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ORIGINAL ARTICLE

Hsp90 regulates processing of NF-κB2 p100 involving protection of NF-κB-inducing kinase (NIK) from autophagy-mediated degradation

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NF-κB-inducing kinase (NIK) is required for NF-κB activation based on the processing of NF-κB2 p100. Here we report a novel mechanism of NIK regulation involving the chaperone 90 kDa heat shock protein (Hsp90) and autophagy. Functional inhibition of Hsp90 by the anti-tumor agent geldanamycin (GA) efficiently disrupts its interaction with NIK, resulting in NIK degradation and subsequent blockage of p100 processing. Surprisingly, GA-induced NIK degradation is mediated by autophagy, but largely independent of the ubiquitin-proteasome system. Hsp90 seems to be specifically involved in the folding/stabilization of NIK protein, because GA inhibition does not affect NIK mRNA transcription and translation. Furthermore, Hsp90 is not required for NIK-mediated recruitment of the α subunit of IκB kinase to p100, a key step in induction of p100 processing. These findings define an alternative mechanism for Hsp90 client degradation and identify a novel function of autophagy in NF-κB regulation. These findings also suggest a new therapeutic strategy for diseases associated with p100 processing.

Keywords: autophagy, geldanamycin, Hsp90; NF-κB2, NIK, proteasome-independent degradation

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Introduction

The non-canonical NF-κB pathway, based on processing of the *nf-kb2* gene product p100 to generate p52, plays an important role in the function and development of the secondary lymphoid tissues, as well as in autoimmune disease progression and tumorigenesis [1-3]. Whereas the precursor protein p100 serves as a potent inhibitor of NF-κB, the processed product p52 is an important functional member of NF-κB [4]. Thus, the functions of p100 processing are two-fold: one is to relieve p100-mediated inhibition and liberate specific NF-κB dimers, and the other is to generate functional p52 NF-κB.

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Abbreviations: 5-aminoimidazole-4-carboxamide 1-b-D-ribofuranoside (AICAR); B-cell activating factor (BAFF); CD40 ligand (CD40L); geldanamycin (GA); 90 kDa heat shock protein (Hsp90); IκB kinase (IKK); α subunit of IκB kinase (IKKα); lymphotoxin beta (LTβ); NF-κB-inducing kinase (NIK); tumor necrosis factor receptor-associated factor 3 (TRAF3)

The processing of p100 is a tightly regulated event. Induction of this process strictly depends on NF-κB-inducing kinase (NIK) and its downstream kinase IKKα (α subunit of IκB kinase, also named IKK1) [5, 6], and is mediated only by a limited number of stimuli, such as lymphotoxin beta (LTβ), B-cell activating factor (BAFF) and CD40 ligand (CD40L) [2, 6-10]. One important function of these stimuli is to prevent basally translated NIK protein from undergoing degradation mediated by the tumor necrosis factor receptor-associated factor 3 (TRAF3) [11-13]. Stabilized NIK in turn specifically activates and recruits IKKα into the p100 complex via serines 866 and 870 of p100 [14]. After recruitment into the p100 complex, activated IKKα phosphorylates serines 99, 108, 115, 123 and 872 of p100 [14]. The phosphorylation of these specific serines results in ubiquitination and subsequent processing of p100 mediated by the β-TrCP ubiquitin ligase and 26S proteasome, respectively [6, 15, 16].

By controlling the stability of various signaling regulatory proteins, the proteasome regulates various signaling pathways in addition to p100 processing. For example, almost all the known clients of the molecular chaperone

Hsp90 (90 kDa heat-shock protein), including many important kinases and transcription factors, are found to be degraded by the proteasome when Hsp90 is inactivated by its specific inhibitor geldanamycin (GA), an anti-tumor drug [17-19]. On the other hand, the role of macroautophagy (simply referred to as autophagy hereafter) in the regulation of Hsp90 clients and specific signaling pathways remains largely unknown, although this mode of lysosome-dependent degradation is well-known to be the primary mechanism other than the proteasome employed for protein degradation within eukaryotes [19]. Additionally, whether Hsp90 is involved in p100 processing has not been determined yet.

Here, we find that NIK is a novel client of Hsp90, which interacts with Hsp90 through its C-terminal regulatory domain. GA inhibition of Hsp90 disrupts NIK/Hsp90 interaction and leads to NIK degradation and inhibition of p100 processing. Notably, the GA-induced NIK degradation is independent of both ubiquitination and proteasome but is mediated by autophagy. However, Hsp90 is not required for the transcription/translation of NIK mRNA. Furthermore, Hsp90 is not required for NIK-mediated IKKα recruitment into the p100 complex either. Thus, Hsp90 regulates p100 processing indirectly via controlling stability of NIK protein. These findings establish a link among autophagy, Hsp90 and non-canonical NF-κB signaling for the first time, and also suggest a therapeutic strategy for diseases associated with p100 processing.

Materials and Methods

Expression vectors and reagents

Expression vectors encoding p100 and NIK have been described previously [6, 20]. The anti-NIK (H248), anti-Hsp90 (F-8) and anti-p53 (FL-393) antibodies were from Santa Cruz Biotechnology, Inc. The antibody used for detecting mouse NIK protein (#4994) was from Cell Signaling Tech. The anti-actin (AAN01) and anti-HA (12CA5) antibodies were from Cytoskeleton Inc. and Roche Molecular Biochemicals, respectively. The antibody recognizing the N-terminus of p100 (anti-p100N) was described previously [16]. The monoclonal antibodies for human CD40 and Myc were prepared from hybridomas G28-5 and 9E10, respectively [12]. 5-aminoimidazole-4-carboxamide 1-b-D-ribofuranoside (AICAR) and MG132 were from Biomol and Calbiochem, respectively [21].

Cell culture and transfection

Human B-cell line Ramos RG69, mouse M12.4.1 B cell stably infected with pCLXSN-NIK-HA, mouse fibroblasts ts20, Atg5 knockout mouse embryonic fibroblasts (MEFs) and TRAF3 knockout MEFs were gifts from Drs L Covey, SC Sun, HL Ozer, N Mizushima and G Cheng, respectively. Human kidney 293 cells were described previously [22]. 293, ts20 and MEF cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Ramos RG69 and M12.4.1 cells were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 10 mM 2-mercaptoethanol [11, 12]. ts20 cells were usually maintained at 35 °C instead of 37 °C. To inactivate E1 in the ts20 cells, the culture temperature was shifted to 39 °C. 293, ts20 and MEF cells were transfected with DEAE-Dextran and LipofectAMINE 2000 (Invitrogen), respectively [23].

Immunoblotting and immunoprecipitation

Whole-cell lysates were prepared by lysing the cells in radioimmuoprecipitation assay buffer (RIPA buffer) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF). For immunoblotting (IB) assays, the whole-cell lysates (~30 μg) were fractionated by SDS-PAGE, transferred to nitrocellulose membranes and subjected to IB using the indicated antibodies. To detect MG132-induced recovery of Akt from GA-mediated degradation, the whole-cell lysates were made by the RIPA buffer containing 1% SDS [24]. For immunoprecipitation (IP) assays, the whole-cell lysates (~1 000 μg) were diluted to 1 ml using RIPA buffer, incubated for 30 min at 4 °C in the presence or absence of GA, followed by incubating with the indicated antibodies for 1 h and with 30 μl protein-A-agarose for another 2 h at 4 °C. The agarose beads were washed three times with RIPA buffer, and the bound proteins were eluted by 2×SDS loading buffer and subjected to SDS-PAGE and IB analysis [25].

Polysome and RNA isolation

Ramos RG69 cells were treated for 2 h with anti-CD40 antibody (10 μg/ml) or GA (2 μM) or left untreated. Twenty percent of treated or untreated cells (2×10^7 cells/each group) were pelleted and employed as a source for total RNA using Trizol reagent (Invitrogen). The remaining cells were incubated with cycloheximide (100 μg/ml) for 15 min, followed by cytoplasm extraction. The cytoplasmic extract was then loaded onto a linear 10-45% (w/w) sucrose gradient and centrifuged for 2 h 30 min at 36 000 r.p.m. with a Beckman SW-41 rotor. After centrifugation, the gradient was fractionated and its absorbance at 254 nm was determined continuously by an Isco UA-5 monitor as described before [12, 21]. The polysome-containing fractions were pooled and subjected to phenol extraction. RNA was precipitated with ethanol and dissolved in DEPC-treated water.

Real-time reverse transcription -PCR analysis

Two micrograms of total RNA or RNA isolated from polysomes were reverse-transcribed for real-time reverse transcription (RT)-PCR using the following primers:

NIK, forward 5'-CCCACCTTTTCAGAAGCATT, reverse 5'-CATTTTGCCCTCTGTAGCATGG;
p100, forward 5'-TGCCATTGTGTTCCGGACA, reverse 5'-TGTTTGGAATCAGACACGTCCC;
GAPDH, forward 5'-GCAAATTCCATGGCACCGT, reverse 5'-TCGCCCCACTTGATTTTGG.

Real-time PCR assays were performed with an ABI Prism 7900HT sequence detection system using the SYBR Green PCR Core Reagent (Applied Biosystems, Foster City, CA, USA) [12, 21].

Results

Hsp90 is required for both NIK protein stabilization and p100 processing

Since GA, a specific inhibitor of Hsp90, has been used for tumor treatment in phase II clinical trials [18, 26], we investigated whether GA inhibition of Hsp90 blocks p100 processing, a signaling pathway that has recently been linked to tumorigenesis [2, 3]. As shown in Figure 1A, addition of GA efficiently prevented p100 processing induced by non-canonical NF-κB inducing stimuli (BAFF or anti-CD40 antibody) at a concentration as low as 2 μM. Moreover, GA inhibition of Hsp90 also suppressed p100 processing induced by overexpression of NIK, a kinase downstream of ligation of these receptors, in a dose-dependent manner (Figure 1B). Interestingly, GA inhibition of p100 processing was associated with decreased levels of NIK protein (Figure 1B, lower panel). This result is consistent with the molecular chaperone function of Hsp90 in protein folding and stabilization [17], suggesting that Hsp90 regulates p100 processing possibly by stabilizing the NIK protein.

NIK is a novel bona fide client of Hsp90

The results shown above also suggested that NIK is a

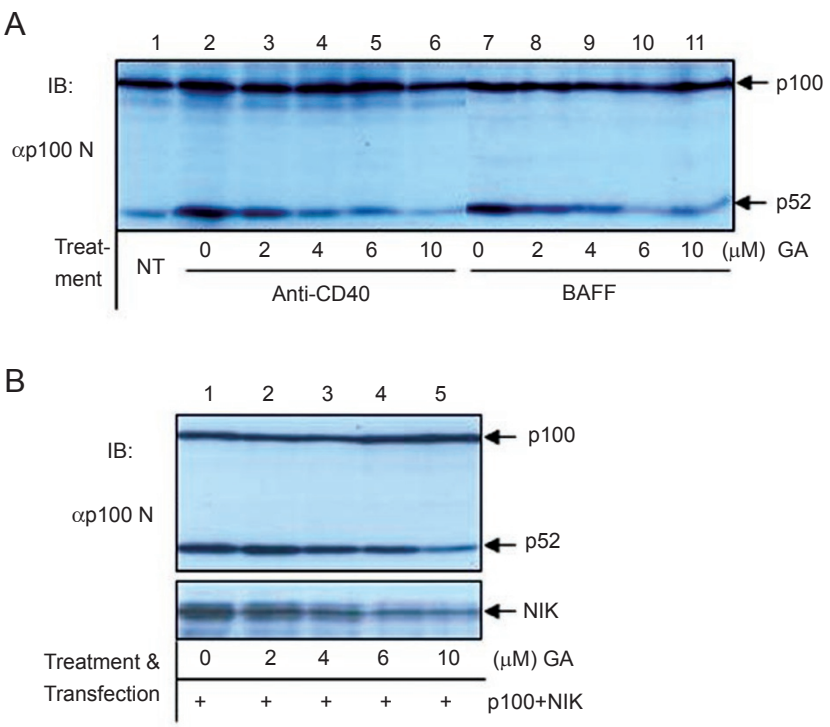

Figure 1 GA inhibition of Hsp90 efficiently prevents p100 processing, which is correlated with decreased levels of NIK protein. **(A)** GA inhibition of p100 processing triggered by receptor ligation. Ramos B cells were treated with BAFF (0.25 μg/ml) or anti-CD40 (10 μg/ml) in the presence of increased amounts of GA for 10 h, followed by IB assays using anti-p100N antibody. Equal amounts of proteins (30 μg) were loaded for each lane. (**B**) GA inhibition of p100 processing induced by overexpression of NIK. 293 cells were transfected with p100 and NIK. Twenty-four hours after transfection, the cells were incubated with the indicated amount of GA for 20 h, followed by IB assays using anti-p100N antibody (upper panel) or anti-NIK antibody (lower panel).

previously unidentified client of Hsp90. To test this possibility, we examined whether NIK forms a complex with Hsp90 by performing co-IP (co-IP) assays. As expected, NIK stably bound to Hsp90 (Figure 2A, lane 2). We also examined whether GA disrupts the NIK/Hsp90 interaction. To do so, we added GA into the cell lysate immediately

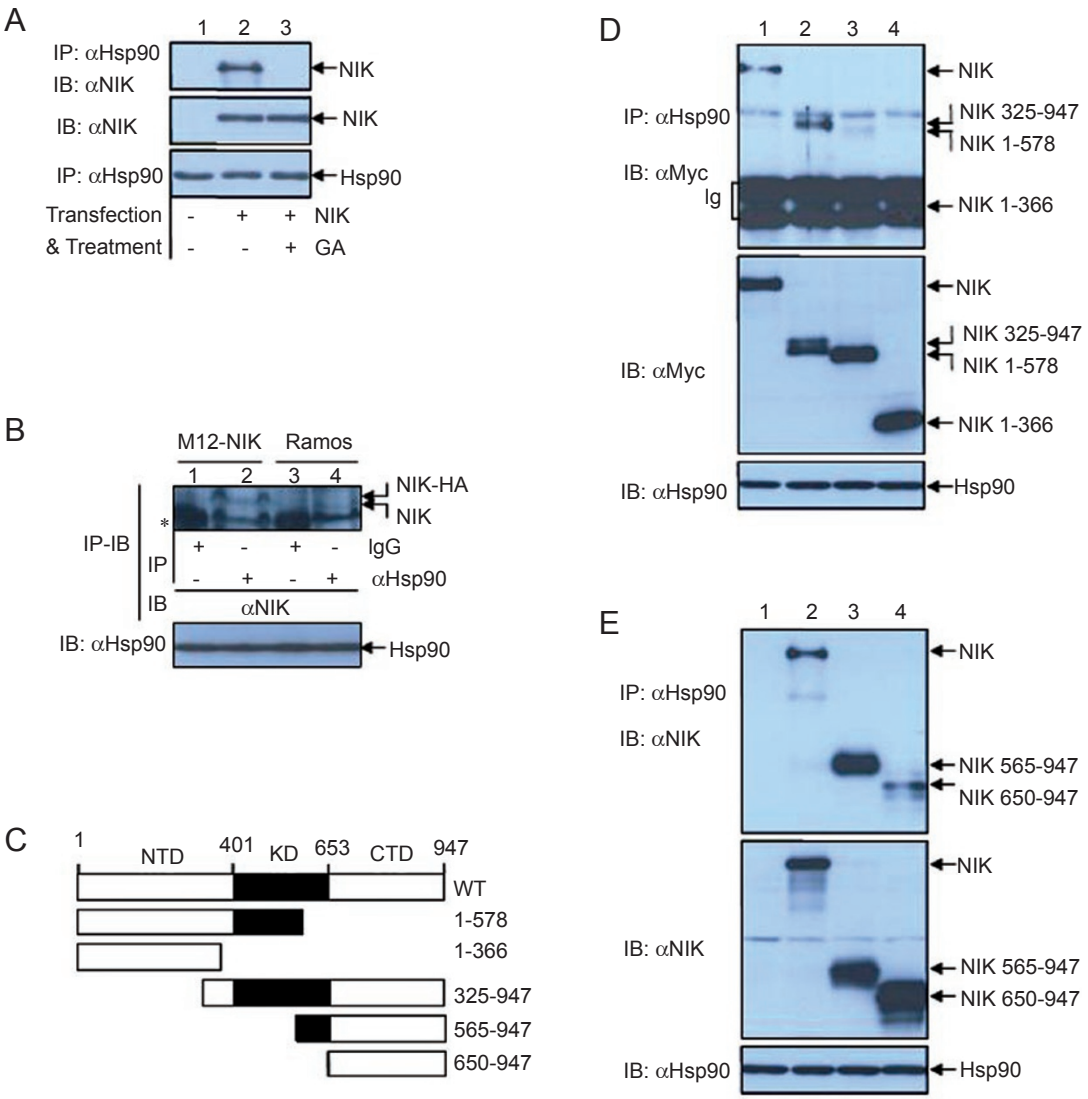

Figure 2 NIK physically interacts with Hsp90, which is sensitive to GA. **(A)** GA inhibition of NIK/Hsp90 interaction. Cell lysates from 293 cells transfected with NIK were immunoprecipitated with anti-Hsp90 antibody in the presence or absence of 10 μM GA, followed by IB using anti-NIK antibody (top panel). Some of the cell lysates were directly subjected to IB for the expression levels of NIK (middle panel) and Hsp90 (bottom panel). **(B)** Interaction between endogenous NIK and Hsp90. Cell lysates from M12.4.1 B cells stably infected with pCLXSN-NIK-HA (labeled as M12-NIK) and CD40L-treated Ramos cells (labeled as Ramos) were immunoprecipitated with anti-Hsp90 antibody (lanes 2 and 4) or control IgG (lanes 1 and 3), followed by IB using anti-NIK antibody (upper panel). Some of the cell lysates were directly subjected to IB for the expression level of Hsp90 (lower panel). The non-specific band was indicated as asterisk (*). Note the CD40L treatment is to prevent NIK from the proteasomal degradation largely mediated by TRAF3 (see Figure 7C for details). **(C)** Schematic picture of NIK and its mutants, showing the N-terminal domain (NTD), the kinase domain (KD), and the C-terminal domain (CTD). **(D and E)** Mapping of domains within NIK responsible for binding to Hsp90. 293 cells were transfected with the indicated NIK mutants, followed by IP using anti-Hsp90 antibody and IB using anti-Myc antibody **(D)** or anti-NIK C-terminal antibody **(E)** to detect the co-immunoprecipitated NIK and NIK mutants (top panel). The expression levels of Hsp90, NIK or NIK mutants were also detected by IB (lower panels). The multiple bands detected for NIK mutants were due to constitutive phosphorylation at their C-terminal region (20).

before co-IP assay, because GA efficiently triggers NIK protein degradation *in vivo* (Figure 1B). Importantly, GA efficiently blocked the interaction between NIK and Hsp90 (Figure 2A, lane 3). To confirm the NIK/Hsp90 interaction under more physiological conditions, we repeated the co-IP assays using NIK protein from mouse B cells stably expressing a low level of HA-tagged NIK or using endogenous NIK protein from human B cells [11, 12]. Consistently, both stably expressed and endogenous NIK form a complex with endogenous Hsp90 (Figure 2B, lanes 2 and 4, respectively). Taken together, these results clearly indicated that NIK is a novel client of Hsp90.

To identify the region within NIK protein responsible for the interaction with Hsp90, we repeated the co-IP assay using various NIK C- or N-terminal deletion mutants (Figure 2C). Deletion of 368 amino acids from the C-terminal end of NIK largely prevented its interaction with Hsp90 (Figure 2D, lane 3). Further deletion from this end completely disrupted the interaction between NIK and Hsp90 (Figure 2D, lane 4). In contrast, deletion of up to 324 amino acids from the N-terminal end of NIK had no effect on the NIK/Hsp90 interaction (Figure 2D, lane 2). These results suggested that the C-terminal regulatory domain (CTD) of NIK is required for its interaction with Hsp90, while the N-terminal regulatory domain and the middle kinase domain are largely dispensable for this association. In further support, the CTD alone was sufficient to bind to Hsp90 (Figure 2E, lane 4). Thus, the C-terminal regulatory domain of NIK is involved in its association with Hsp90.

NIK degradation induced by GA is independent of the ubiquitination and proteasome system

Since it has been reported so far that GA-induced degradation of Hsp90 clients is mediated by the ubiquitin-proteasome pathway [17-19], we first investigated the role of ubiquitination in GA-mediated degradation of NIK. To achieve this goal, we utilized the temperature-sensitive (ts) mutant cell line (ts20) expressing a heat-labile ubiquitin-activating enzyme (E1) and thus defective in the ubiquitin pathway upon heat shock [27]. Surprisingly, heat shock failed to prevent GA-induced NIK degradation in these mutant cells (Figure 3A, lane 4). This was not due to inefficient inactivation of E1 by heat shock, because it completely blocked p53 degradation (lane 6). It is worth to note that the heat-shock condition we used did not cause NIK dissociation from Hsp90 (Figure 3B), suggesting NIK was not denatured under this condition. These results demonstrated that the ubiquitin system is not required for GA-induced NIK degradation.

These results also suggested that NIK degradation by GA might be independent of the proteasome, although the proteasome can mediate either ubiquitin-dependent or

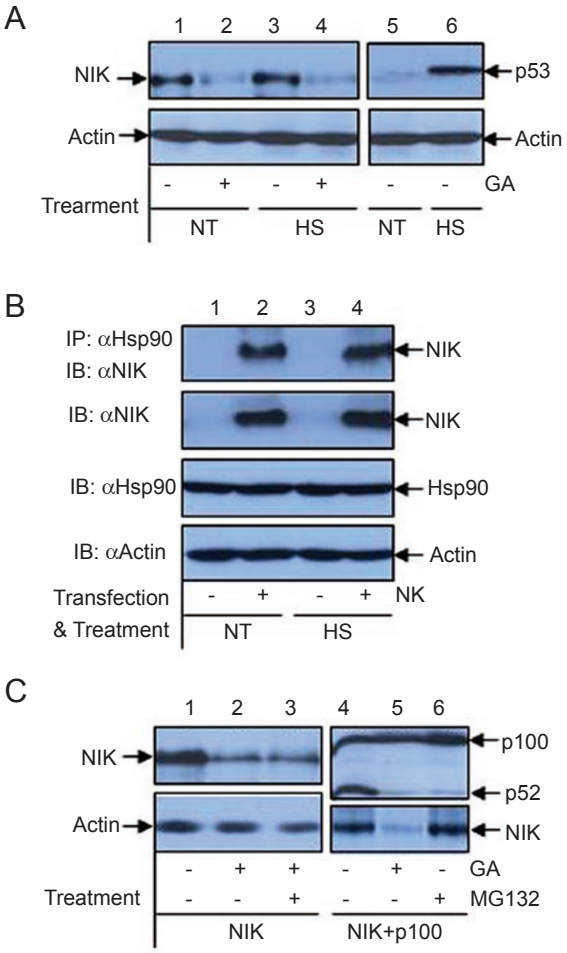

Figure 3 GA-induced NIK degradation does not involve the ubiquitin-proteasome system. **(A)** Ubiquitination system is dispensable for GA-induced NIK degradation. NIK or mock transfected ts20 cells were incubated at 35 °C (labeled as NT) or 39 °C (labeled as HS) in the presence of 10 μM GA (+) or DMSO (–) for 20 h, followed by IB using NIK antibody (lanes 1-4) or anti-p53 antibody (lanes 5-6). **(B)** Heat shock fails to block the NIK/Hsp90 interaction. Cell lysates from normal or heat shocked cells transfected with NIK-expressing or empty vector were immunoprecipitated with anti-Hsp90 antibody, followed by IB using anti-NIK antibody (top panel). Some of the cell lysates were directly subjected to IB for the expression levels of NIK (middle panel) and Hsp90 (bottom panel). **(C)** The proteasome is dispensable for GA-induced NIK degradation. 293 cells transfected with NIK alone (lanes 1-3) or both NIK and p100 (lanes 4-6) were incubated with 10 μM GA (+) or DMSO (–) in the presence or absence of 25 μM MG132 for 20 h, followed by IB using the indicated antibodies.

-independent proteolysis [28]. To address this important issue, we inhibited the proteolytic activity of the proteasome using MG132, a specific inhibitor of the proteasome. Consistent with the essential role of the proteasome in p100

processing, addition of MG132 efficiently inhibited processing of p100 (Figure 3C, lane 6). However, the addition of MG132 failed to block GA-induced NIK degradation (lane 3). Of note, the mechanisms mediating inhibition of p100 processing by GA and MG132 are different, as evidenced by that NIK expression was suppressed by GA (upper panel, lane 3) while MG132 alone failed to do so (lower panel, lane 6). As a matter of fact, MG132 treatment alone actually increased NIK protein levels (clearer in short exposure, data not shown; also see Figure 7). Collectively, these studies clearly demonstrated that NIK degradation in the absence of Hsp90 function is independent of both ubiquitination and proteasome, further suggesting that an unidentified mechanism is responsible for the degradation of this new Hsp90 client.

GA-mediated NIK degradation is largely mediated by autophagy

To define the novel mechanism by which NIK is degraded in the absence of Hsp90 function, we examined the possible role of autophagy, since autophagy is the other major system responsible for protein degradation in addition to the proteasome [19, 21]. As shown in Figure 4A, AICAR, an inhibitor of autophagy [21, 29], efficiently prevented NIK degradation induced by GA (top panel, lane 3). The role of AICAR is specific, because it failed to protect Akt, a well-known client of Hsp90, from GA-mediated degradation (middle panel, lane 3). Consistent with previous studies showing that Akt degradation induced by GA depends on the proteasome [24], proteasome inhibition by MG132 rescued Akt from GA-induced degradation (middle panel, lane 4). These studies suggested that autophagy may be responsible for the GA-mediated degradation of NIK.

To validate these biochemical studies, we utilized the Atg5-deficient cells. Atg5 is essential for autophagosome formation, and knockout of Atg5 blocks autophagy [30]. Consistent with the results shown above, knockout of Atg5 also significantly inhibited GA-triggered degradation of NIK (Figure 4B, top panel, lane 4). In sharp contrast, Atg5 is not required for GA-induced Akt degradation, because Akt was still degraded in the Atg5 null cells (Figure 4B, middle panel, lane 4). Thus, NIK degradation in the absence of Hsp90 is mediated by autophagy. This evidence also indicated that autophagy may function as an alternative mechanism for Hsp90 client degradation when Hsp90 function is lost.

Hsp90 is not required for NIK transcription or translation

Although our biochemical and genetic data clearly demonstrated that GA treatment induces autophagy-mediated degradation of NIK protein, we also examined any potential effects of GA on NIK mRNA transcription and translation by performing the real-time RT-PCR and polysome fractionation analysis. As a control, the transcription and translation of p100 mRNA was also examined. In agreement with the fact that CD40L can activate canonical NF-kB to induce p100 expression, we found that CD40 antibody treatment dramatically enhanced p100 mRNA transcription and subsequent protein translation (Figure 5, column 4). However, GA treatment hardly influenced NIK mRNA transcription and translation (column 2). Taken together, these studies strongly suggested that Hsp90 regulation of NIK biosynthesis is largely, if not completely, at the protein level.

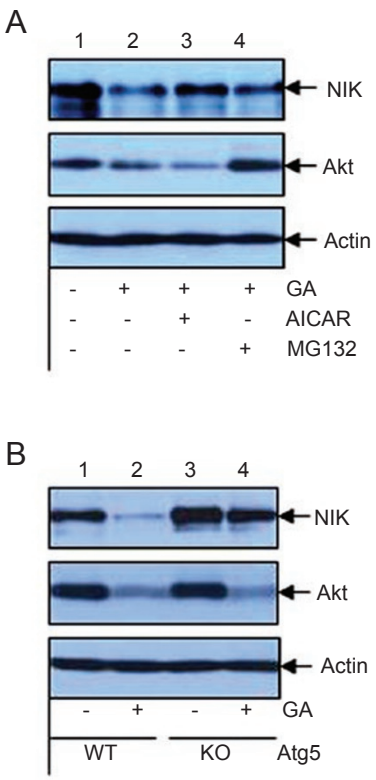

Figure 4 NIK protein degradation induced by GA is largely mediated by autophagy. **(A)** AICAR, an inhibitor of autophagy, blocks GA-induced NIK degradation. 293 cells transfected with NIK or Akt were incubated with 10 μM GA (+) or DMSO (–) in the presence of AICAR (1 mM), or MG132 (25 μM) for 20 h, followed by IB to detect protein levels of NIK (top panel) or Akt (middle panel). The protein level of actin (bottom panel) was detected as a loading control. **(B)** GA-induced degradation of NIK is blocked in Atg5 deficient cells. Atg5 wild type or null cells transfected with NIK or Akt were incubated with 5 μM GA (+) or DMSO (–) for 20 h, followed by IB as described in **(A)**.

Hsp90 is dispensable for NIK-mediated IKKα recruitment to p100

In addition to its function in protein stabilization, Hsp90 may also play other important roles in cell signaling. For example, Hsp90 has been found to be involved in signaling complex assembly [31]. We thus also examined whether Hsp90 is required for the formation of NIK/IKKα/p100 complex, a step serving as a molecular switch for p100 processing [12, 14]. Since NIK nucleates the signaling complex by functioning as an adaptor to bind to both IKKα and p100 [14], we first checked the effect of GA on the NIK/IKKα and NIK/p100 interactions. Although it could efficiently disrupt the interaction between NIK and Hsp90 (Figure 2A), GA had no effect on NIK binding to IKKα or p100 (Figure 6A and 6B, lanes 3). Not surprisingly, NIK recruitment of IKKα to p100 was also resistant to GA treatment (Figure 6C, lane 3).

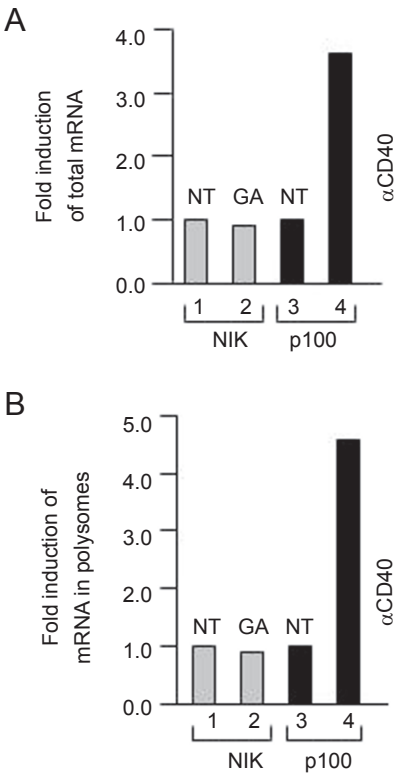

Figure 5 GA treatment does not change the transcription or translation of NIK mRNA. **(A)** GA addition has no obvious effect on NIK RNA transcription. Ramos B cells were either untreated or treated for 10 h with DMSO, GA (2 μM) or anti-CD40 (10 μg/ml), followed by RNA extraction. NIK and p100 mRNAs were quantitated by real-time RT-PCR. The amount of NIK and p100 mRNA was normalized to the level of GAPDH mRNA. The values represented fold change in mRNA abundance relative to the DMSO-treated sample (arbitrarily set as one-fold) and were averages of three independent experiments. **(B)** GA addition has no obvious effect on NIK protein translation. Polysomes were isolated from the Ramos B cells treated as in **(A)**, followed by RNA extraction. The mRNA levels of NIK and p100 in the polysomes were quantitated by real-time RT-PCR as described in **(A)**.

Hsp90-mediated maturation is upstream of TRAF3-mediated proteasomal degradation of NIK protein

In resting cells, NIK protein is usually undetectable (Figure 7A, lane 1), at least partially due to TRAF3-mediated proteasomal degradation [11-13]. Hence, it seems that NIK is regulated by both autophagic and proteasomal degradation. However, the potential link between these two degradation mechanisms is unknown. To address this important issue, we investigated the effect of GA on NIK in both TRAF3 wild type and knockout cells in the presence or absence of MG132. Consistent with previous findings [11-13], MG132 treatment or TRAF3 deficiency could stabilize NIK in the absence of GA (Figure 7A, lane 3 and 7B, lane 1, respectively). However, GA addition could diminish the effect of MG132 treatment or TRAF3 knockout (Figure 7A and 7B, lanes 4 and 2, respectively). Of note, the constitutive processing of p100 caused by TRAF3 deficiency was also blocked by GA (Figure 7B, lane 2). These results suggested that Hsp90-mediated maturation of NIK protein precedes the TRAF3-midated proteasomal degradation.

Discussion

The proteasome and autophagy are two highly conserved mechanisms that are primarily employed for protein degradation within eukaryotes. It is generally believed that autophagy is in principle a non-selective, bulk degradation system of long-lived proteins and organelles through the lysosome; while the proteasome specifically degrades short-lived proteins, including regulatory proteins and misfolded proteins caused by the absence of Hsp90 function. Our recent studies showed that Hsp90 inhibition leads to autophagic but not proteasomal degradation of IKK [21]. The data presented in the current study demonstrate that NIK, a novel client of Hsp90, is also degraded by autophagy when Hsp90 function is inhibited. This study thus provides the second piece of evidence showing that Hsp90 clients may be targeted for degradation by a mechanism different from the proteasome with autophagy serving as the alternative mechanism.

Previous studies have clearly demonstrated that newly synthesized NIK protein is rapidly eliminated by TRAF3-mediated proteasomal degradation [11-13]. It seems that proteins other than TRAF3 may also contribute to the proteasomal degradation of NIK protein, since inhibition

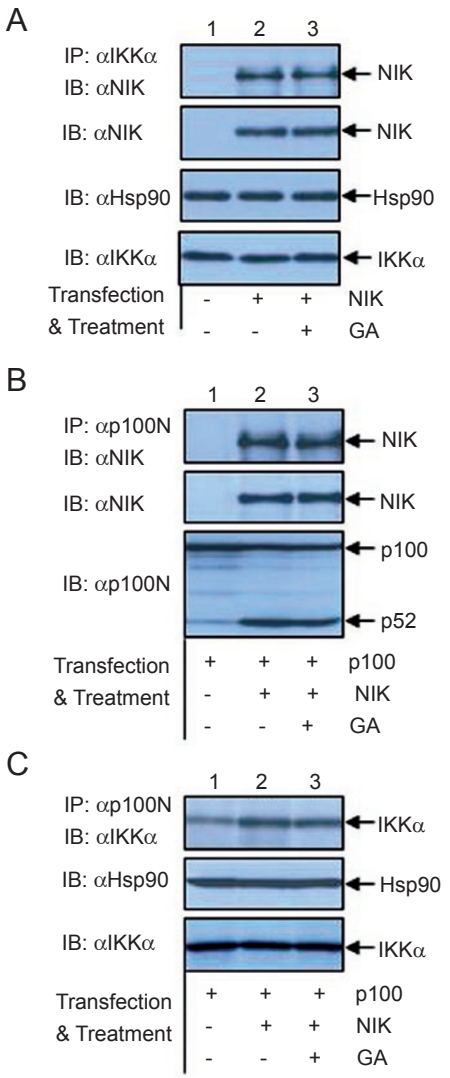

Figure 6 GA disruption of NIK/Hsp90 interaction has no effect on NIK-mediated recruitment of IKKα to p100. **(A)** GA inhibition of Hsp90 does not affect the NIK/IKKα interaction. 293 cells were transfected with an empty vector or the vector expressing NIK, followed by IP using anti-IKKα antibody in the presence or absence of GA (10 μM) and IB using anti-NIK (top panel). Direct IB was also performed to check the expression levels of transfected NIK (2nd panel from the top), endogenous Hsp90 (3rd panel) and IKKα (bottom panel). **(B)** GA inhibition of Hsp90 does not affect the NIK/p100 interaction. 293 cells were transfected with the indicated constructs, followed by IP using anti-p100 antibody in the presence or absence of GA (10 μM) and IB using anti-NIK (top panel). Direct IB was also performed to check the expression levels of NIK (middle panel) and p100 (bottom panel). **(C)** GA inhibition of Hsp90 does not affect NIK-mediated IKKα recruitment to p100. 293 cells were transfected with the indicated constructs, followed by IP using anti-p100N antibody in the presence or absence of GA (10 μM) and IB using anti-IKKα (top panel). The expression levels of Hsp90 (middle panel) and IKKα (bottom panel) were also examined by direct IB. The NIK and p100 expression levels were similar to those shown in **(B)**.

of the proteasome by MG132 could further significantly increase NIK protein levels in TRAF3 knockout cells (Figure 7B). But more importantly, Hsp90-mediated folding and maturation of nascent NIK protein is prior to the TRAF3-mediated proteasomal degradation, or in other words, the NIK protein degraded by the TRAF3-activated proteasome is already maturated (Figure 7). Based on these findings, a model of NIK regulation is presented in Figure 7C. In brief, Hsp90 binds to newly synthesized NIK protein, which promotes the correct folding, maturation and/or conformation maintenance of NIK. When Hsp90 function is absent, such as upon inhibition by GA, the nascent NIK protein cannot be folded correctly and/or the mature NIK protein cannot maintain the correct conformation, resulting in its degradation via the autophagy pathway. In resting cells, however, the mature NIK protein associated with Hsp90 is still rapidly degraded by the proteasome, which is mediated by TRAF3 and possibly also by other proteins such as TRAF2. The non-canonical NF-κB stimuli, such as BAFF, LTβ and CD40L, somehow lead to TRAF3 degradation and/or its dissociation from NIK, thereby protecting NIK from proteasomal degradation. The NIK protein that has escaped from both autophagic and proteasomal degradation then functions as the molecular switch for p100 processing.

Like the proteasomal proteolysis, autophagic degradation has also been linked to the pathogenesis of various disorders, particularly tumorigenesis [19, 32-34]. Current models suggest that autophagy generally contributes to tumor suppression, and defects in autophagy facilitate or even lead to oncogenesis. The responsible mechanisms, however, remain unclear. As over-activation of the non-canonical NF-κB signaling has been found in various tumors [1-3], our study provides one possible explanation for the tumor suppression function of autophagy: by specifically targeting signaling regulatory proteins (such as NIK and IKK) for degradation, autophagy negatively regulates signaling pathways (such as NF-κB pathways) that contribute to tumorigenesis.

Another important aspect of these data is the identification of Hsp90 as an essential component for the non-canonical NF-κB signaling. In addition to IKK regulation [21], another important function of Hsp90 is to regulate NIK, whose expression functions as a molecular switch in p100 processing and ultimate activation of the alternative NF-κB signaling [12, 14]. Although it is not required for NIK mRNA transcription and translation (Figure 5), Hsp90 is necessary for NIK expression and subsequent p100 processing (Figure 1). Thus, the role of Hsp90 in controlling NIK expression is to protect NIK protein from autophagic degradation by facilitating its correct folding and maturation.

Although this is the first demonstration of a link of Hsp90

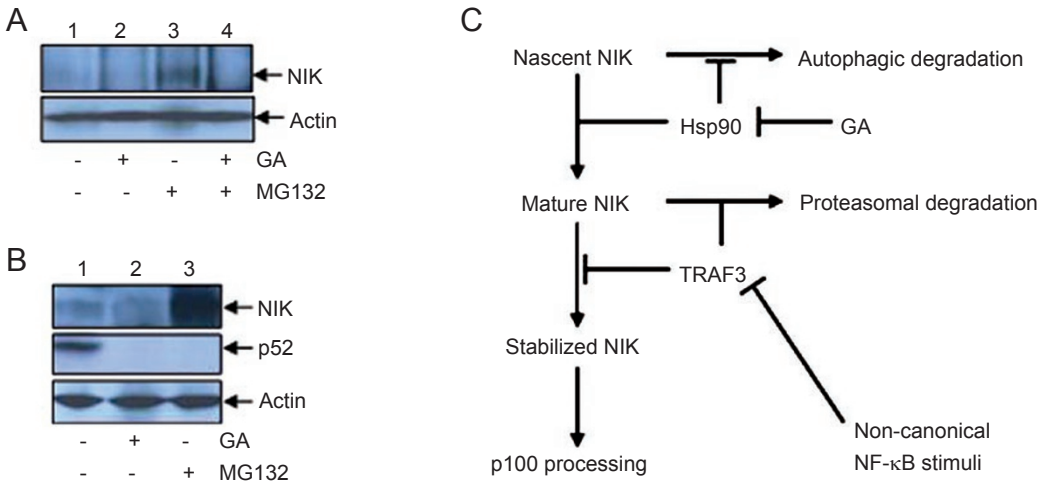

Figure 7 Hsp90 function is required for the increase of NIK protein mediated by the proteasome inhibition or TRAF3 deficiency. **(A)** GA inhibition of Hsp90 prevents the MG132-induced increase of NIK protein. Wild-type MEFs were treated with the indicated drugs for 20 h, followed by IP-IB using anti-NIK antibody (upper panel) for endogenous NIK expression. Some of the cell lysates were directly subjected to IB for the expression level of actin (lower panel). **(B)** GA inhibition of Hsp90 prevents NIK protein increase and p100 processing caused by TRAF3 deficiency. TRAF3 null MEFs were treated with GA (10 μM), MG132 (25 μM), or DMSO for 20 h, followed by IB to detect the expression levels of endogenous NIK (top panel), p52 (middle panel) and actin (bottom panel). **(C)** Dual regulation of NIK by autophagic and proteasomal degradation. In resting cells, newly synthesized NIK proteins bind to Hsp90 for their maturation and/or conformation maintenance, with any free NIK being degraded via autopagy-lysosome pathway due to failure of correct folding and/or conformation maintenance. However, the mature NIK proteins, which are still associated with Hsp90, are rapidly captured by TRAF3 and quickly degraded by the proteasome. In response to the non-canonical NF-κB stimuli, NIK proteins are somehow freed from TRAF3 and therefore being stabilized. The stabilized NIK proteins then induce p100 processing for the activation of the non-canonical NF-κB signaling (see text for details).

to the non-canonical NF-κB signaling, the importance of Hsp90 for activation of the canonical NF-κB pathway has already been suggested [35, 36]. Mechanistic studies showed that RIP and IKK, two important components for NF-κB activation, are Hsp90 clients. Like most Hsp90 clients, RIP is degraded by the proteasome when its interaction with Hsp90 is disrupted by GA [36]. Similarly, GA treatment also leads to IKK degradation. However, the GA-induced IKK degradation is not through the proteasome but rather mediated by autophagy [21], which resembles the NIK degradation induced by GA. Interestingly, disruption of Hsp90/IKK association abolishes the recruitment of IKK to the tumor necrosis factor (TNF) receptor I (TNFR-I) and subsequent activation of IKK induced by TNF, a prototypic stimulator of the canonical NF-κB signaling [31]. Surprisingly, disruption of the Hsp90/NIK interaction does not affect NIK binding to its downstream factors IKKα or p100 and subsequent recruitment of IKKα to p100, essential steps for p100 processing (Figures 2 and 6). Moreover, NIK degradation induced by GA is mediated by autophagy but not by the proteasome (Figures 3 and 4). It therefore seems that different mechanisms are applied by Hsp90 to regulate the canonical and non-canonical NF-κB pathways.

In addition to NF-κB pathways, Hsp90 function is also required for activation of several other signaling pathways that have been linked to tumorigenesis [17]. Due to its significance, Hsp90 is a therapeutic target of tumor treatment. As a matter of fact, Hsp90 has been identified as a specific target of the novel anti-tumor drug GA [18]. Thus, inhibition of the non-canonical NF-κB activation pathway by GA may be a critical component of the anti-tumor activity of this drug, at least for some tumors. Reasonably, GA may be therapeutically effective for those tumors and autoimmune diseases associated with deregulated p100 processing.

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