Signal-responsiveness of IκB kinases is determined by Cdc37-assisted transient interaction with Hsp90

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The IκB kinase (IKK) holocomplex, containing the kinases IKKα, IKKβ and the scaffold NEMO (NF-κB essential modifier), mediates activation of NF-κB by numerous physiological stimuli. Heat shock protein 90 (Hsp90) and the co-chaperone Cdc37 have been indicated as additional subunits, but their specific functions in signal transduction are indistinct. Using an RNAi approach, we demonstrate that Cdc37 recruits Hsp90 to the IKK complex in a transitory manner, preferentially via IKKα. Binding is conferred by N-terminal as well as C-terminal residues of Cdc37. Cdc37 is essential for the maturation of de novo synthesized IKKs into enzymatically competent kinases, but not for assembly of an IKK holocomplex. Mature IKKs, T-loop phosphorylated after stimulation either by receptor-mediated signaling or upon DNA damage, further require Hsp90-Cdc37 to generate an activated state. Thus, the present data denote Hsp90-Cdc37 as a transiently acting essential regulatory component of IKK signaling.

NF-κB maintains key functions in various biological and pathological processes, including immune and inflammatory reactions, development, proliferation, apoptosis, stress responses and oncogenesis (1-3). Three major NF-κB activating pathways can be distinguished. In the first, canonical pathway a broad range of extracellular stimuli, including bacterial pathogens, antigens, mitogens and inflammatory cytokines induce diverse intracellular cascades, which activate the IκB kinase (IKK) complex. IKK-mediated phosphorylation triggers IκB and p105 polyubiquitination by the SCFβTrCP E3 ligase complex and subsequent proteasomal destruction, resulting in the release of p50-, p65- and c-Rel-containing heterodimers (1,4). With much slower kinetics, a second, non-canonical NF-κB pathway induces C-terminal processing of NF-κB2/p100 to generate p52-containing complexes (5). Finally, a “nuclear-to-cytoplasmic” pathway promotes NF-κB activation in response to DNA damage (6).

The IKK complex has a large apparent molecular weight of 700-900 kDa and contains the kinases IKKα and IKKβ as well as the regulatory non-enzymatic scaffold protein IKKγ aka NEMO (NF-κB Essential Modifier) (1,7). Stoichiometric analyses indicated equimolar content of IKKγ and kinase molecules (8,9) in the IKK complex, where a tetramer of IKKγ is thought to bind to two kinase dimers (10). The scaffold protein ELKS has been proposed as a further regulatory component of cellular IKK complexes (11). IKK activation is dependent on phosphorylation at activation loop (T-loop) serines, either by upstream IKK kinases (IKKs) or by auto-phosphorylation. The activation process involves catalytic, non-destructive Lys-63-linked polyubiquitination (UbK63) of IKKγ as well as UbK63 binding by IKKγ (12,13). Moreover, conformational changes by induced protein interactions may also be a mechanism to stimulate IKK activity. A number of regulatory proteins have been suggested to interact with IKK components (reviewed in (1,7)). Amongst them the chaperones Hsp90 and Cdc37 were proposed as stoichiometric subunits of an IKK holocomplex (14).

Heat-shock protein 90 (Hsp90), which represents 1-2 % of cytosolic protein, acts as molecular chaperone and requires ATP binding and hydrolysis to maintain its function (15-17). Hsp90 client proteins are preferentially cellular signal transducers, such as protein kinases and transcription factors. Hsp90 promotes their
proper folding, assembly, and transportation across different cellular compartments (18-21). Hsp90 is regulated through sequential and cooperative binding of a set of co-chaperones that link Hsp90 to distinct classes of client proteins. The mammalian homologue of the budding yeast cell cycle control protein Cdc37 is one of such co-chaperones (22,23). The immunophilin FK506-binding protein 51 (FKBP51), also suggested as a regulator of NF-κB activity (24), is another co-chaperone that binds to Hsp90 via a tetratricopeptide repeat (TPR) domain (18,25). Due to the complex biological functions of Hsp90 and its co-chaperones, knockout studies may not be readily informative and in fact result in lethal phenotypes (26,27).

Studies using the Hsp90-specific ATPase-inhibitor geldanamycin (GA) initially proposed a requirement of Hsp90 for NF-κB activation (14,28-30). Hsp90 interacts with IκB kinases and signaling proteins of the NF-κB pathway, including MEKK3, NIK, RIP1, TAK1 and TBK1 (14,24,31-33). Prolonged inhibition of Hsp90 by GA causes proteasomal degradation of IKK components and RIP1 (14,24,29,31,34). Hence, it is unclear to which extent Hsp90 inhibition affects NF-κB pathways at the level of IKK versus that of upstream kinases, and if chaperones control the signaling process per se or have homeostatic functions.

In the present study we investigated how Hsp90 and Cdc37 interact with the IKK signalosome, utilizing RNA interference. With a compositional analysis, we demonstrate that the IKKα, β, γ core complex is not associated with additional stoichiometric subunits. Cdc37 recruits Hsp90 to the IKK complex in a transitory manner, preferentially via IKKα and is phosphorylated by both, IKKα and IKKβ. Interaction with the chaperone complex is essential to promote functional maturation of de novo synthesized IκB kinases. Moreover, Hsp90-Cdc37 activity is required to trigger catalytic activation of T-loop phosphorylated IKKs following stimulation with TNF-α or DNA damage. These results provide evidence that the chaperone complex acts on the IKK complex in the process of its activation.

**Experimental Procedures**

*Cell culture and transfection*- HeLa and 293 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (PAA), supplemented with 10% fetal calf serum (FCS), 1mM sodium pyruvate and 100 U/ml penicillin/streptomycin. 17DMAG was purchased from InvivoGen. 293 cells were transiently transfected by calcium phosphate precipitation using standard protocols. HeLa cells were transfected with DNA constructs using Lipofectamine 2000 (Invitrogen). Synthetic siRNA were transfected in final concentrations between 0.5 nM and 10 nM. Lipid mediated transfection was carried out with atufect01 (36). For sequence information see supplemental data. Where indicated, cells were additionally transfected with DNA constructs 48 hours after siRNA transfection using Lipofectamine 2000. Cells were lysed 72 h after siRNA transfection. Full length human IKKα, IKKβ and IKKγ cDNAs were cloned with N-terminal Flag epitope into pcDNA3 or with N-terminal Myc-epitope into pRK5. Kinase-dead mutants of IKKα and IKKβ carry a K44A mutation (37). N-terminally Flag-tagged full length, truncated or point mutated human Cdc37 was cloned into pcDNA3.

**Immunoprecipitation, Western blotting, Kinase assay and Electrophoretic mobility shift assay (EMSA)**- Immunoprecipitations and western blots were performed in accordance with standard procedures. Cells were lysed in 100 mM NaCl, 25 mM Tris pH8, 0.2% NP-40, 10% glycerol, supplemented with complete protease inhibitor cocktail (Roche), 10 mM NaF, 8 mM β-glycerophosphate, 0.2 mM Na3VO4, 1 mM DTT. For antibodies, see supplemental data. Kinase assays and electrophoretic mobility shift assays were performed as described previously (38). Intensities of signals for IκBα protein (Western blots) phosphorylated GST-IκBα (kinase assays) and NF-κB-DNA complexes (EMSA) were quantified with TINA 2.0.

**Reporter gene assay**- 293 cells were transfected per 6-well with 0.25 µg of pRL-TK (Promega), 3 ng of 6x NF-κB-luc (39), and 2.5 µg of pCDNA3. Where indicated, 0.8 µg of Cdc37 expression construct and 0.7 µg Flag-IKKβ was transfected together with pCDNA3 vector up to 2.5 µg. 24 hours post transfection, the cells were stimulated with 40 ng/ml TNFα for 4 h, as indicated, and luciferase activity was determined using a dual luciferase assay kit (Promega).

**Gel filtration**- ~2×10^7 HeLa cells were lysed in a 500-µl volume of 50 mM HEPES, pH 7.5,
150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 1 mM DTT, 10 mM NaF, 8 mM β-glycerophosphate, 0.1 mM orthovanadate, 10% glycerol plus complete protease inhibitor cocktail (Roche). Gel filtration chromatography was carried out on a Superose 6 column (Amersham Pharmacia Biotech) as described previously (8).

Metabolic labeling- HeLa cells were labeled for 1 h with 70 µCi/ml [35S]methionine (38) and lysed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 1 mM DTT, 10 mM NaF, 8 mM β-glycerophosphate, 0.1 mM orthovanadate, 10% glycerol plus complete protease inhibitor cocktail (Roche). Extracts were precleared with protein A-Sepharose for 1 h and incubated with anti-IKKγ antibodies overnight at 4 °C. Finally, protein A-Sepharose was added for 3 h. Precipitates were washed five times with lysis buffer and boiled SDS loading buffer. The supernatant was applied on a SDS-PAGE and analyzed by autoradiography.

RESULTS

Hsp90-Cdc37 interacts with the core IKK complex in a transitory manner. To determine the role of Hsp90 and Cdc37 in IKK signaling we analyzed the mode of their association with the IKK holocomplex. IKKγ and IKKα precipitation from lysates of [35S]methionine labeled HeLa cells revealed only three prominent interacting species (Fig. 1A). Silencing of single IKK components with specific siRNAs verified the observed species as IKKα, IKKβ and IKKγ (Fig. 1B). In contrast, silencing of Cdc37 or FKBP51, suggested as stoichiometric components (Hsp90-Cdc37) or regulatory co-factor (FKBP51) of the IKK-complex (14,24) did not interfere with the pattern of IKKγ co-precipitated proteins from metabolically labeled cells (Fig.1B).

As a second approach, cell lysates were analyzed by size-exclusion chromatography. IKKα, IKKβ and IKKγ were identically eluted in fractions 12-16 with a peak in fraction 14 (Fig. 1C) with an apparent molecular mass between 700 and 900 kDa, as described before (8,40,41). Upon gel filtration, Hsp90 (fractions 18-22), Cdc37 (fractions 24-28), and FKBP51 (fractions 22-26) eluted with distinct smaller sizes compared to the IKK complex (Fig. 1C). Moreover, TNFα stimulation did not change this gel filtration profile of Cdc37 and Hsp90 (Fig. S1). Because small amounts of Cdc37 and Hsp90 trailed into the IKK fractions, a stoichiometric contribution of Cdc37 and Hsp90 to the IKK-complex could still be possible if Cdc37 and Hsp90 were present in high molar excess over the IKK components. However, quantification of the molar ratio of the endogenous proteins indicated only a roughly 2-3 fold molar excess of Cdc37 over IKKβ (Fig. S2). These data reveal that only a small fraction of cellular IKK-complexes interacts with Cdc37 and Hsp90 at a given time, considering that Hsp90 is largely recruited to IKKs via Cdc37 (see below). Surprisingly, our data gave no indication that the previously proposed essential subunit ELKS (105 kDa) (11) is part of the IKK complex. (Fig. 1A, B and C). In fact, further investigations, addressing IKK binding and NF-κB activation (data not shown), could not support ELKS as an essential, generally associated regulatory subunit of the IKK complex.

As expected for a stoichiometric component, silencing of IKKα - in contrast to ELKS, Cdc37 or FKBP51 - caused a clearly discernible decrease of the apparent size of the residual IKKγ containing complex (Fig. 1D), indicating a different structure or oligomeric composition. The siRNA-mediated knockdown of indicated proteins (Fig. 1B and D) was controlled by western blotting (Fig. 1E). Taken together, we conclude that the chaperones are not stable stoichiometric components of the IKK complex, but rather associate transiently.

Cdc37 preferentially interacts with IKKα to recruit Hsp90 and FKBP51 to the IKK complex. Although the importance of Hsp90 for IKK activation pathways seems unambiguous, it is not understood, how the chaperone-complex interacts with the IKK complex. Previous studies, using ectopically expressed IKK deletion mutants and GST pull-down assays, indicated that Cdc37-Hsp90 complexes bind to the kinase domains of IKKα and β (14). Indeed, both Cdc37 and Hsp90 were co-precipitated with anti-IKKγ antibodies, indicating association with the endogenous IKK complex (Fig. 2A). Silencing of Cdc37 expression disrupted binding of
Hsp90 to IKKs. Residual Hsp90 may reflect incomplete Cdc37 depletion. Notably, loss of IKKα but not IKKβ interfered with Hsp90-Cdc37 recruitment, suggesting that preferentially IKKα bridges Cdc37 and Hsp90 to the IKK complex. Protein interaction studies in cells transiently expressing Flag-tagged Cdc37 together with Myc-tagged IKKα and Myc-IKKβ confirmed the highly preferential binding of Cdc37 to IKKα (Fig. 2B). Remarkably, a kinase domain mutant (K44A) of both IKKs bound with much higher affinity to Cdc37 compared to wild type (Fig. 2B), suggesting that structural alterations of the kinase domain influence Cdc37 interaction. A double band of Cdc37 was detected when cotransfected with wild-type IKKs, suggesting that structural alterations of the kinase domain influence Cdc37 interaction. A double band of Cdc37 was detected when cotransfected with wild-type IKKs, suggesting that structural alterations of the kinase domain influence Cdc37 interaction. A double band of Cdc37 was detected when cotransfected with wild-type IKKs, suggesting that structural alterations of the kinase domain influence Cdc37 interaction. A double band of Cdc37 was detected when cotransfected with wild-type IKKs, suggesting that structural alterations of the kinase domain influence Cdc37 interaction.

To investigate the mode of Hsp90-Cdc37-IKKα interaction in more detail, we transiently expressed Flag-tagged Cdc37 mutant constructs together with Myc-tagged IKKα. In co-immunoprecipitation studies all deletion mutants were defective in Hsp90 association (Fig. 2D). Surprisingly, N-terminal (ΔN) as well as C-terminal deletion mutants (ΔC1, ΔC2) could efficiently bind to IKKα, indicating two independent IKKα binding sites on Cdc37 (Fig. 2D). One of these seems to be located in a central region of Cdc37, since binding of ΔC1 to IKKα was diminished. In sum, these data confirm Cdc37 as an adaptor between Hsp90 and IKKα.

Strict requirement of Hsp90-Cdc37 for activation of ectopically expressed IkB kinases. To define the functional role of Hsp90 and Cdc37 for the IKK activation process, we directly manipulated Hsp90 and Cdc37 levels in cells. Co-transfection of IKKβ together with Cdc37 robustly increased NF-κB dependent transcription (Fig. 3A). Moreover, Cdc37 amplified activation loop phosphorylation of Myc-IKKα or IKKβ (Fig. 3B). In turn, silencing of endogenous Cdc37 abolished T-loop phosphorylation and kinase activity of transfected IKKβ without affecting IKK expression levels (Fig. 3C). Down modulation of Hsp90α and Hsp90β likewise resulted in reduced kinase activity, but to a minor extent, due to incomplete knockdown (data not shown).

Ectopically expressed IKKs respond in a much more sensitive manner to modulation of Cdc37 expression than preformed cellular IKK complexes in TNFα-induced cells, as judged by luciferase reporter (Fig. 3A) and kinase assays (Fig. S4). Taken together, these observations strongly suggest that newly synthesized IKKs require Hsp90-Cdc37 to achieve an inducible conformation.

Cdc37 is required for the formation of functionally competent endogenous de novo synthesized IKKs. The fact that Hsp90-Cdc37 mediates maturation of ectopically expressed IKKs into a catalytically competent conformation alludes to similar functions for de novo synthesized endogenous IKKs. Thus, we analyzed Hsp90-Cdc37 dependent IKK activity in a situation where the role of de novo synthesis of endogenous IKKα and IKKβ can be addressed.

In agreement with prior findings (Fig. 3A, Fig. S4), down modulation of Cdc37 alone did not affect TNFα induced NF-κB activation, as judged by NF-κB DNA binding activity and IκBα degradation (Fig. 4A). While silencing of IKKβ caused a robust inhibition of in vitro kinase activity (Fig. S4), only a moderate inhibition of TNFα induced IκBα degradation and concurrently a medium decrease in NF-κB DNA binding could be observed (Fig. 4A). This discrepancy might be explained by different sensitivities of these assays, e.g. different contribution of residual IKKβ towards endogenous substrate compared
to excess GST-\textit{IxB}α used in the \textit{in vitro} kinase assay. Intriguingly, combined siRNA-mediated depletion of Cdc37 and IKKβ significantly inhibited \textit{IxB}α degradation and NF-κB DNA-binding activity in a synergistic manner. We assume that under these conditions a major portion of residual IKKs results from de novo synthesis, which in turn requires the contribution of Hsp90-Cdc37 as shown for ectopically expressed IKKs (Fig. 3).

For further verification, a 2-step transfection strategy (at day 0 and day 3) was used, to achieve long-term knockdown of Cdc37 (Fig. 4B, lanes 4, 5, 8, 9, 12, 13). IKKα and IKKβ were silenced in parallel in a single step (day 0). In general, protein expression is recovered 5 or 6 days after siRNA transfection, thereby allowing the observation of IKK de novo synthesis and complex assembly. Hence, cells were stimulated with TNFα at day 4 to 6 and lysates were examined for protein expression or kinase activity (Fig. 4B). At day 4, IKKα and IKKβ protein expression was strongly reduced, thereby preventing kinase activation (lanes 3-6). At day 5 and day 6 IKKα and IKKβ expression levels increased (lanes 7-14), which in fact led to the production of new, TNFα inducible IKK complexes in control cells (Lanes 7, 10, 11, 14). In contrast, loss of Cdc37 strongly diminished kinase activity of newly formed IKK complexes (lanes 8, 9, 12, 13).

To test if chaperone activity is required for IKK complex assembly, we analyzed lysates of day 6 (lane 13) by gel filtration. Indeed, silencing of Cdc37 expression did not affect IKK complex assembly (Fig. 4C). In addition, we silenced Cdc37 expression and co-transfected Flag-IKKα, β and γ in parallel. Neither IKK protein expression (Fig. S5A), nor complex assembly (Fig. S5B) was affected. Nevertheless, loss of Cdc37 again resulted in a significant reduction of IKK auto-phosphorylation and kinase activity (Fig. S5A). Taken together, these results establish an important function for Hsp90-Cdc37 in IKK maturation. Hsp90-Cdc37 interacts with de novo synthesized \textit{IxB} kinases to generate functionally competent IKK complexes.

\textit{Inhibition of signal-induced activation of steady state IKKs requires a robust block of Hsp90-Cdc37 activity.} Ectopic Cdc37 expression only marginally interfered with TNFα induced NF-κB activation (Fig. 3A). In line with this, neither silencing of Cdc37 and Hsp90 nor of IKKα, which simultaneously abrogates binding of Cdc37 and Hsp90 (Fig. 2A), strongly affected IKK activation (Fig. 4A, Fig. 5A and Fig. S4). At first sight, these observations are in conflict with studies suggesting that GA inhibits IKK activation (14,29). However, significantly lower concentrations of the geldanamycin derivative 17-DMAG are required to inhibit NF-κB DNA binding activity and \textit{IxB}α degradation (Fig. 5A) upon silencing of either Hsp90α/β or Cdc37 (Fig. 5B). Apart from confirming the target specificity of 17-DMAG, these data also demonstrate a cooperative function of Hsp90 and Cdc37 in TNFα-induced activation of mature IKKs. Due to the abundant expression level of Hsp90, a more robust inhibition of Hsp90-Cdc37 activity is needed to down modulate signal-induced IKK and NF-κB activation.

\textit{Hsp90-Cdc37 acts downstream of T-loop phosphorylation in TNFα or DNA damage induced IKK activation.} To investigate whether Hsp90-Cdc37 acts preferentially on receptor-induced IKK cascades, we analyzed a mechanistically different signaling pathway, which promotes IKK and NF-κB activation in response to DNA damage (6). Remarkably, pharmacological inhibition of Hsp90 nearly abolished DNA damage induced NF-κB DNA binding activity under conditions where protein expression of clients such as IKKs or RIP1 was unaffected (Fig. 6A). As shown for receptor mediated signaling, singular knockdown of Cdc37 or Hsp90 was inefficient to disrupt DNA damage induced NF-κB activation (data not shown).

The fact that inhibition of Hsp90 interferes with both receptor-mediated and DNA damage induced NF-κB activation, suggests that the chaperone directly acts on the IKK complex. Consequently, we assayed for IKK T-loop phosphorylation, which is the prerequisite for IKK activation in both experimental settings (Fig. 6B). Short pre-treatment (4h or 2 h) with 17-DMAG significantly impaired TNFα- or DNA damage- induced IKK activity. In contrast, stimulus-dependent IKK T-loop phosphorylation was unaffected. These data reveal that kinase activity is not spontaneously augmented upon T-loop phosphorylation. Rather, a subsequent Hsp90-Cdc37 dependent process is required to generate a catalytically
activated IKK complex, likely by mediating conformational alterations.

**DISCUSSION**

The chaperone complex Hsp90-Cdc37 has been suggested to stably associate with the IKK signalosome and to confer important regulatory functions in NF-κB signaling, such as IKK recruitment to TNF receptors (14). Here we present evidence for a more general requirement of Hsp90-Cdc37 in IKK signaling (Fig. 7). Hsp90-Cdc37 interacts with the IKK complex in a transitory manner to promote two distinct functions. De novo synthesized IKKs depend on Hsp90-Cdc37 to achieve an enzymatically competent state. Upon stimulation either by receptor mediated signaling or in response to DNA damage, mature IKKs again require Hsp90-Cdc37 following their T-loop phosphorylation to attain full catalytic activation. RNAi of Cdc37 and Hsp90 interfere with IKK maturation, while inhibition of the final IKK activation step was only achieved by a more robust pharmacological block, indicating different threshold levels for the requirement of chaperone activities.

As a starting point, we investigated the composition of the proposed IKK holocomplex with approaches that allow stoichiometric predictions, such as gel filtration chromatography and IKK-co-precipitation from metabolically labeled cells. Thus far, only IKKα, β and γ have been conclusively demonstrated as IKK subunits (8,9,41). In fact, our studies gave no evidence for Hsp90, Cdc37 or ELKS as bona fide components of the IKK complex (Fig. 1). These findings are contradictory to prior gel filtration experiments, showing co-elution of Hsp90, Cdc37 and ELKS as bona fide components of the IKK complex (Fig. 1). These findings are contradictory to prior gel filtration experiments, showing co-elution of Hsp90, Cdc37 and ELKS as bona fide components of the IKK complex (11,14). The latter studies analyzed purified IKK complexes, and did not address the stoichiometric composition of endogenous complexes. The assumption that IKK and Hsp90-Cdc37 form stoichiometric, stable complexes was supported by gel filtration experiments, where GA-treatment resulted in a decrease of the apparent size of the IKK complex (14). However, extended exposure to GA causes ubiquitin-mediated proteasomal degradation of IKKα and β (29) which results in altered migration of the IKK complex, as observed after down modulation of IKKα (Fig. 1D). Therefore, we conclude that the suggested IKK holocomplex consists exclusively of the established IKK subunits, which transiently interact with a broad range of regulatory proteins, such as Hsp90-Cdc37.

The present study indicates a preferential interaction between Hsp90-Cdc37 and IKKα (Fig. 2). Structural domains, defining the assembly of Hsp90-Cdc37-client complexes have been identified recently. The central region of Cdc37 (amino acids 128-282) contains the Hsp90 binding domain (reviewed in (23,25)). In fact, partial deletion of this region disrupts Hsp90 recruitment to IKKs (Fig. 2D). The binding of Cdc37 to client proteins is more complex, since either the N-terminal portion (amino acids 1-126) or a central region (amino acids 191-195) has been proposed as crucial domains (42-44). Binding of IKKα is conferred by N-terminal as well as C-terminal regions of Cdc37, suggesting two independent binding sites (Fig. 2D). For the recognition of client proteins by Hsp90-Cdc37, two mechanisms have been suggested. Based on phage display analysis a GXFG motif has been identified that is part of the canonical glycine-rich loop (GXGXXG) of protein kinases (45). Hsp90-Cdc37 was also shown to bind preferentially to positively or neutrally charged αC-β4 loop regions of client kinases (32). Both regions are in proximity within the kinase domains of IKKs, which contain the binding site for the Hsp90-Cdc37 complex (14). Interestingly, kinase dead mutants of both IKKs bind with strongly increased affinity to Cdc37 compared to the wild type proteins (Fig. 2B). In both kinases, the mutated residue (Lys44) might alter the local structure and affect recognition of the flanked GXFG and αC-β4 motifs at position 22 and 61, respectively. Although the co-IP data suggest highly preferential association with IKKα, it is obvious that Cdc37 is capable of interacting functionally with IKKβ as well, because Flag-Cdc37 is equivalently phosphorylated at serine 300 upon co-expression with either kinase (Fig. 2 and Fig. S3). Phosphorylation of Cdc37 might function as part of a feedback mechanism, which alters the mode of interaction of Cdc37 with Hsp90 or with functional, correctly folded IKKs.

Hsp90 chaperone complexes primarily trigger functional maturation and activation of client proteins, although the molecular mechanisms are rather enigmatic (18,23,25). The present study reveals two modes of action, for how Hsp90-Cdc37 acts on the IKK
signalosome, namely maturation of \textit{de novo} synthesized IκB kinases and assistance during the catalytic cycle (Fig. 7). It has to be noted that modulation of Cdc37 expression affects T-loop auto-phosphorylation of ectopically expressed IKKs, which mechanistically differs from the signal-induced T-loop phosphorylation of mature endogenous IKKs by IKK kinases (Fig. 3). Ectopically expressed IKKs require Hsp90-Cdc37 for proper maturation, which allows T-loop auto-phosphorylation and subsequent full catalytic activation. Likewise, \textit{de novo} synthesized endogenous IKKs require Cdc37 to gain a functionally active conformation, which then allows activation by upstream kinases (Fig. 4).

Previous studies, utilizing the Hsp90 inhibitor geldanamycin, have suggested that Hsp90-Cdc37 is required for TNFα dependent recruitment of the IKK complexes to TNF receptor 1 (14). However, extended Hsp90 inhibition strongly decreases expression of RIP1 (14,31,32), an essential component in this process (12). Notably, short-time inhibition of Hsp90 impaired IKK kinase activation by various receptor-mediated signaling cascades without affecting expression levels of IKK subunits or signaling proteins (Fig. 7) (29). Chaperone activity is not only needed for receptor-mediated, but also for DNA damage induced IKK activation, which is a mechanistically distinct pathway (Fig. 6)(6,7). Thus, Hsp90-Cdc37 acts as a generally required component in the activation process of the IKK complex. Signal-induced IKK activation depends on T-loop phosphorylation as an initial essential event. We could demonstrate that Hsp90 inhibition interferes with the activation of IKK kinase activity at a level downstream of T-loop phosphorylation (Fig. 6). In fact, the activation loop phosphorylation may not be the immediate activation step but rather the entry into a catalytic cycle, which primes IKK for activation. Induction of Ser/Thr kinases in general may involve conformational alterations (46,47) and a contribution of Hsp90-Cdc37 to these processes has been discussed (48).

Accordingly, we propose that Hsp90-Cdc37 assists the IKK signalosome to undergo conformational changes that take place during the kinase activity cycle, after the T loops have been phosphorylated. Future structural analyses of the Hsp90-Cdc37-IKK interaction will be important to reveal further mechanistic details.

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Footnotes

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Figure Legends

Fig. 1. Cdc37, Hsp90, FKBP51 and ELKS are not constitutively associated components of the IKK complex. (A) HeLa cells were pulse labeled with [35S] methionine and cell extracts were immunoprecipitated using IKKα, IKKγ or isotype control antibodies. (B) HeLa cells were transfected with different siRNAs as indicated and pulse labeled with [35S] methionine. Cell extracts were immunoprecipitated using IKKγ or isotype control antibodies. (C) HeLa cell extracts were supplied to a Superose 6 gel filtration column and the fractions were analyzed by Western blotting. Migration of marker proteins is indicated. (D) HeLa cells were transfected with siRNAs as indicated and extracts were analyzed by gel filtration and subsequent Western blotting for IKKγ. (E) Down modulation of the indicated proteins was analyzed by Western blotting. The specific bands for ELKS and FKBP51 in (C) and (E) are marked by *.

Fig. 2. Cdc37 preferentially binds to IKKα and connects Hsp90 and FKBP51 to the IKK complex. (A) HeLa cells were transfected with siRNA against the indicated proteins. The IKK complex was precipitated with an IKKγ antibody and co-IP of Hsp90 and Cdc37 was analyzed by Western blotting. Equal amounts of cell extracts (Input) were immunoblotted to determine protein expression levels. (B) 293 cells were transfected with Flag-Cdc37 and different Myc-IKK constructs as indicated. K/A, kinase-inactive K44A mutant. Lysates were precipitated with Flag-antibody. Precipitates were analyzed for Myc-IKK, Hsp90 and Flag-Cdc37 by Western blotting. Expression levels in the lysates were also determined (Input). (C) HeLa cells were transfected with indicated siRNAs and lysates were precipitated with an IKKγ antibody. Co-IP of FKBP51 was analyzed by Western blotting. Cell extracts (Input) were immunoblotted to determine expression levels of IKKα, IKKβ and Cdc37. The specific band for FKBP51 is marked by *. (D) 293 cells were transfected with Flag-Cdc37 wild type or deletion constructs along with Myc-IKKα. Lysates were precipitated with Flag-antibody. Precipitates were analyzed for Myc-IKK, Hsp90 and Flag-Cdc37 by Western blotting.

Fig. 3. Ectopically expressed IKKs require Hsp90-Cdc37 for kinase activity. (A) 293 cells were transfected either with empty vector or expression vector for Cdc37, together with 6xNF-κB-luc and pRLTK as internal standard. Cells were either co-transfected with IKKβ expression vector or stimulated with TNFα, and NF-κB activation was measured in a luciferase reporter assay. Shown is a representative of 3 independent experiments. (B) 293 cells were transfected with expression vectors for Flag-Cdc37 and Myc-IKKα or Myc-IKKβ. The activation-loop phosphorylation of Myc-IKKs was detected by immunoblotting with a phospho-specific IKK antibody. Expression levels of ectopically expressed proteins were controlled with respective antibodies. (C) HeLa cells were first transfected with siRNAs against the indicated proteins and 48 h later with Flag-IKKβ. Lysates were immunoprecipitated with Flag antibody and kinase activity was determined in an in vitro kinase assay. IKK activation loop phosphorylation, Flag-IKKβ expression and down-modulation of Cdc37 and IKKα protein levels were analyzed by Western blotting.

Fig. 4. Cdc37 is required for the activity of de novo synthesized IκB kinases. (A) HeLa cells were transfected with siRNAs as indicated and treated with TNFα for 10 minutes or left untreated. Left, cell extracts were used for EMSA and Western blotting. Right, IκBα protein expression levels before and after TNF stimulation were determined by western blotting and quantified. Expression relative to un-stimulated controls (%) is indicated. (B) Two-step transfection of HeLa cells with different siRNAs was performed as indicated. 4 to 6 days after transfection cells were treated with TNFα for 10 minutes. Cell extracts were immunoprecipitated with an IKKγ antibody and analyzed for kinase activity. The fold TNF-induction of phosphorylated GST-IκBα compared to non-stimulated cells is indicated. Protein expression levels (Input) were determined by Western blotting. (C) The indicated cell lysate was applied to a Superose 6 gel filtration column and the fractions were analyzed for IKKβ and γ expression.

Fig. 5. Inhibition of TNFα induced steady state IKKs requires a robust inhibition of Hsp90-Cdc37. (A) HeLa cells were transfected with siRNAs against Hsp90α and Hsp90β or Cdc37 and subsequently treated for 15 h with different doses of 17-DMAG. Finally, cells were stimulated with TNFα for 10
minutes or left untreated. Lysates were used for EMSA and Western blotting to monitor DNA binding activity and IκBα degradation. The amount of IκBα was quantified in comparison to non-TNF treated samples. (B) Protein levels of Hsp90α, Hsp90β and Cdc37 were determined by Western blotting.

Fig. 6. Hsp90-Cdc37 is required for NF-κB activation in response to DNA damage and acts downstream of T-loop phosphorylation. (A) HeLa and 293 cells were treated with 17-DMAG (500 µM) as indicated, γ-irradiated (10 Gray) and further incubated for 2 h. Lysates were used for EMSA. The fold induction of NF-κB DNA binding activity was quantified. (B) HeLa cells were incubated with 17-DMAG (500 µM) as indicated. For DNA damage induction cells were treated according to (A). For receptor mediated activation cells were treated with TNFα for 10 min. Lysates were immunoprecipitated with an IKKγ antibody and assayed for kinase activity, activation-loop phosphorylation and binding of IKK subunits. Fold induction of P-GST-IκBα and P-IKK compared to non-treated cells is indicated. Expression levels (input) of IKKα, IKKβ, IKKγ and RIP1 were analyzed by Western blotting.* The panel shows different exposure times for TNFα treatment and γ-irradiation.

Fig. 7. Hsp90-Cdc37 is a crucial regulator of the IKK signalosome. De novo synthesized IKK subunits pass through an assembly and maturation process, which generates a kinase competent IKK complex. Mature IκB kinases can be induced upon DNA-damage or receptor–mediated signaling cascades via phosphorylation at activation loop (T-loop) serines. Then, pre-activated IKK enter the catalytic cycle to achieve a kinase active conformation, a process that presumably includes conformational rearrangements. Finally, termination of IKK activity occurs. The IKK signalosome requires chaperone activity at three different levels: IKK homeostasis (1) (29), functional maturation of newly assembled IKK complexes (2) and assistance during the kinase activity cycle (3).
Figure 1

A

Isotype control
anti-IKKγ

IKKβ
IKKα
IKKγ

- 130 kD
- 100 kD
- 72 kD
- 55 kD
- 40 kD

B

UT
siIKKα
siIKKβ
siIKKγ
siCdc37
siFKBP51
siELKS

IP: anti-IKKγ

IKKβ
IKKα
IKKγ

- 130 kD
- 100 kD
- 72 kD
- 55 kD

C

MW (kDa)

669
440
232

IKKα
IKKβ
IKKγ
Cdc37
Hsp90
FKBP51
ELKS

D

WB: IKKγ

siIKKα
siCdc37
siFKBP51
siELKS

E

UT
siIKKα
siIKKβ
siIKKγ
siCdc37
siFKBP51
siELKS

IKKα
IKKβ
IKKγ
Cdc37
FKBP51
ELKS

*
Figure 2

A

| IP: IKKγ | UT | siCdc37 | siCdc37 | siCdc37 | siCdc37 | siControl |
|---------|----|---------|---------|---------|---------|-----------|
| IP      | Hsp90 | Cdc37 | IKKγ |
| Input   | I KKα | IKKβ | IKKγ |

B

Flag-Cdc37  -  +  +  +  +
Myc-IKKβ    +  +  -  -  -
Myc-IKKβ K/A -  -  +  -  -
Myc-IKKα    -  -  -  +  -
Myc-IKKα K/A -  -  -  -  +

D

Cdc37

|     | 1 | 164 | 165 | 222 | 378 |
|-----|---|-----|-----|-----|-----|
|     |   | WT  | ΔC1 | ΔC2 | ΔN  |
Figure 3

A

![Bar graph showing fold induction](image)

B

| Myc-IKKα | Myc-IKKβ | + | + | Flag-Cdc37 |
|----------|----------|---|---|------------|
|          |          |   |   | P-IKK      |
|          |          |   |   | Myc-IKK    |
|          |          |   |   | Flag-Cdc37 |

C

| Flag-IKKβ |
|-----------|
| UT | siCdc37-1 | siCdc37-3 | siIkkα | siControl |
| KA | GST-IkBα |
|    | P-IKK     |
|    | Flag-IKK  |
|    | Flag-Cdc37|
|    | IKKα      |
Figure 4

A

| Treatment | UT | UT | siIKKα | siIKKβ+siCdc37 | siCdc37 |
|-----------|----|----|--------|----------------|---------|
| TNFα      | -  | +  | ++     | +              | ++      |
| EMSA      |    |    |        |                |         |
| Input     |    |    |        |                |         |
| IKKα      |    |    |        |                |         |
| IKKβ      |    |    |        |                |         |
| Cdc37     |    |    |        |                |         |

B

| Treatment | day 4 | day 5 | day 6 |
|-----------|-------|-------|-------|
| siIKKβ/siIKKα | ++   | ++   | ++   |
| siCdc37-1  | +    | +    | +    |
| siCdc37-2  | +    | +    | +    |
| siCdc37-3  | +    | +    | +    |
| TNFα       | -    | +    | +    |
| KA         | 1    | 27   | 4     |
|            | 2    | 3    | 1     |
|            | 22   | 8    | 13    |
|            | 23   | 11   | 9     |
|            | 38   |      |       |

C

| siIKKα/siIKKβ+siCdc37_3 (day 6) |
|----------------------------------|
| Fraction | I | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 |
| IKKβ     | - |
| IKKγ     |   |
Figure 5

A

| 17-DMAG (nM) | sicontrol | siHsp90α/β | siCdc37 |
|-------------|----------|------------|--------|
| -           | 10 20 50 200 | - 10 20 50 200 | - 10 20 50 200 |

TNFα

| + + + + + | + + + + + | + + + + + |

EMSA

| + | + | + |

WB

| 100 12 8 15 36 100 | 100 5 12 77 166 169 | 100 24 44 44 93 90 |

% expression

p50

B

siCdc37

Hsp90α

NF-κB

Hsp90β

Cdc37

IkBα

% expression

p50
Figure 6

A

|          | HeLa | 293 |
|----------|------|-----|
| γ-Irr.   | -    | +   |
| 17-DMAG (h) | - 1 3 6 | - 1 3 6 |

NF-κB

Fold

|     | 1,0 | 4,3 | 3,0 | 1,0 | 0,5 | 1,0 | 7,2 | 2,8 | 1,2 | 0,4 |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|     | 1,0 | 3,8 | 3,1 | 1,0 | 2,2 | 3,3 |

IP

|       | IKKβ | IKKα | IKKγ |
|-------|------|------|------|

Input

|      | IKKα  | IKKβ  | IKKγ  |
|------|-------|-------|-------|

B

17-DMAG (h) - - 4 - - 2
TNF-α - + - - -
γ-Irr. - - - + +

KA*

|     | 1,0 | 10,2 | 4,3 | 1,0 | 6,0 | 2,2 |

GST-IκBα

Fold

|     | 1,0 | 3,8 | 3,1 | 1,0 | 2,2 | 3,3 |

IP

|       | IKKβ | IKKα | IKKγ |
|-------|------|------|------|

Input

|      | IKKα  | IKKβ  | IKKγ  |
|------|-------|-------|-------|
Figure 7

IKK biosynthesis → "new" IKK complex → mature IKK complex → IKK competent → IKK active

1. Hsp90
2. Cdc37

IKK p

DNA damage → receptor mediated activation

IKK p

dephosphorylation → phosphorylation intramolecular activation

IKK p

pre-activated
Signal-responsiveness of IκB kinases is determined by Cdc37-assisted transient interaction with Hsp90
Michael Hinz, Meike Broemer, Seda Col Arslan, Albrecht Otto, Eva-Christina Mueller, Rudolf Dettmer and Claus Scheidereit

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