The Journal of Immunology

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J Immunol 2007; 179:6291-6296; ;
doi: 10.4049/jimmunol.179.9.6291
http://www.jimmunol.org/content/179/9/6291

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Heat Shock Protein 90 Associates with Monarch-1 and Regulates Its Ability to Promote Degradation of NF-κB-Inducing Kinase[1]

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Monarch-1/NLRP12 is expressed in myeloid cells and functions as a negative regulator of inflammation by inducing proteasome-mediated degradation of NF-κB-inducing kinase. Monarch-1 is a member of the *CATERPILLER* gene family, also known as the nucleotide-binding domain leucine-rich repeat gene family. This family shares strong structural homology to major immune regulators expressed in lower organisms, including plants. In plants, these disease-resistance proteins (R proteins) sense pathogenic insult and initiate a protective response to limit pathogen growth. To perform this role, many R proteins require the highly conserved chaperone molecule, heat shock protein (Hsp) 90. Using a two-dimensional gel/mass spectrometry system, we detected the association of the nucleotide-binding domain leucine-rich repeat protein Monarch-1 with heat shock proteins. Further analysis indicates that analogous to plant R proteins, Hsp90 is required for Monarch-1 activity. In human monocytes, Monarch-1 associates with Hsp90, and these complexes are sensitive to treatment with specific Hsp90 inhibitors. Disruption of these complexes results in rapid degradation of Monarch-1 via the proteasome and prevents Monarch-1-induced proteolysis of NF-κB-inducing kinase. This demonstrates that Hsp90 is a critical regulator of Monarch-1 anti-inflammatory activity. ***The Journal of Immunology,*** **2007, 179: 6291–6296.**

Inflammation is a dynamic protective response that must be controlled at both the initiation and resolution phase as improper regulation underlies many human diseases. Nucleotide-binding domain (LRR) leucine-rich repeat (NLR)[4] proteins (previously known as CATERPILLER, NAIP, CIITA, HET-E, and TP1 domain-LRR (NACHT-LRR) or NOD-LRR) play a critical role in this regulation (1–4). The importance of NLR proteins in controlling inflammation is highlighted by strong linkage of the NLR proteins CIITA, NOD2, and cryopyrin/NALP3/ to human immunodeficiency and autoinflammatory diseases (5). Yet, despite the critical role of NLR proteins in human health, relatively little is known regarding their molecular regulation.

NLR proteins share a strong structural and functional homology to a class of disease-resistance proteins (R proteins) that comprise a major immune response system in the plant kingdom. These plant proteins function as molecular sensors that mediate the containment of a broad range of pathogens including bacteria, viruses, fungi, parasites, nematodes, and insects (6, 7). Recent evidence suggests that a critical component in R protein-mediated defense responses is heat shock protein (Hsp) 90. This evolutionarily conserved molecular chaperone associates with a subset of proteins, deemed "client proteins," to promote their maturation and stability.

The Hsp90 chaperone cycle is a multistep process where client proteins first form an early complex with Hsp70. An intermediate complex then forms with the incorporation of Hsp90. Within this intermediate complex, the client protein is transferred from Hsp70 to Hsp90. Finally, Hsp70 dissociates from the complex and the client protein remains bound to Hsp90 in an activation-competent state (reviewed in Ref. 8). Pharmacologic inhibition of Hsp90 prevents the transfer of client proteins to Hsp90 and stalls this chaperone cycle at the intermediate stage. In the absence of Hsp90 binding, the client protein remains bound to Hsp70 and is degraded by the proteasome (9–11). Notably, the majority of known Hsp90 client proteins are signaling molecules such as kinases and transcription factors (reviewed in Ref. 12).

Monarch-1 is an understudied NLR protein with a unique function. Previously, we demonstrated that Monarch-1 serves as a novel attenuating factor of inflammation by destabilizing NF-κB-inducing kinase (NIK), which results in suppression of noncanonical NF-κB activation (13). In the present study, we demonstrate that this activity is regulated by Hsp90. We find that Monarch-1 associates with Hsp90 in a stable, functionally competent state. In the presence of Hsp90 inhibitors, this association is lost resulting in degradation of Monarch-1 via the proteasome. This rescues NIK

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Received for publication March 22, 2007. Accepted for publication August 16, 2007.

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[1] This work was supported by National Institutes of Health Grants AI063031, DE016326, DK38108, SERCEB A1-02-031, and a Sandler Program in Asthma Research Senior Investigator Award (to J.P,-Y.T.). J.D.L. was supported by The American Cancer Society. J.C.A. was supported by National Institutes of Health Training Grant T32-A1007273-22.

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[4] Abbreviations used in this paper: NLR, nucleotide-binding domain leucine-rich repeat; R protein, disease-resistance protein; Hsp, heat shock protein; NIK, NF-κB-inducing kinase; Ha, hemagglutinin; Hsc, heat shock cognate; 2D, two dimensional; MS, mass spectometry; GA, geldanamycin; TAK1, TGF-β-activated kinase 1; LRR, leucine-rich repeat; NACHT, NAIP, CIITA, HET-E, and TP1 domain; NALP3, NACHT-, LRR-, and PYD-containing protein.

from Monarch-1-induced proteolysis, demonstrating that Hsp90 performs an integral role in regulating Monarch-1 activity.

Materials and Methods

Cell lines

HEK293T cells (GenHunter) were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS and 100 mg/ml penicillin and 100 mg/ml streptomycin. Undifferentiated THP-1 cells were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 mg/ml penicillin, and 100 mg/ml streptomycin. THP-hemagglutinin (Ha)-Mon1 and THP-EV cells have been previously described (13).

Primary cell isolation

PBMC were isolated from whole blood (American Red Cross) using a Ficoll-Hypaque gradient (ICN Pharmaceutical-Cappel). To enrich human primary adherent monocytes, PBMC were plated in serum-free RPMI 1640 (Invitrogen Life Technologies) and allowed to adhere for 2 h at 37°C; at this time, nonadherent cells were removed and adherent cells were washed with sterile PBS in triplicate. Cells were cultured in RPMI 1640 supplemented as described above.

Abs and reagents

Anti-Hsp70 (W27), anti-heat shock cognate (Hsc) 70 (B-6), anti-Hsp90 (H-114), anti-TGF-β-activated kinase 1 (TAK1; C-9), anti-NIK (H-248), and anti-actin-HRP (C-11) Abs were obtained from Santa Cruz Biotechnology. Anti-Ha Abs (12CA4 and 13F10) were obtained from Roche Applied Science. Purified anti-mouse I-A^d (control Ig) was obtained from BD Pharmingen. Rabbit polyclonal anti-CagA (b-300, control Ig) was obtained from Santa Cruz Biotechnology. Normal rabbit serum was obtained from Vector Laboratories. Rabbit polyclonal anti-Monarch-1 and mouse monoclonal anti-Monarch-1 have been described previously (14). Geldanamycin (GA), radicicol, and MG132 were obtained from Calbiochem. TLR2 agonist Pam3Cys4 was obtained from Invivogen.

Two-dimensional (2D) gel electrophoresis and mass spectometry (MS)

HEK293T cells seeded in 10-cm plates were transfected with 5 μg of Flag-tagged Monarch-1 or pcDNA control vector. Twenty-four hours later, the cells were lysed in 0.5% CHAPS and protein complexes were immunoprecipitated for 18 h with M2 agarose. Protein complexes were dissociated in an 8 M urea solution and individual proteins separated by 2D gel electrophoresis. Briefly, protein eluate was loaded on 18-cm immobilized pH gradient strips (pH 4–7) and separated by isoelectric point for a total of 58,000 V-h. The strips were transferred to SDS-PAGE gels (10%; 19 $\times$ 18 cm) and the proteins were then separated by molecular mass. Silver-stained gels were analyzed and spots unique to precipitates derived from Monarch-1-transfected cells were excised from the gel and analyzed by MALDI-MS as described previously (15). Protein identities were established using the MASCOT search engine (Matrix Sciences) with the following settings: peptide mass tolerance of 0.1 Da, zero missed cleavage sites, one fixed modification of carboxymethyl cysteine, one variable modification of methionine oxidation, and no restrictions on protein molecular mass or isoelectric point. The protein identities reported received a Mowse score greater than the significance threshold ($p < 0.05$).

Immunoprecipitations and Western blot analysis

HEK293T cells seeded in 6-well plates were transfected with 1 μg of Ha-tagged Monarch-1 plasmid described previously using Fugene 6 (Roche). Twenty hours later, the cells were lysed in 1% Triton X-100, 150 mM NaCl, 50 mM Tris (pH 8) supplemented with protease inhibitor mixture (Roche). Samples were immunoprecipitated with 2 μg of the indicated Ab and rotated end-over-end for 18 h. Ab complexes were captured by the addition of protein A/G agarose beads (Pierce) for an additional 2 h. The beads were washed three times in lysis buffer, eluted into boiling sample reducing buffer, and separated by SDS-PAGE. Proteins were transferred to nitrocellulose (Bio-Rad), probed with the indicated primary Ab, and visualized by chemiluminescence (Pierce).

To examine stably expressed proteins in THP-1 cells, THP-Ha-Mon1 and THP-EV cells were seeded at a density of 8 $\times$ 10^6/25-cm^2 tissue culture flask. To examine endogenous Monarch-1 protein, THP-1 cells were seeded at a density of 2 $\times$ 10^7 in 75 cm^2 tissue-culture flasks; primary adherent monocytes were seeded at 1 $\times$ 10^7 in 25-cm^2 flasks. Samples were immunoprecipitated with 2 μg of the indicated Ab or 10 μl of Monarch-1 rabbit antisera. Cell lysis and Western blot analysis were performed as described above.

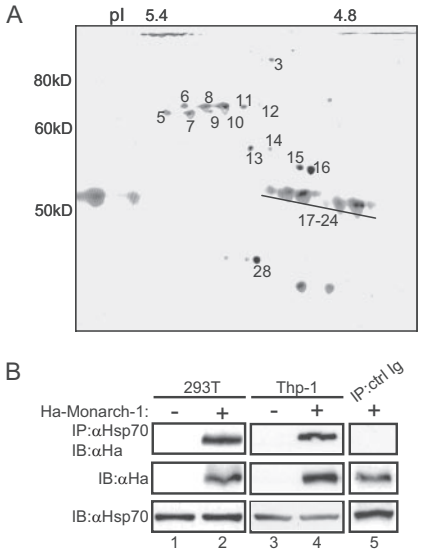

FIGURE 1. Monarch-1-interacting proteins include Hsp70. *A*, HEK293T cells were transfected with empty vector or Flag-Monarch-1. Cell lysates were immunoprecipitated with anti-Flag Abs and protein complexes were fractionated by 2D gel electrophoresis. Individual proteins were visualized by silver stain and those unique to Monarch-1-transfected cells were excised and analyzed by MALDI MS. Protein identities are described in Table I. *B*, HEK293T cells transfected with an empty control vector (*lane 1*) or Ha-Monarch-1 (*lane 2*), or THP-1 monocytic cells stably transfected with an empty control vector (*lane 3*) or Ha-Monarch-1 (*lane 4*) were lysed and protein complexes were immunoprecipitated with an anti-Hsp70 Ab. Following fractionation by SDS-PAGE, Western blots were probed with an anti-Ha Ab to detect Monarch-1. Lysate controls show the presence of Hsp70 in all lanes and Ha-Monarch-1 in the expected lanes (*2* and *4*). In *lane 5*, lysates from THP-1 cells stably expressing Ha-Monarch-1 were immunoprecipitated with an isotype control Ab, mouse anti-I-A^d, and Western blots do not show nonspecific binding of Monarch-1. These data are representative of at least three independent experiments.

Results

Monarch-1-interacting proteins include Hsp70

NLR proteins assemble into large, multiprotein complexes that serve as functional platforms to promote downstream activities such as transcription regulation and IL-1β processing (1). To begin to identify the protein complexes formed by Monarch-1, HEK293T cells were transfected with Flag-Monarch-1 or a pcDNA control vector and cellular lysates were immunoprecipitated with anti-Flag Abs. Captured protein complexes were resolved by 2D gel electrophoresis. The gels were stained and protein spots unique to Monarch-1-transfected cells were identified (Fig. 1*A*). These spots were excised and processed for MALDI-MS (Table I). Protein identities were determined by comparing the resulting peptide mass fingerprints to the MASCOT search engine. Among the proteins that achieved high confidence scores were members of the Hsp70 family.

Mammalian Hsc/Hsp70 binds to a wide range of newly synthesized proteins in unstressed cells (16). To confirm the association of Monarch-1 with Hsp70, we performed coimmunoprecipitation experiments. HEK293T cells were transfected with Ha-Monarch-1 or pcDNA control vector, and endogenous Hsp70 complexes were immunoprecipitated from cellular lysates. Western blots were then probed with anti-Ha to detect Monarch-1 (Fig. 1*B*, *lanes 1–2*). In

Table I. *Summary of Hsp70 proteins identified by MALDI-TOF MS*[a]

Spot Number	Accession	Protein Identity	Matched/Total Peptides	% Coverage
5	AAA52697	Hsp70	7/16	12
7	AAD21816	Hsp70.1	4/9	7
8	BAD96505	Hsp70 protein 8 isoform 1 variant	7/20	10
10	NP_006588	Hsp70 protein 8 isoform 1	11/16	20

[a] Protein spots shown above achieved a Mowse score above the significance threshold ($p < 0.05$).

agreement with the results obtained from 2D gels (Fig. 1*A*), Monarch-1 coprecipitated with endogenous Hsp70. Identical results were obtained from THP-1 monocytes stably transfected with Ha-tagged Monarch-1 (THP-Ha-Mon1) (Fig. 1*B*, *lanes 3–4*). As Monarch-1 is expressed exclusively in cells of myeloid lineage (17, 18), this is a more physiologically relevant model system. Monarch-1 was not detected in control samples that were immunoprecipitated with an isotype-matched Ab (Fig. 1*B*, *lane 5*).

Monarch-1 associates with endogenous Hsp90

The molecular chaperone Hsp70 is an essential component of the Hsp90 multichaperone complex. This complex aids in the maturation and stabilization of a select set of client proteins, predominantly signaling molecules (12). We identified Hsp70 as a Monarch-1-interacting protein, and combined with the important role of Hsp90 in plant R protein function, we hypothesized that Monarch-1 also associates with Hsp90. To test this hypothesis, we transfected HEK293T cells with Ha-Monarch-1 or pcDNA control vector and endogenous Hsp90-containing complexes were immunoprecipitated. Western blots were then probed with anti-Ha to detect Monarch-1. Monarch-1 coprecipitated with endogenous Hsp90 but not in control samples using an isotype-matched Ab (Fig. 2). Importantly, this association was also found in THP-Ha-Mon1 monocytic cells, demonstrating that Monarch-1 forms molecular complexes with Hsp90 in a more relevant model system (Fig. 2, *lanes 3–4*).

Hsp90 inhibition alters the dynamic association of Monarch-1 with Hsp70 and Hsp90

Pharmacologic inhibition of Hsp90 prevents the transfer of client proteins to Hsp90. This then leads to the accumulative association of client proteins within Hsp70 complexes (8). The observation that Monarch-1 associated with both Hsp70 and Hsp90 led us to hypothesize that Monarch-1 serves as an Hsp90 client protein. If this is the case, treatment of cells with the ansamycin antibiotic GA, a specific Hsp90 inhibitor, would result in reduced association of Monarch-1 with Hsp90 and increased association with Hsp70. To test this, we treated THP-Ha-Mon1 cells with GA and then immunoprecipitated endogenous Hsp70 or Hsp90 complexes at multiple time points over a 6-h period (Fig. 3*A*). Western blots were probed with anti-Ha to detect coprecipitated Monarch-1. After 2 h of GA treatment, Monarch-1 was barely detectable in Hsp90-containing complexes. Notably, Monarch-1 levels decreased with GA treatment, suggesting that the stability of Monarch-1 is dependent upon Hsp90 activity. However, because Monarch-1 levels were comparable at 1- and 6-h post-GA treatment (*lanes 2* and *5*), the loss of Monarch-1/Hsp90 complex formation was due to Hsp90 inhibition and not due to decreased levels of Monarch-1 protein.

In sharp contrast to its association with Hsp90, the association of Monarch-1 with Hsp70 increased after 2 h of GA treatment and

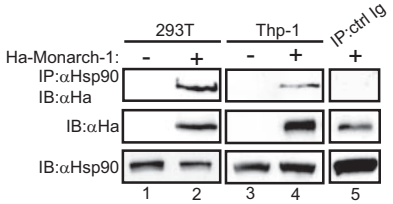

FIGURE 2. Monarch-1 interacts with Hsp90. HEK293T cells transfected with an empty control vector (*lane 1*) or Ha-Monarch-1 (*lane 2*), or THP-1 cells stably transfected with an empty vector (*lane 3*) or Ha-Monarch-1 (*lane 4*) were lysed and protein complexes were immunoprecipitated with an anti-Hsp90 Ab. Following fractionation by SDS-PAGE, Western blots were probed with an anti-Ha Ab to detect Monarch-1. Lysate controls show the presence of Hsp90 in all lanes and Ha-Monarch-1 in the expected lanes (*2* and *4*). In *lane 5*, lysates from THP-1 cells stably expressing Ha-Monarch-1 were immunoprecipitated with an isotype control Ab, rabbit anti-CagA, and Western blots do not show any nonspecific binding of Monarch-1. These data are representative of at least three independent experiments.

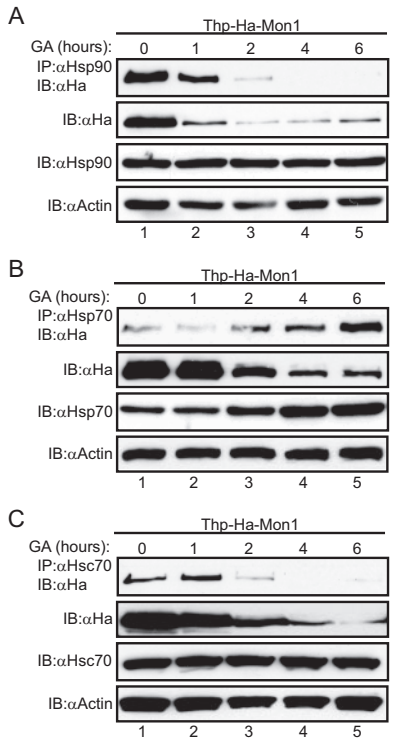

FIGURE 3. Hsp90 inhibition alters the association of Monarch-1 and Hsp. THP-Ha-Mon1 monocytes were treated over the course of 6 h with 0.2 μM of the Hsp90 inhibitor, GA. Lysates were immunoprecipitated with anti-Hsp90 Abs (*A*), anti-Hsp70 Abs (*B*), and anti-Hsc70 Abs (*C*), and fractionated by SDS-PAGE. Western blots were probed with an anti-Ha Ab to detect Monarch-1. Immunoblots were performed with the indicated Abs to monitor protein expression. All panels are representative of at least three independent experiments.

strengthened throughout the 6-h time course (Fig. 3*B*). This agrees with other studies where GA treatment causes an increase in association of the Hsp90 client protein with Hsp70 (19–21). Also in line with previous reports (22–25), a minimal increase in Hsp70 levels was detected upon inhibition of Hsp90. These results indicate that Hsp90 inhibition leads to the accumulation of Monarch-1 within Hsp70-containing molecular complexes and supports our hypothesis that Monarch-1 is processed through the Hsp90 chaperone cycle.

In addition to Hsp70, we also analyzed the association between Monarch-1 and Hsc70. In contrast to Hsp70, which is induced upon cell stress, Hsc70 is constitutively expressed and performs multiple functions distinct from Hsp70 (26). Also in contrast to Hsp70, the association between Monarch-1 and Hsc70 decreased upon treatment of cells with GA (Fig. 3*C*). Thus, the pattern of association was similar to that seen with Hsp90. These results suggest different roles for Hsp70 and Hsc70 in Monarch-1 function and demonstrate that the association between Monarch-1 and Hsc70 is dependent upon Hsp90 activity. Taken together, these results demonstrate that Monarch-1 is a substrate of the Hsp90 multichaperone complex.

Endogenous Monarch-1 stability in THP-1 cells and primary monocytes is dependent upon Hsp90 activity

In addition to altering the dynamic association of client proteins with Hsp70 and Hsp90, inhibition of Hsp90 also results in degradation of the client protein (8). Consistently, Western blot analysis of cellular lysates indicated that Monarch-1 levels decreased upon Hsp90 inhibition. In fact, in THP-Ha-Mon1 cells, Monarch-1 protein levels were dramatically reduced after only 1 h of treatment with GA (Fig. 3). This was also observed upon treatment of THP-Ha-Mon1 cells with radicicol, an Hsp90 inhibitor that is structurally dissimilar and chemically unrelated to GA (Fig. 4*A*) (27).

To ensure that the effect of Hsp90 inhibition on Monarch-1 levels was not due to overexpression of a tagged protein, we next analyzed endogenous Monarch-1 levels in wild-type THP-1 monocytes. In agreement with the results obtained with tagged Monarch-1, endogenous Monarch-1 protein levels were significantly reduced after 1 h of treatment with GA (Fig. 4*B*), demonstrating that Monarch-1 stability is regulated by Hsp90.

Next, we examined endogenous Monarch-1 levels in the presence of GA in primary human monocytes. Endogenous Monarch-1 is expressed at very low levels in monocytes (18). In these experiments, Monarch-1 was first immunoprecipitated with a Monarch-1-specific rabbit polyclonal Ab and then Western blots were probed with a Monarch-1-specific mouse mAb (Fig. 4*C*). In agreement with the results obtained from THP-1 cells, Monarch-1 levels declined after 1 h of GA treatment and were undetectable following 2.5 h of treatment. Control samples using normal rabbit serum in the immunoprecipitation confirmed the specificity of the Monarch-1 band. Together, these data demonstrate that in human monocytes, Monarch-1 stability is dependent upon Hsp90 activity.

Hsp90 inhibition results in proteasome-mediated degradation of Monarch-1

It is generally believed that Hsp90 inhibition leads to proteasome mediated degradation of Hsp90 client proteins (9–11). To determine whether the reduction of Monarch-1 protein levels upon GA treatment was due to proteasome mediated degradation, we treated THP-Ha-Mon1 cells with GA in the presence or absence of the proteasome inhibitor, MG132. In agreement with the results presented above, Monarch-1 protein levels declined after 1 h of treatment with GA. In contrast, however, Monarch-1 protein levels remained stable in cells that were pretreated with proteasome inhibitor (Fig. 5). No change was observed in the cellular levels of TAK1, demonstrating that these treatments were not globally affecting signaling molecules. In addition, no change was observed in cellular levels of Hsp90 or actin under these treatment conditions. Together, these results demonstrate that Hsp90 controls Monarch-1 stability and upon Hsp90 inhibition, Monarch-1 is degraded via the proteasome.

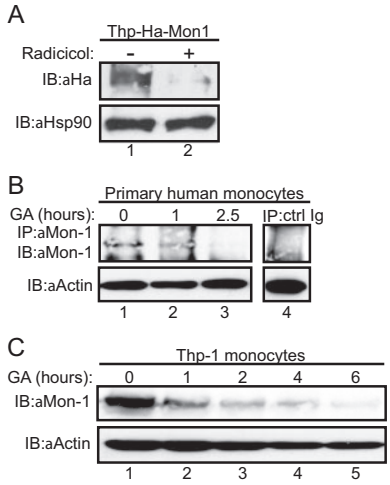

FIGURE 4. Endogenous Monarch-1 stability is dependent on Hsp90 activity. *A*, THP-Ha-Mon1 monocytes were treated with 0.1 μM of the Hsp90 inhibitor, radicicol, for 1 h. Lysates were fractionated by SDS-PAGE. Western blots were probed with an anti-Ha Ab to detect Monarch-1. Control immunoblots were probed with an anti-Hsp90 Ab to ensure equal loading. *B*, Human THP-1 monocytes were treated over the course of 6 h with 0.2 μM GA and lysates were fractionated by SDS-PAGE. Western blots were probed with a mouse anti-Monarch-1 Ab to detect endogenous Monarch-1. Control immunoblots were probed with an anti-actin Ab to ensure equal loading. *C*, Human primary adherent monocytes were enriched from PBMC by adherence and treated with 0.2 μM GA for the indicated times. To detect endogenous Monarch-1, lysates were immunoprecipitated with a rabbit anti-Monarch-1 Ab, fractionated by SDS-PAGE, and Western blots were probed with a mouse anti-Monarch-1 Ab. Control immunoprecipitations were performed with normal rabbit serum and Western blots were probed with a mouse anti-Monarch-1 Ab. Control immunoblots were probed with an anti-actin Ab to ensure equal loading. Each panel is representative of at least three independent experiments.

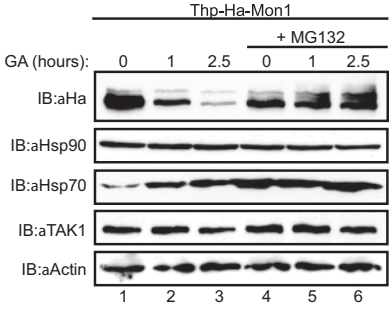

FIGURE 5. Inhibition of Hsp90 induces proteasome-mediated degradation of Monarch-1. THP-Ha-Mon1 human monocytes were treated with 0.2 μM GA for the indicated times in the absence (*lanes 1–3*) or presence (*lanes 4–6*) of the proteasome inhibitor, MG-132 (10 μM). Lysates were fractionated by SDS-PAGE and immunoblots were probed with an anti-Ha Ab to detect Monarch-1. Lysate controls show protein levels of Hsp90, Hsp70, TAK1, and actin. These data are representative of at least three independent experiments.

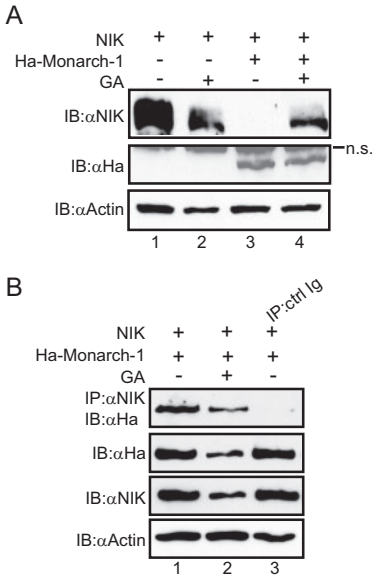

FIGURE 6. Hsp90 is required for Monarch-1-induced NIK degradation. *A*, HEK293T cells were transfected with NIK and in the indicated lanes, Ha-Monarch-1. Cells were treated for six hours with 0.1 μM GA. Lysates were fractionated by SDS-PAGE and Western blots were probed with an anti-NIK Ab. Monarch-1 was detected in the expected lanes using an anti-Ha Ab; n.s., nonspecific band. Control immunoblots were probed with an anti-actin Ab. *B*, HEK293T cells were transfected with NIK and Ha-Monarch-1 and treated the following day for 6 h with 0.2 μM GA, where indicated. Protein complexes were immunoprecipitated from cellular lysates with an anti-NIK Ab (*lanes 1–2*), fractionated by SDS-PAGE, and Western blots were probed with an anti-Ha Ab to detect Monarch-1 in NIK-containing complexes. In *lane 3*, lysates were immunoprecipitated with an isotype control Ab, rabbit anti-CagA and Western blots do not show any nonspecific binding of Monarch-1. Monarch-1 was detected in cellular lysates using anti-Ha, NIK was detected using an anti-NIK Ab, and control immunoblots were probed with an anti-actin Ab. Each panel is representative of at least three independent experiments.

Hsp90 regulates Monarch-1-induced NIK degradation

Recently, we demonstrated that Monarch-1 suppresses the production of proinflammatory cytokines and chemokines (13, 14). One mechanism by which Monarch-1 performs this function is by associating with and destabilizing NIK (13). NIK is degraded when coexpressed with Monarch-1, thus providing a measurable function of Monarch-1. To examine the role of Hsp90 in Monarch-1-induced NIK degradation, NIK and Monarch-1 were coexpressed in HEK293T cells in the presence or absence of GA. NIK levels were then monitored by Western blot analysis. In cells expressing NIK alone, inhibition of Hsp90 caused a slight reduction in NIK levels (Fig. 6*A*, *lane 2*). This agrees with an earlier report indicating that NIK is an Hsp90 client protein and NIK levels are reduced upon Hsp90 inhibition (28). In agreement with our previous report, coexpression of Monarch-1 and NIK resulted in the near ablation of NIK protein (Fig. 6*A*, *lane 3*). However, GA treatment restored NIK levels to those observed in cells treated with GA in the absence of Monarch-1 (Fig. 6*A*, compare *lanes 2* and *4*), thus demonstrating that Hsp90 activity is required for Monarch-1-induced NIK degradation.

Monarch-1 and NIK associate to form molecular complexes (13). Therefore, we next sought to determine whether the inability to induce NIK degradation in the presence of GA was due to a loss of association. Monarch-1 and NIK were coexpressed in HEK293T cells and coimmunoprecipitation experiments were performed. Although Monarch-1 and NIK protein levels were both reduced by an incubation with GA (see lysate controls, *rows 2* and *3*), there was sufficient residual protein expression that permitted the examination of their interaction (Fig. 6*B*), in the absence (*lane 2*) or presence (*lane 3*) of GA. The cells were treated with GA for 6 h, NIK complexes were immunoprecipitated and Western blots were probed with anti-Ha to detect associated Monarch-1. Interestingly, the association of Monarch-1 with NIK was not affected by Hsp90 inhibition, suggesting that this chaperone is not required for Monarch-1 to form molecular complexes with NIK. Monarch-1-induced NIK degradation, however, was prevented upon Hsp90 inhibition (Fig. 6*A*), thus demonstrating that Hsp90 is required for the functional activity of Monarch-1.

Discussion

NLR proteins are rapidly emerging as important mediators of innate and adaptive immune signaling. Yet, despite recent reports describing the physiologic role of NOD2, NALP3, and IPAF/NLRC4 in the response to numerous ligands, relatively little is known concerning the molecular events that regulate these proteins. Recently, we demonstrated that the NLR protein, Monarch-1, functions as a negative regulator of NF-κB activation through its association with NIK (13). In this report, we show Monarch-1 requires Hsp90 for both its stabilization as well as its negative regulatory activity.

Hsp90 is a highly conserved chaperone molecule that plays a critical role in the stability and function of many signaling proteins. These client proteins generally follow a pathway where upon translation they associate with Hsp70 to achieve proper folding conformation. The client protein is then transferred to Hsp90 where it is held in a functionally active state. Upon the addition of Hsp90 inhibitors, the client protein no longer associates with Hsp90, but instead remains in a complex with Hsp70 and undergoes proteasome-mediated degradation (12). In this study, we show that Monarch-1 follows the same mechanism as reported Hsp90 client proteins. We found that upon Hsp90 inhibition with GA, Monarch-1 proteins dissociated from Hsp90 and, simultaneously, accumulated within Hsp70 complexes. As a result, Monarch-1 protein levels rapidly decreased due to degradation via the proteasome.

These results are analogous to those reported for NLR structural homologues found in the plant kingdom. In *Arabidopsis,* inhibition of Hsp90 reduces steady-state levels of the R proteins RPS2 and RPM1 (29). Consequently, the pathogen-induced defensive response is attenuated and disease resistance conferred by these proteins is impaired (29–31). Similarly, in *Nicotiana benthamiana,* virus-induced gene silencing of Hsp90 results in the loss of resistance mediated by R proteins PRF (against *Pseudomonas syringae*), RX, and N (against tobacco mosaic virus) (32, 33). Thus, Hsp90 is critical for disease resistance in plants as this chaperone regulates both the stability and function of multiple R proteins.

Similar to these R proteins, in this report, we demonstrate that, in addition to regulating Monarch-1 stability, Hsp90 also controls Monarch-1 function. Although Monarch-1 still bound NIK, it no longer induced NIK degradation in the presence of an Hsp90 inhibitor. Thus, while Hsp90 is required for the negative regulatory function of Monarch-1, it is not required for Monarch-1 to form molecular complexes with NIK. A similar observation has been made for the Hsp90 client protein, Raf. In this report, inhibition of Hsp90 reduced cytoplasmic Raf levels but did not prevent Raf from binding downstream signaling proteins (34). These results suggest that Hsp90 activity is required for the processing steps that function downstream of the Monarch-1-NIK complex formation to promote degradation of the kinase. Future studies will elucidate

whether Hsp90 regulates the association of Monarch-1-NIK complexes with ubiquitin-conjugating enzymes and/or the proteasome degradation complex.

In addition to our findings regarding Monarch-1, two recent reports have demonstrated a role for Hsp90 in the regulation of other NLR family members. During the review of this manuscript, Hsp90 was shown to be critical for the proinflammatory activity of NOD1, NOD2, IPAF/NLRC4, and cryopyrin/NALP3/NLRP3 (35). Furthermore, inhibition of Hsp90 resulted in proteasome-mediated degradation of NOD1 and cryopyrin/NALP3/NLRP3 (35, 36), demonstrating that similar to Monarch-1, Hsp90 also serves to stabilize these NLR proteins. Together with our results, these findings suggest that a role for Hsp90 in regulating NLR stability and function represents a common feature of this family.

In summary, in this report we used an unbiased proteomic approach to show that Monarch-1 associates with Hsp70, and a further investigation shows that it also associates with Hsp90. These two Hsp regulate protein stability, and Hsp90 in particular is required for the stability of numerous inflammatory signaling molecules (12, 28). The effect of Hsp90 on Monarch-1 was studied using specific Hsp90 inhibitors, and was observed not only in experiments investigating Monarch-1 protein derived from transfected expression plasmids, but also with endogenous Monarch-1 protein in both a monocytic cell line and primary human monocytes. Our results indicate that Hsp90 protects Monarch-1 from proteasome-mediated degradation, and further regulates the function of Monarch-1. These findings suggest an evolutionary conserved regulation of mammalian NLR proteins by Hsp90 that is strikingly similar to the regulation of plant R proteins.

Disclosures

The authors have no financial conflict of interest.

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