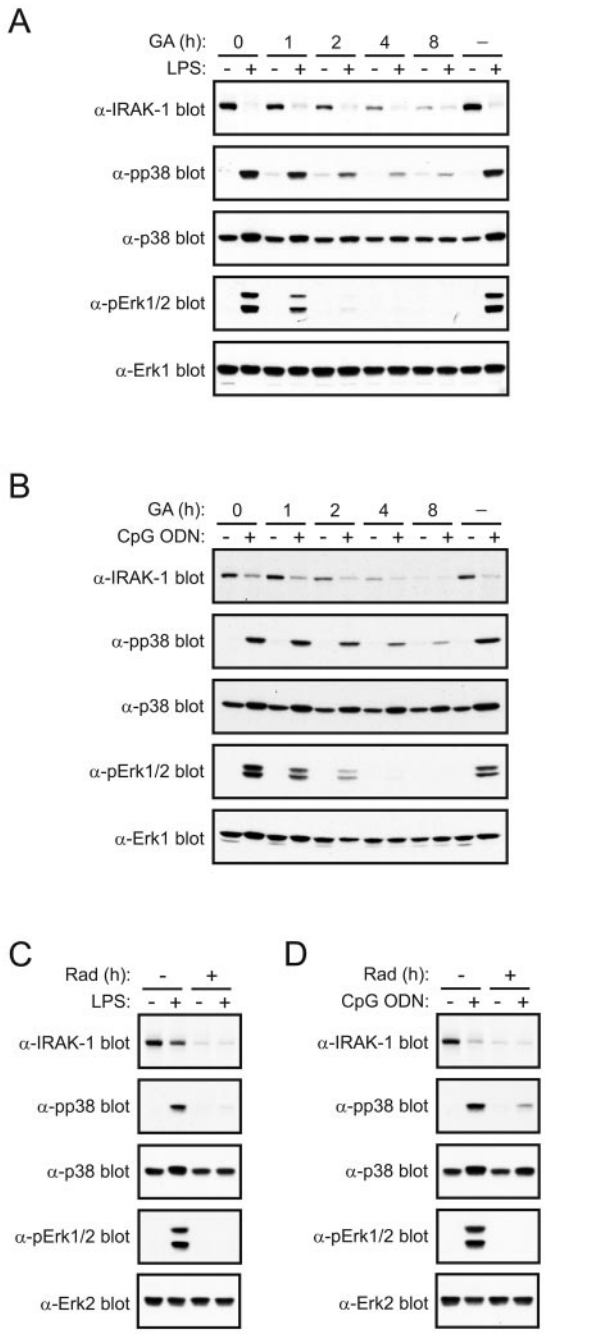

FIG. 7. **Inhibition of Hsp90 suppresses the activation of MAP kinases by TLR ligands.** *A* and *B*, BMMs were pretreated with 2 μM GA for the indicated times or 0.1% Me_2SO for 8 h (−). The cells were then stimulated with 100 ng/ml LPS (*A*) or 2 μM CpG ODN (*B*) for 30 min or left unstimulated. The BMMs were lysed, and the activation states of p38 MAP kinase and Erk1/2 were assessed by Western blotting with phospho-specific anti-p38 MAP kinase (*α-pp38 blot*) and anti-Erk1/2 (*α-pErk1/2 blot*) antibodies, respectively. *C* and *D*, BMMs were pretreated with 2 μM RAD or 0.1% Me_2SO for 8 h (−) and then stimulated with 100 ng/ml LPS (*C*) or 2 μM CpG ODN (*D*) for 30 min or left unstimulated. The BMMs were lysed, and the activation states of p38 MAP kinase and Erk1/2 were assessed by Western blotting; *ODN*, oligonucleotide ODN1860.

BMMs with LPS or CpG DNA. As shown in Fig. 8*A*, LPS-induced expression of *TNFα* mRNA transcripts was suppressed at least 10-fold by prior treatment of BMMs with GA. Likewise, the pretreatment of BMMs with RAD resulted in a profound reduction in *TNFα* gene expression in response to LPS stimulation. The ability of CpG DNA to induce *TNFα* gene expression was also compromised in BMMs that had been pretreated with GA or RAD (Fig. 8*A*). TLR ligand-induced expression of *IL-1β* mRNA transcripts was reduced similarly in BMMs pretreated with either GA or RAD (Fig. 8*B*). Treatment of BMMs with GA or RAD had no effect on the basal expression levels of *TNFα* or *IL-1β* mRNA transcripts (Fig. 8, *A* and *B*).

DISCUSSION

TLRs play crucial roles in innate immunity. However, excessive and prolonged activation of macrophages by TLR ligands is harmful to the host and even potentially fatal (*e.g.* septic shock). Tolerance, a phenomenon whereby macrophages become desensitized to subsequent exposure to bacterial products (*e.g.* LPS) is an adaptive mechanism that exists to limit the magnitude of the inflammatory reaction that occurs during bacterial infections (39). One molecular explanation for tolerance in macrophages is the down-regulation of IRAK-1 expression (18, 40). Thus in this study, we sought to identify proteins that interact with IRAK-1 because such proteins may regulate the expression or function of IRAK-1 and hence control the magnitude of the inflammatory response of macrophages to TLR ligands. Using a proteomic-based approach, we have identified a cohort of molecular chaperones and co-chaperones including Hsp90 and Cdc37, which bind to IRAK-1 (Fig. 1).

The biochemical importance of the interaction of Hsp90 with IRAK-1 was established directly by demonstrating that two structurally dissimilar Hsp90 inhibitors, namely GA and RAD, promoted the degradation of IRAK-1 in both 293T cells and

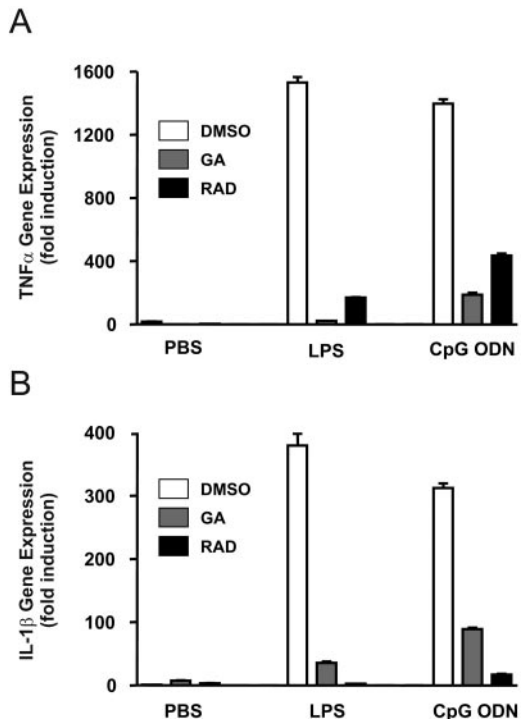

FIG. 8. **Hsp90 inhibition reduces TLR-mediated inflammatory cytokine mRNA expression.** BMMs were pretreated with 2 μM GA (*gray bar*), 2 μM RAD (*black bar*), or 0.1% Me_2SO (*DMSO*) (*white bar*) for 8 h and then stimulated with phosphate-buffered saline (*PBS*), 20 ng/ml LPS, or 0.5 μM CpG ODN for 60 min. Total RNA was isolated, and relative mRNA expression levels of TNFα (*A*) and IL-1β (*B*) were measured by real-time PCR.

primary mouse macrophages (Figs. 2 and 3). Investigation of the mechanism of Hsp90-mediated protein folding has revealed that it acts in concert with Hsp70 and various co-chaperones (see Ref. 22 for review). The first step in the paradigm derived from these studies is the association of Hsp70 with the client protein to form "early" folding complexes. The subsequent binding of Hsp90 and its co-chaperone Hop leads to the formation of "intermediate" complexes with further maturation of the complex accompanied by the loss of Hsp70 and Hop from the complex and the inclusion of other Hsp90 co-chaperones (*e.g.* Cdc37). Significantly, we found that the interaction of Hsp70 and Hop with IRAK-1 increased in response to Hsp90 inhibition, whereas the interaction of Cdc37 with IRAK-1 was abolished under the same conditions. Inhibition of Hsp90 activity with GA has been shown previously to suppress the ability of Cdc37 to bind client protein kinases (29, 41, 42). Therefore, our data suggest that the interaction of Cdc37 with IRAK-1 is essential for the transition of IRAK-1 from "early/intermediate" protein-folding complexes to mature folding complexes (see Fig. 2). Moreover, the expression level of Cdc37 may dictate the rate at which IRAK-1 transitions from one complex to the next (Fig. 4). Thus it can be concluded that the interaction of Cdc37 with IRAK-1 is a key regulatory step in the Hsp90-mediated folding of IRAK-1 into a conformation that is able to participate in TLR signaling.

The ability of Hsp90 inhibitors to reduce the half-life of endogenous IRAK-1 in macrophages is a particularly significant finding, because it indicates that, in addition to being required for the *de novo* folding of nascent IRAK-1 into a stable conformation, Hsp90 and Cdc37 are also necessary for the on-going stabilization and function of IRAK-1 (see Fig. 9, *be-*

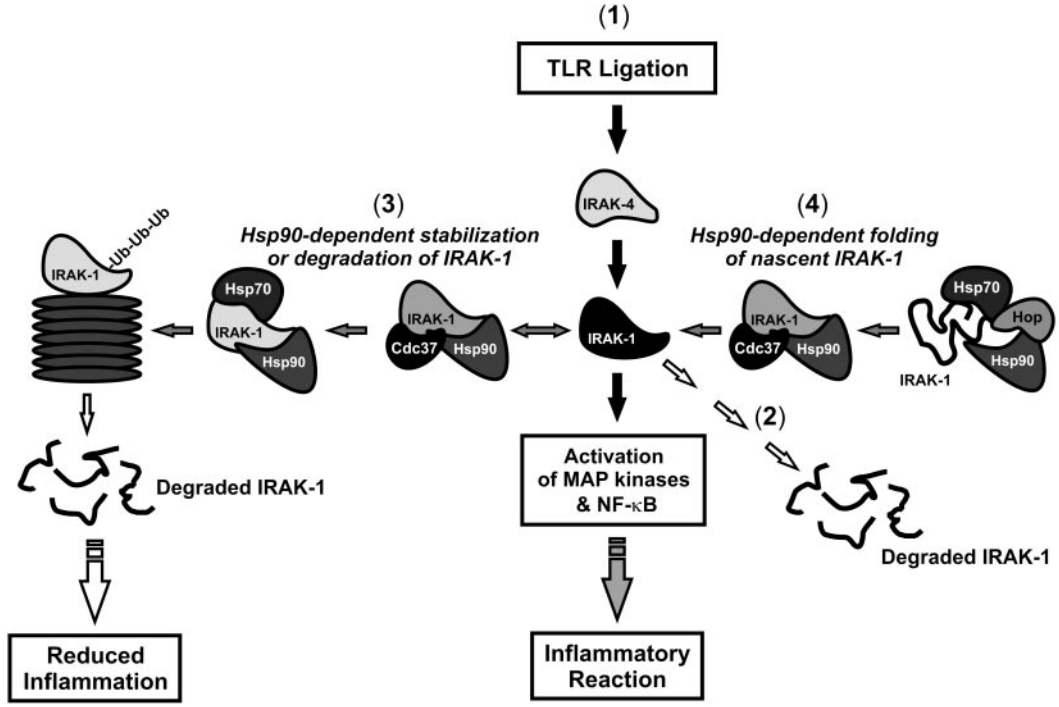

FIG. 9. **A model in which an Hsp90·Cdc37 chaperone module influences TLR-mediated macrophage activation by governing the on-going levels of functional IRAK-1.** *1*, TLR ligation leads to the activation of IRAK-4 and IRAK-1 with subsequent activation of MAP kinases (*e.g.* p38 MAP kinase), NF-κB and expression of proinflammatory mediators (*e.g.* TNFα and IL-1β). *2*, TLR-mediated activation of IRAK-1 leads to its ubiquitination and degradation. *3*, the ability of TLR ligands to activate macrophages is dependent on Hsp90 and Cdc37 maintaining IRAK-1 in a stable and functional conformation. However, they would not physically sequester functional IRAK-1 and prevent its participation in TLR signaling. The level of maintenance required by IRAK-1 would be expected to increase under stressful conditions (*e.g.* elevated temperature during fever). When the level of maintenance provided by the Hsp90·Cdc37 chaperone module becomes insufficient to maintain IRAK-1 in a stable and functional conformation, it would associate with Hsp70 and ultimately be degraded by the proteasome. This in turn would limit the capacity of TLRs to activate downstream signaling targets of IRAK-1 and dampen the inflammatory response of the macrophage. *4*, the ability of macrophages to respond to repeated TLR ligation requires the synthesis of new IRAK-1. Thus the Hsp90·Cdc37 chaperone module could also contribute to tolerance by controlling the rate at which nascent IRAK-1 is folded into a functional conformation.

low). Size-exclusion chromatography revealed that only a relatively small subfraction of IRAK-1 was associated with Hsp90 and Cdc37 under steady-state conditions, even when IRAK-1 overexpressed in 293T cells. Thus the stable expression of functional IRAK-1 in cells is likely to depend on it interacting with Hsp90 and Cdc37 in a highly dynamic rather than static fashion.

The loss of IRAK-1 expression in macrophages following inhibition of Hsp90 severely compromised the ability of LPS (TLR4 ligand) and CpG DNA (TLR9 ligand) to trigger the activation of MAP kinases and expression of inflammatory cytokine genes (*e.g. TNFα* and *IL-1β*) (Figs. 7 and 8). Intriguingly, LPS- and CpG DNA-induced activation of Erk1/2 appeared more sensitive to inhibition by GA than p38 MAP kinase activation. This finding suggests that the IRAK-1-dependent signaling flux required to activate Erk1/2 is greater than that for p38 MAP kinase. Alternatively, the inhibition of Hsp90 may also result in the inactivation of another protein required for the activation of Erk1/2. Raf-1 is a potential candidate for such a protein, because it has been reported previously to mediate the activation of Erk1/2 by LPS (43, 44) and is dependent on Hsp90 and Cdc37 for its activity (24, 45).

Our current findings are in agreement with other reports describing the ability of GA to suppress the inflammatory response of macrophages to LPS and CpG DNA (46–49). Moreover, they provide a novel molecular basis for the inhibitory effect of GA on TLR-mediated macrophage activation. Vega and De Maio (49) conclude that the amelioration in the inflammatory response of macrophages treated with GA to LPS was due to decreased surface expression of the LPS co-receptor CD14. However, the findings we have presented here provide an additional explanation for the amelioration in the response of GA-treated macrophages to LPS, namely GA-induced degradation of IRAK-1. The internalization of CpG DNA by macrophages was shown by Zhu and Pisetsky (47) to be unaffected by GA. Hence, the ability of GA and RAD to inhibit the activation of macrophages by CpG DNA is likely to be attributed again primarily to the degradation of IRAK-1.

The dependence of IRAK-1 for maintenance from Hsp90 and Cdc37 would be predicted to increase under stressful conditions and potentially have important physiologic implications. Heine *et al.* (50) have reported previously that LPS up-regulates Hsp90, Hsp70, and Hop expression in macrophages. However, although we also found that the expression of these proteins was elevated in BMMs stimulated with LPS and/or incubated at a febrile temperature (*e.g.* 39.5 °C), no change in Cdc37 expression was observed.[2] Therefore, the expression level of Cdc37 in macrophages might become rate-limiting under stressful conditions (*e.g.* bacterial infection leading to fever) and compromise the ability of Hsp90 to maintain IRAK-1 in a functional conformation. Thus based on our novel findings, we propose a model in which Hsp90 and Cdc37 act together as a molecular "safety valve" to limit the extent of macrophage activation during infection (Fig. 9). In this model, Hsp90 and Cdc37 would interact with IRAK-1 in a highly dynamic fashion in order to maintain IRAK-1 in a functional conformation but they would not physically sequester functional IRAK-1 and prevent its involvement in TLR signaling. The level of chaperone support required by IRAK-1 would be dictated by the severity of the inflammatory reaction (*i.e.* level of cellular stress). When the expression level (or co-chaperone activity) of Cdc37 is no longer sufficient to mediate the maintenance of IRAK-1 by Hsp90, IRAK-1 would unfold, associate with Hsp70, and ultimately be degraded by the proteasome. This in turn would limit the capacity of TLRs to activate downstream signaling targets of IRAK-1 and lead to a dampened inflammatory response (Fig. 9).

The dependence of IRAK-1 on Hsp90 and Cdc37 for its folding into a functional conformation is also likely to have implications for our current understanding of tolerance in macrophages. Because IRAK-1 is degraded following macrophage activation by TLR ligands (*e.g.* LPS, CpG DNA, and lipopeptide), newly synthesized IRAK-1 must be folded into a functional conformation before it can participate in further TLR-mediated signaling (Fig. 9). Several recent studies (18, 40, 51–53) have revealed that tolerance in macrophages might be explained by a reduction in the expression and/or functional activity of IRAK-1. Thus Hsp90 and Cdc37 could contribute to tolerance by directly controlling the rate at which nascent IRAK-1 is folded into a functional conformation. The proinflammatory cytokine interferon-γ (IFN-γ) is able to suppress tolerance in macrophages, possibly by increasing IRAK-1 expression and/or function (40). Given that the co-chaperone activity of Cdc37 is regulated by CK2-mediated phosphorylation (54, 55) and IFN-γ enhances CK2 activity in macrophages (56), it is tempting to speculate that IFN-γ could in part suppress tolerance in macrophages by increasing Cdc37 function. It will clearly be of interest to establish the effect of proinflammatory (*e.g.* IFN-γ and granulocyte/macrophage colony-stimulating factor) and anti-inflammatory (*e.g.* IL-4 and IL-10) cytokines on Cdc37 function in macrophages.

Raf-1 and IκB kinase are two additional protein kinases involved in TLR signaling that also depend on Hsp90 and Cdc37 for their functions (45, 57). Therefore, the ability of Hsp90 and Cdc37 to directly influence the activity of multiple components of TLR signaling pathways (*e.g.* IRAK-1, Raf-1, and IκB kinase) may represent a fundamental mechanism for governing the extent of macrophage activation. As proposed above, integration of TLR signaling and Hsp90-mediated protein-folding pathways could effectively act as a molecular safety valve to limit macrophage activation during severe bacterial infection and hence prevent or at least reduce tissue injury and destruction.

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[2] G. M. Scholz, unpublished observations.

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